BREAST CYST FLUID FORMATION, STUDIED
BIOCHEMICALLY AND CYTOCHEMICALLY

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A Thesis submitted for the degree of
Doctor of Philosophy of the University of Surrey
The purpose of this study was to test the hypothesis that Breast Cyst Fluid (BCF) is a secretion from the "pink" epithelial cells found to line such cysts.

A series of BC fluids specially collected over a period of 18 months from a local hospital clinic, as well as routinely sent samples, were investigated for their cell content, proteins, and various chemical parameters compared with milk and serum. Histological and cytological material was examined by light and electron microscopy with various stains, and by immunohistochemical techniques using an antiserum raised against whole BCF.

The aspirates could be divided into two groups based on cell content and cellulose acetate electrophoresis of the fluid. These methods correlated well. The lack of immunoglobulins in one group was examined and possible reasons for this suggested.

The chemical content was found to be very variable; for all parameters a large range was found, and for most, levels were close to those of serum. The exceptions are urate, cholesterol and potassium which tended to be high. Cholesterol often reached enormous levels and was found to be mainly present in a particulate fraction of the whole fluid.

The particulate fraction was separated by density gradient centrifugation and analysed for cholesterol, protein, and various membrane marker enzymes, which suggested a possible origin from cell membranes.

The antiserum to BCF was found to contain antibodies to 2 BCF-specific proteins after absorption to remove antibodies to serum and milk proteins. The specific proteins were found by

*In this study, "cell membranes" is taken to mean membranes from cell organelles generally and does not refer solely to plasma membrane.*
immunohistochemistry to locate in the Golgi complex of the 'pink' cell, and also in apocrine glands from axillary skin.

Whilst the process of secretion was never observed, electron microscopy showed the 'pink' cell to have secretory characteristics.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>anti-(x)</td>
<td>Antiserum to x</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BCF</td>
<td>Breast cyst fluid</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>CAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein iso-thiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>HE</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>MFGM</td>
<td>Milk fat globule membrane</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid – Schiff</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>p.m.</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>r.e.r.</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SC</td>
<td>Secretory component</td>
</tr>
<tr>
<td>SGOT</td>
<td>Glutamate-oxalacetate transaminase</td>
</tr>
<tr>
<td>SGPT</td>
<td>Glutamate-pyruvate transaminase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) methylamine</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Summary</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations list</td>
<td>iii</td>
</tr>
</tbody>
</table>

## INTRODUCTION

1 STRUCTURE AND FUNCTION OF THE HUMAN BREAST  
1.1 Development  
1.2 Gross structure  
1.3 Montgomery glands  
1.4 Histology and Cytology  
1.4.1 Epithelium  
1.4.2 Myoepithelium  
1.5 Lactation  
1.6 Secretion of milk  
1.6.1 Lipids  
1.6.2 Protein  
1.6.3 Lactose  
1.7 Immunoglobulins in milk  
1.8 Membranes in milk  
1.9 Secretion and absorption in non-lactating breast  
1.10 Involution  
1.10.1 Post-lactational involution  
1.10.2 Senile involution  
1.11 Cyclical changes in breast tissue  
2 CYSTIC DISEASE OF THE BREAST  
2.1 Clinical presentation  
2.2 Management  
2.3 Aetiology  
2.3.1 Lobular sclerosis  
2.3.2 Cystic lobular sclerosis  
2.3.3 Cystic lobular degeneration  
2.4 Tension cysts  
2.5 Hormones  
2.6 Incidence  
3 'PINK' CELLS  
3.1 Morphology  
3.1.1 Microscopic
3.1.2 Ultramicroscopic

3.2 Aetiology
   3.2.1 Degeneration
   3.2.2 Hormonal variation

3.3 Incidence

3.4 Histochemistry

4. SKIN APOCRINE GLANDS
   4.1 Embryology
   4.2 Morphology
   4.3 Cells
      4.3.1 Duct
      4.3.2 Secretory cells
   4.4 Ultrastructure of apocrine gland cells
      4.4.1 Abnormal mitochondria
      4.4.2 Large dark granules
      4.4.3 Microbodies
      4.4.4 Microvesicles
   4.5 Secretion and excretion by skin apocrine glands
   4.6 Composition of apocrine gland secretion

5 DIRECTION OF RESEARCH
   5.1 Objectives
   5.2 Means

MATERIALS AND METHODS
1 SPECIMEN COLLECTION
   1.1 Breast cyst fluid
   1.2 Serum
   1.3 Urine
   1.4 Tissue samples

2 ELECTROPHORETIC METHODS
   2.1 Cellulose acetate
      2.1.1 PAS staining
   2.2 Acrylamide gel
      2.2.1 Disc
      2.2.2 Flat bed
   2.3 SDS gel
   2.4 Molecular weight determinations by SDS gel
   2.5 Isoelectric focussing
6.3.1 Persijn and van der Slik method 111
6.3.2 Method entailing phosphate assay 112
6.3.3 Validation of assay 112
6.3.4 Stability of enzyme 113
6.4 Na+, K+-dependent ATPase 113
6.5 Glucose-6-phosphatase 114
6.6 Acid phosphatase 115
6.7 Alkaline phosphatase 116
6.8 Alkaline phosphodiesterase 116
6.9 Acid phosphodiesterase 117
6.10 Cathepsin D 117
6.10.1 Validation of assay 118
6.10.2 Activation and stability of the enzyme 119
6.11 Succinate dehydrogenase 120
6.12 Amylase 120
6.13 Xanthine oxidase 121
6.14 Lactoperoxidase 122
6.15 AutoAnalyzer 122
7 CENTRIFUGATION METHODS 124
7.1 High-speed centrifugation without a gradient 125
7.2 Flotation method 125
7.3 Density gradient centrifugation 125
7.3.1 Gradient formation 125
7.3.2 Sample preparation and addition 126
7.3.3 Centrifugation and fractionation 127
7.3.4 Analysis of fraction 128
8 CYTOLOGY 130
8.1 Cells from BCF 130
8.2 Histological sections 131
8.3 Staining methods 131
8.3.1 Haematoxylin and eosin 131
8.3.2 May-Grünwald-Giemsa 132
8.3.3 Gram's stain 132
8.3.4 Papanicolaou's stain 133
8.3.5 PAS stain 134
8.3.6 Diastase-PAS 134
8.3.7 Perls' Prussian Blue stain 134
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3.8</td>
<td>Alcian Blue stain</td>
</tr>
<tr>
<td>8.3.9</td>
<td>Phosphotungstic acid haematoxylin</td>
</tr>
<tr>
<td>8.3.10</td>
<td>Schmorl's method</td>
</tr>
<tr>
<td>8.3.11</td>
<td>Chrome alum haematoxylin</td>
</tr>
<tr>
<td>8.3.12</td>
<td>Perls' method (for sections)</td>
</tr>
<tr>
<td>8.3.13</td>
<td>Turnbull's method</td>
</tr>
<tr>
<td>8.4</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>8.4.1</td>
<td>Fixation</td>
</tr>
<tr>
<td>8.4.2</td>
<td>Embedding</td>
</tr>
<tr>
<td>8.4.3</td>
<td>Sectioning</td>
</tr>
<tr>
<td>8.4.4</td>
<td>Staining</td>
</tr>
<tr>
<td>8.4.5</td>
<td>Carbohydrate stain</td>
</tr>
<tr>
<td>8.4.6</td>
<td>Negative stain</td>
</tr>
<tr>
<td>8.4.7</td>
<td>Examination and photography</td>
</tr>
<tr>
<td>8.5</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>8.6</td>
<td>Microbiology</td>
</tr>
<tr>
<td>9</td>
<td>PHOTOGRAPHIC METHODS</td>
</tr>
<tr>
<td>9.1</td>
<td>Photography</td>
</tr>
<tr>
<td>9.2</td>
<td>Processing</td>
</tr>
<tr>
<td>9.3</td>
<td>Printing</td>
</tr>
</tbody>
</table>

## RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PATIENT AND CYST DATA</td>
</tr>
<tr>
<td>1.1</td>
<td>Patient data</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Age</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Parity</td>
</tr>
<tr>
<td>1.2</td>
<td>Cyst physical data</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Volume</td>
</tr>
<tr>
<td>1.2.2</td>
<td>BCF colour</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Site</td>
</tr>
<tr>
<td>2</td>
<td>CYTOLOGY</td>
</tr>
<tr>
<td>2.1</td>
<td>Cells from breast cyst fluid</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Other cell types</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Relationship of cell type to BCF electrophoresis</td>
</tr>
<tr>
<td>2.2</td>
<td>Histological sections</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Cystic breast tissue</td>
</tr>
</tbody>
</table>
4.4 Triglycerides 213
4.5 Phospholipids 213
4.6 Nucleic acids 213
4.7 Lactose 213
4.8 Uric acid 213
4.9 Inorganic ions 215
4.10 Carcinoembryonic antigen 215
4.11 Hormonal steroids 216

5 ENZYME ASSAYS 219
5.1 Validation of phosphate assay 219
5.2 5'-Nucleotidase 220
   5.2.1 5'-Nucleotidase assay - validation 222
   5.2.2 Stability of 5'-Nucleotidase 222
5.3 Acid phosphatase 222
5.4 Alkaline phosphatase 224
5.5 Cathepsin D 224
   5.5.1 Validation of assay 224
   5.5.2 Stability and activation of cathepsin D 225
5.6 Amylase 225
5.7 Xanthine oxidase 225
5.8 Lactoperoxidase 227
5.9 AutoAnalyzer 227

6 CENTRIFUGATION METHODS 228
6.1 High speed centrifugation 228
   6.1.1 5'-Nucleotidase 228
   6.1.2 Cathepsin D 228
   6.1.3 Cholesterol 230
6.2 Flotation method 230
   6.2.1 5'-Nucleotidase 230
   6.2.2 Cathepsin D 231
   6.2.3 Cholesterol 231
6.3 Density gradient centrifugation 233
   6.3.1 Breast cyst fluids 233
   6.3.2 Milk 242
   6.3.3 Blood plasma 244
<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Patient data</td>
<td>245</td>
</tr>
<tr>
<td>2  Proteins</td>
<td>245</td>
</tr>
<tr>
<td>3  Particles</td>
<td>249</td>
</tr>
<tr>
<td>4  Chemical components</td>
<td>253</td>
</tr>
<tr>
<td>5  The evidence for secretion of BCF</td>
<td>258</td>
</tr>
<tr>
<td>6  Conclusions</td>
<td>263</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>266</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>280</td>
</tr>
</tbody>
</table>
INTRODUCTION
I. STRUCTURE AND FUNCTION OF HUMAN BREAST

1.1 DEVELOPMENT

About the sixth week of embryonic life, the ectoderm of the foetus becomes thickened along the "milk lines" running from axilla to groin. The epithelial cells have the ability to grow into the mesenchyme at any point on this line to eventually form the breast bud. In the human, this usually occurs at one point on each line, although supernumary breasts or nipples do occur. At these points, the epithelial cells form a small cluster from which approximately 15 to 25 separate cords of epithelial cells push into the mesenchyme. Each of these develops into a separate compound exocrine gland. During foetal life, the cords branch to some extent, developing equally in male and female.

As puberty approaches, the female gland begins to develop and enlarge. The increase in size is largely due to fat accumulating in the connective tissue. There is some increase in glandular tissue, but probably not beyond the duct stage, i.e. not secretory units. These changes are largely due to oestrogen produced by the maturing ovarian follicles under the stimulation of gonadotrophins, mainly FSH, from the anterior pituitary.

1.2 GROSS STRUCTURE

The mature breast is dome-shaped with a circular pigmented skin area 2 to 3 cm in diameter, the areola mammae, located at its centre (Fig.1). It has three major structures; the skin, subcutaneous tissue, and corpus mammae.

The skin is thin and elastic and adheres to the fat-laden subcutaneous tissue. It has hair, and sebaceous and exocrine sweat glands. The areola mammae is pigmented reddish brown and surrounds
Fig. 1  Gross structure of the human mammary gland.
the nipple. It enlarges during pregnancy and lactation, and contains sweat glands, sebaceous glands not attached to hair, and a specialised gland type called Montgomery's glands which have ducts opening onto the skin surface at Morgagni's tubercle. The nipple contains the 15 to 25 milk duct outlets, and abundant sebaceous and apocrine glands, again with no hair.

The corpus mammae consists of parenchyma set in a stroma of connective tissue and fat with blood vessels, nerves and lymphatics. The parenchyma comprises of the ductular-lobular-aveolar structures. The 15 to 25 lobes, each served by one of the major milk ducts, are arranged like the spokes of a wheel converging on the nipple. Each lobe is subdivided into 20 to 40 lobules, which each contain 10 to 100 alveoli (secretory units). These are approximately 0.12 mm in diameter, the main ducts being 2 mm, enlarging to 5 to 8 mm in the lactiferous sinuses at the nipple base.

The alveoli are enveloped in a collagenous sheath, forming the basement membrane, and this continues round the collecting duct. This sheath is a loose type of collagen and contains small blood vessels and lymphatics. The lobule is surrounded by a thicker denser collagen envelope. These are called intralobular and interlobular connective tissue, respectively.

1.3 MONTGOMERY GLANDS

These are phylogenetically derived from skin apocrine glands. They open onto the areolar skin in the form of a funnel shaped terminal duct, closely connected with hair papillae and sebaceous glands, which open into the infundibular part of the duct. They undergo hypertrophy during pregnancy, and are hormonally stimulated in the newborn. Their
physiological function is unclear, but they may be analogous to the accessory tissue of sexual glands and function as a scent organ. Like breast tissue they undergo post-menopausal involution, unlike apocrine glands which do regress in old age but seemingly not in relation to hormones (Montagna, 1959; Hurley & Shelley, 1960).

1.4 HISTOLOGY AND CYTOLOGY

The epithelium of the lactiferous ducts at their orifices is like that which covers the nipple, i.e. squamous. Deeper in the nipple, this becomes a double layer of columnar epithelial cells resting on a basement membrane. The main ducts branch to form intralobular ducts. The ducts are surrounded by a very cellular connective tissue continuous with the papillary layer of the dermis which abuts directly on the epidermis. These are set in a matrix of substantial non-cellular connective tissue, extending down from the reticular layer of the dermis, to separate and tightly hold the lobes and lobules. The larger of these bundles are called the "suspensory ligaments of Cooper". They also contain accumulations of fat.

The walls of the intralobular ducts are generally composed of two layers of epithelial cells, having pale cytoplasm and pale oval nuclei, resting on a basement membrane.

The resting breast probably consists only of a duct system. In pregnancy, this develops and branches further, and secretory alveoli form at the ends of ductules within lobules. The intralobular connective tissue becomes very thin, due to stretching, but nonetheless contains many blood vessels. The alveoli contain a single layer of columnar cells. Epithelial development is finished by the end of the sixth month, and the continued size increase of the breast is due to
secretion.

In the resting breast, the ducts and ductules are lined by two main types of cell: a layer of epithelial cells surrounded peripherally by a discontinuous layer of myoepithelial cells in the ductules, and a continuous layer in the ducts (Ahmed, 1978).

4.1 Epithelium

Bässler (1970) has described two types of epithelial cells: a dark cell (A-cells) and a clear type (B-cells). The two are morphologically similar, having numerous free ribosomes, scattered vesicles of rough endoplasmic reticulum (r.e.r.), a few mitochondria, and a small Golgi complex displaying no signs of secretory activity. Membrane-bound lipid bodies, glycogen particles and lysosomes may be present. Tonofilaments are located randomly, and the apical plasma membrane has microvilli. The nucleus is generally oval, central, with variable invaginations.

The dark A-cells are eosinophilic, and contain numerous ribosomes causing the dark appearance of the cytoplasm. They tend to have a more secretory character than light cells. The light B-cells are the major type and probably represent a different functional stage (Ozzello, 1971), or even a stem cell from which both A-cells and myoepithelial cells arise. The cytoplasm is transparent, and the nucleus has more equal chromatin distribution than that of A-cells.

4.2 Myoepithelium

This forms a basket-like network round alveoli, and runs along the long axis of the ducts. It can be demonstrated with Masson's trichrome or silver stains, and its function is to cause ejection of milk when stimulated to contract by oxytocin - a posterior pituitary hormone. The cells lie between the epithelium and the basement
membrane, and, under normal conditions, do not approach the lumen. The cytoplasm contains numerous fine filaments (5.7 nm in diameter) along the cell axis, resembling the myofilaments of smooth muscle.

1.5 LACTATION

In pregnancy, there is considerable development of the breast, especially the lobulo-alveolar system. The ducts proliferate and branch, and alveoli are formed on the ends of the terminal ducts. The alveoli are lined by a single layer of cells, surrounded by a myoepithelial network. The basal region of the alveolar cells abuts on the myoepithelial cells or the basement membrane, and is indented to form clefts between the cell plasma membrane and the basal lamina (Ahmed, 1978). This may be to allow increased surface area for uptake of nutrients.

The epithelial cell nucleus is round or oval, large, and may be indented. There is a well developed rough endoplasmic reticular system, arranged in parallel and stretching from the base of the cell almost to the apex, often arranged around mitochondria. The Golgi complex is very prominent and located in the supranuclear area, and consists of parallel flattened smooth-membrane stacks, with many small vesicles and a few vacuoles. Mitochondria are numerous and of variable size and shape. (Cowie & Tindal, 1971).

1.6 SECRETION OF MILK

Milk is produced by the breast after parturition. For the first three days the secreted material is rich in protein, especially immunoglobulins, and lipid; but the levels gradually reduce as more copious quantities of mature milk are produced. The process of
secretion begins in the third trimester of pregnancy, but until full secretion is commenced the quantity produced is very small.

The suppression of full lactation during pregnancy is generally believed to be due to oestrogen and progesterone, produced by the ovaries and placenta, acting on mammary epithelium and the anterior pituitary. Progesterone is believed to inhibit prolactin secretion and action. After parturition, and loss of the placenta, the inhibitory levels of these hormones are removed thus promoting lactation (Cowie & Tindal, 1971). In mice, lactose synthesis is observed to be inhibited by suppression of lactalbumin (part of the lactose-producing enzyme system) by progesterone (Turkington & Hill, 1969).

The main constituents of milk are protein, both serum-derived and milk-specific; lipid, mostly triglyceride; lactose; ions, Na\(^+\), K\(^+\) and Ca\(^{2+}\); and water. The mode of secretion of these compounds has been controversial (Cowie & Tindal, 1971) but there is now general agreement on the basic mechanisms.

The synthesis of the milk fat globule takes place in the lactating epithelial cell, as does synthesis of lactose and the milk-specific proteins.

1.6.1 Lipids

More than 95% of milk lipid is triglyceride contained in milk fat globules. The fatty acids are derived from triglycerides of chylomicrons and low density lipoproteins from serum, and de novo synthesis in mammary tissue (Patton & Keenan, 1975). The triglycerides are synthesised in the r.e.r. (Stein & Stein, 1967), and forming milk fat droplets are consistently seen in r.e.r. at the basal end of the cell.

The forming droplet, which may have a bounding membrane, increases in size as it moves, by an unexplained mechanism, to the
apex of the cell. One possible explanation is the distortion of the cell produced by distension of the alveolus by secretion (cells flattened) and relaxation after milk ejection (cells elongated). The droplet is then gradually pushed out of the cytoplasm, becoming enfolded by plasma membrane which surrounds the droplet. The formed globule is thus released when the plasma membrane rejoins beneath it (Fig. 2). The free milk fat globule therefore consists of a core of milk fat surrounded by a milk fat globule membrane. Keenan et al. (1970) have shown this membrane to be derived from plasma membrane on the basis of their similarity of composition of phospholipids, cholesterol esters, fatty acids, and protein.

The attraction of the droplet to the plasma membrane is another unresolved problem. Patton and Fowkes (1967) suggested sufficient attractive force would arise from London-Van der Waals forces, when the membrane lipid separation was 2 nm, to snap the droplet across the membrane. Wooding (1971), on ultrastructural evidence, disagreed, never finding the membrane droplet separation to be less than 10 - 20 nm. He suggested an alternative mechanism whereby Golgi vesicles surround the droplet and by fusing with the plasma membrane, provide the excess membrane to allow the droplet to cross easily the cell boundary.

I.6.2 Protein

Milk contains at least 18 serum proteins, some only in trace amounts (Hanson & Johansson, 1970), and at least 13 proteins specific for milk. These latter are synthesized in the r.e.r. of the lactating epithelium. They then proceed to the Golgi complex by a mechanism which is uncertain but probably involves the flow of membranes from r.e.r. to Golgi complex. In this organelle, the r.e.r. membrane is transformed to Golgi-type membrane and the addition of carbohydrate
Fig. 2  Secretory epithelial cell of the lactating mammary gland.
to secretory glycoproteins is completed. The membrane-flow hypothesis suggests that the now complete proteins are packaged in Golgi vesicles which bud off from the cisternae, the membrane by now having been transformed to plasma membrane. The vesicles then move to the cell apex and fuse with the plasma membrane, releasing their contents into the alveolar lumen. The membrane flow hypothesis is supported by the fact that Golgi membrane has a phospholipid composition intermediate between that of r.e.r. and plasma membrane (Keenan & Morré, 1970).

Also, in rats, the time for $^{14}$C cholesterol to move from blood to milk (where cholesterol is almost entirely in the form of membranes) is 17 hours (Easter 1971). The author suggests that this seems too long for simple equilibration of the cholesterol with plasma membrane (p.m.) and probably represents the time for its incorporation into r.e.r. and the conversion to p.m. However, whilst this might fit in with the membrane-flow hypothesis, it is hard to reconcile with the fact that r.e.r. is low in cholesterol compared with p.m. (Thinès-Sempoux, 1974; Morré et al., 1974).

1.6.3 Lactose

This carbohydrate of milk is synthesised from glucose in the Golgi complex by an enzyme system consisting of two proteins, A and B. The A protein is galactosyl transferase, a Golgi enzyme which catalyses the addition of carbohydrate to glycoproteins; the B protein is $\alpha$-lactalbumin, a milk protein which interacts with the A protein to specify glucose as the substrate for galactose addition:

$$\text{UDP-galactose} + \text{glucose} \xrightarrow{\text{galactose synthetase, (i.e. A+B)}} \text{lactose} + \text{UDP}. $$

In the absence of $\alpha$-lactalbumin, lactose is only found in small amounts (as in colostrum) and thus the protein controls the synthesis. Brew (1969) has suggested that the reason for the secretion of
α-lactalbumin into milk is to allow rapid control of lactose synthesis since part of the enzyme thus has a high throughput. The lactose, being unable to pass through the membrane of Golgi vesicles, is then secreted with the protein. It has been suggested that the effect of lactose synthesis is to draw water osmotically into the Golgi complex (Linzell & Peaker, 1972) to act as a carrier for secretions and form the aqueous phase of milk.

I.7 IMMUNOGLOBULINS IN MILK

The relative concentrations of IgG, and IgA are not the same in milk as in blood. In the latter, IgG predominates whereas in milk IgA is the major immunoglobulin, as in all secreted fluids. Therefore, since the breast stroma contains mainly IgG, which is presumably serum derived, the immunoglobulins in milk do not arrive by simple diffusion. Also, the IgA in milk is in a different form from that in plasma, and IgM, which is also found in milk, has a much higher molecular weight than IgG and occurs in the stroma in lower concentrations than IgG.

Using immunofluorescence on saline-extracted tissue, Brandtzaeg (1977) has shown that immunoglobulin-producing cells are present in the epithelium of secretory tissues, and that 70 to 97% of them produce IgA. The IgA from these immunocytes is the dimeric form, rather than the serum-type monomer, linked by a polypeptide called the "J-chain".

In milk, as with other secretions, the IgA dimer is bound by a small glycoprotein called 'secretor piece' or 'secretory component' (SC). This makes the molecule much more stable and resistant to proteolysis. The secretory component is shown by immunohistochemistry in rabbit mammary gland to occur in the Golgi complex and vesicles at the apical end of the epithelial cells. IgA however is found only in the
vesicles, suggesting SC is produced in the cells and IgA is produced extracellularly (Kraehenbuhl et al., 1975). Antigenic properties by the same technique suggest that SC is in free form in the Golgi complex, but bound to IgA in the apical cytoplasm (Brandtzaeg, 1974).

Based on this and other evidence, Brandtzaeg (1977) has proposed a model for immunoglobulin transport at epithelial surfaces. (Fig. 3).

1. SC produced by the epithelial cells accumulates in the Golgi complex, where some final addition of carbohydrate may take place.

2. The SC is distributed to the cell cytoplasm and plasma membrane.

3. Dimeric IgA, linked by J-chain, is produced by local immunocytes.

4. The membrane-associated SC acts as an epithelial receptor for the dimeric IgA. Such SC has an affinity for dimeric IgA probably due to characteristics imparted by the J-chain.

5. The complex is mobilised to the lateral region of the p.m.

6. Disulphide bridges are formed to stabilise the complex.

7. The complex is transported to the Golgi region by pinocytosis.

8. The quaternary structure is completed and the complex secreted together with free SC.

9. Some complex may pass the tight junction between cell apices and not enter the cytoplasm.

Pentameric IgM may be secreted by the same system, the five monomer molecules are also joined by J-chains. However the complex is not as stable as secretory IgA. The free SC may be secreted in order to maintain the complexes after secretion.

1.8 MEMBRANES IN MILK

Plantz & Patton (1973) in a study of the fractions obtained from skimmed bovine and caprine milks, after centrifugation by differential
Fig. 3  Immunoglobulin secretion at epithelial surfaces according to the model of Brandtzaeg (1977).
and density gradient methods, found that virtually all the lipid phosphorus and unesterified cholesterol is contained in membrane fragments and vesicles. In bovine skimmed milk, 85% of cholesterol is unesterified and therefore the great majority is associated with membranes. They found the membrane fraction to be of density from 1.10 to 1.15 and to contain over half the 5'-nucleotidase activity, and most of the nucleotide pyrophosphatase activity of skimmed milk.

The majority of work on membranes of milk has involved the milk fat globule membrane (MFGM), which was suggested on strong evidence by Keenan et al., (1970) to be derived from plasma membrane of mammary cells. Specimens have mainly been obtained from animals, with few results available for human milk. One such piece of work (Martel-Pradal & Got, 1972) showed 5'-nucleotidase to be absent from human skimmed milk, though other p.m. markers (Mg$^{2+}$-ATPase and alkaline phosphodiesterase) were found. In addition, enzyme markers for Golgi complex, endoplasmic reticulum, and plasma membrane were shown to be present in human skimmed milk. Thus, membrane marker enzymes consistent with the types of membrane found in bovine skimmed milk by Plantz & Patton (1973) are also found in human skimmed milk, with the exception of 5'-nucleotidase. Thines-Sempoux (1974) has suggested inactivation of 5'-nucleotidase in rat liver endocytotic vesicles, which are also derived from plasma membrane, and possibly the same inactivation may occur in human skimmed milk. Martel-Pradal & Got. (1972) also found the Golgi and endoplasmic reticulum markers were present in MFGM preparations from human milk. This might possibly be further evidence for the membrane flow hypothesis.

The fat-free fraction of milk therefore contains membranes (sometimes referred to as the "fluff" fraction) which, like MFGM,
apparently derive from plasma membrane with the possibility of some arising from other sources. Ultrastructural evidence (Stewart et al., 1972; Plantz & Patton, 1973) bears this out and suggests the main constituents to be relatively large structures rather than discrete subunits (also suggested by their exclusion from Sepharose 4B gel) and derived primarily from plasma membrane, including microvilli.

1.9 SECRETION AND ABSORPTION IN NON-LACTATING BREAST

Although secretion is externally evident only after parturition, there is evidence that the mature breast is continuously secreting. Histological studies of normal lobules in mature non-lactating breasts invariably show acini and ducts containing secretion (Bonser et al., 1961). If stained by the periodic acid-Schiff procedure (PAS) those secretions show two components, one brilliant violet, the other a pale pink. This PAS-positive substance is also present in the basement membrane, but not in the normal involuting lobule where presumably secretion has stopped. Alcian blue stain gives the same general picture but differentiates the two components more clearly into a homogenous red material and a blue green mucinous substance. In the resting breast the components are roughly equal, but the mucinous material decreases in pregnancy. Since the secretion does not accumulate and distend the resting breast, nor escape to the exterior, it must be assumed there is an equal process of resorption.

The pathway of absorption has been demonstrated by injecting fluid containing small carbon particles, or a mixture of ferrous ammonium citrate and potassium ferrocyanide (which is precipitated as Prussian blue on acid fixation) into the lactiferous ducts of virgin adult rabbits. Three hours after injection of carbon, small
particles were found in the epithelial cells of ducts and alveoli; after 24 hours, they were in the lymphatics. The same occurred with Prussian blue, but much more rapidly. After five minutes it was in the lymphatics, and after 30 minutes most had left the breast. Bonser et al. (1961) repeated this work using high titre diptheria antitoxin, being a more physiological marker and easier to quantitate. They found that approximately half the antitoxin was absorbed, and absorption continued for at least 24 hours.

Browne (1946) showed that lactosuria is common at the end of pregnancy. This is most easily explained as reabsorption of secretion by the breast. Also, the initial secretion after parturition, colostrum, is high in protein but low in lactose, whereas milk is high in lactose but lower in protein. This would be compatible with reabsorption, the small molecular weight lactose being more easily removed than protein.

Cowie and Tindal (1971) pointed out that milk may also be altered in composition of water and water-soluble constituents in the alveoli and fine ducts. This may be attributable to variations in the osmotic pressure of blood. Linzell and Peaker (1976) add that milk stored in ducts and cisternae apparently remains unchanged for long periods since these structures are impermeable to the main constituents of milk.

I.10 INVOLUTION

Involution is the term used to describe regression of the mammary gland parenchyma. It is of two types; post-lactational and senile, which have different features.

I.10.1 Post-Lactational Involution

This type of involution is sometimes referred to as regression in order to distinguish it from senile involution. It covers the process whereby after cessation of lactation the remaining secreted
milk is removed, and the secretory tissue is decreased. This leaves
the breast tissue in much the same degree of development as before
pregnancy. For obvious reasons most biochemical and ultrastructural
studies of post-lactational involution have been made on animals.

The course of the process varies with the cause of cessation
of lactation, since the total volume and secretory activity of the
parenchyma may decline gradually, following a rise to maximal activity,
as lactation proceeds (Cowie & Tindal, 1971). Thus in enforced stasis
of the gland, or premature weaning, the secretory tissue is at a
different stage of development from that at the normal time of weaning.
The effects of enforced stasis or premature weaning depend upon the
current phase of secretion (i.e. increasing, maximal, or decreasing
activity), and, in the former, upon whether lactation is continued
in other glands of the same animal (i.e. hormonal stimulation of
lactation continues) (Helminen & Ericsson, 1971).

In general, the biochemical changes begin within hours after
cessation of suckling or milking (Cowie & Tindal, 1971). Metabolic
activity, shown by respiratory activity of gland tissue and lactate
accumulation, declines within 8 to 12 hours in animals. There is a
considerable increase in gland weight, lactose and DNA content, and
free ribonuclease and β-glucuronidase; and a reduction in RNA and
anabolic enzymes. The increasing DNA appears to be correlated
with invasion of the tissue by leucocytes, and the enzymes may arise
from ruptured lysosomes. These organelles are greatly involved in
involution; both in autophagocytic processes, which reduce the
cytoplasm of the epithelial cells, and heterophagocytic processes
whereby macrophages remove cell debris and secretion. (Cowie &
Tindal, 1971; Helminen & Ericsson, 1971). Jones (1969) showed the
reduction of enzymes involved in milk synthesis to include glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, UDP-glucose pyrophosphorylase, phosphofructokinase, ATP citrate lyase, and acetyl CoA carboxylase.

The tissue structural changes are similar for all animals, the only variation being the time for completion thereof. This takes 15 days in the mouse, 36 in the rat and 48 days in the goat. For humans, Dawson (1935) estimates the time to be 9 to 12 months.

Initially the alveoli are distended by milk and the epithelium flattened. There follow cytolytic changes which may result in rupture of the alveolar basement membrane (Büssler, 1961), and disposal of cell and secretory products into the interstitial spaces. These may be removed by macrophages and/or via the lymphatic vessels. In enforced milk stasis the cytolysis, increase in lysosomes, and release of lysosomal enzymes, occur to a lesser degree and later than in normal involution (Helminen & Ericsson, 1971).

Dawson (1935) described the histological changes in the human gland as follows (in summary).

1) Distension of acini and rupture of adjacent walls to form large lumina.

2) Distension flattens the epithelium, inhibits secretion, and by interfering with capillary secretion furthers retrogression.

3) Phagocytic cells migrate to the lumen.

4) Secretion is removed by phagocytosis and distension diminishes. Epithelial cells are desquamated and begin to disintegrate. The basement membrane disappears, and the lobule loses definition.

5) Fibrous tissue replaces the disappearing alveoli.

6) Fatty tissue reappears in the stroma around the fifth
month after lactation ceases.

Involution may be delayed or incomplete, especially if pregnancy re-occurs rapidly; and lactating tissue may remain, and can form small cysts (Dawson, 1935).

1.10.2 Senile Involution

The senile type of involution begins around the menopause from the age of about 35 years. It occurs regardless of whether there has been previous lactation.

It is a very uneven process; in the same breast some segments may be fully involuted while others show a normal pattern. The cells lining the tubules and acini are gradually lost, and the basement membrane at the same time thickens and contracts down on the decreasing tubules and acini. Eventually, all that remains of the lobule is a series of rings representing basement membrane. These fuse and the lobule is obliterated, leaving only altered basement membrane which has lost its characteristic staining reactions. At the same time the dense interlobular connective tissue becomes atrophied and sometimes replaced by adipose tissue, often enough to cause the breast to enlarge, despite regression of the parenchyma. The time taken for the process to complete is not known with certainty but probably does not exceed 5 years. At the end, all that remains is the main ducts and their major branches.

In some cases, terminal acini at the periphery of lobules may dilate and join with adjacent acini to form small intralobular cysts. The loose intralobular connective tissue persists round these cysts until they shrink as their epithelium is lost. The pattern is then that of normal involution. The main difference in these cases is that secretion is usually present, unlike normal involution. It is
postulated that this cystic lobular involution may be due to hormonal conditions (Bonser et al., 1961). Hayward and Parks (1958) first suggested this type of involution as a normal, as opposed to pathological, part of involution, and showed how the cysts develop before the menopause, and gradually disappear afterwards.

I.11 CYCLICAL CHANGES IN BREAST TISSUE

The mature human breast undergoes changes during the menstrual cycle. Variations in size and number of microvilli were noted (Waugh & van der Hoeven, 1962), and Fanger and Ree (1974) classified epithelial cells into two types based on ultrastructural differences. Phase I epithelium occurs in the pre-ovulatory phase, is non-secretory, and has free ribosomes, occasional strands of rough endoplasmic reticulum and a small Golgi complex. Phase II epithelium (potentially secretory) occurs predominantly in the post-ovulatory period and has dilated cisternae of rough endoplasmic reticulum, well developed Golgi complex, glycogen deposits and numerous apical microvilli.

Ozzello and Speer (1958), in an histological study of breast biopsy and mastectomy specimens from patients with known menstrual histories, showed cyclical variations of the mucopolysaccharides of breast stroma. They observed no changes in the interlobular connective tissue, which was consistently PAS-positive (neutral mucopolysaccharides) throughout the menstrual cycle. The intralobular stroma showed variations in neutral and acid mucopolysaccharides, stained by PAS and Hale's technique (HS) respectively. In the "resting" (pre-ovulatory) phase the intralobular stroma appeared to be in part PAS- and in part HS-positive, the former predominating somewhat. As the "evolutional" (post-ovulatory) phase began, the HS positivity increased
up to the time of menstruation, while PAS-positive material disappeared more rapidly. This rise in acid mucopolysaccharide was accompanied by an increase in the number of fibroblasts. During the last few days of the post-ovulatory phase, PAS-positive secretory granules appeared in the acinar cells, and similarly staining material in the lumen. Both were diastase-resistant.

In mammary dysplasia these cyclical changes tended to be abolished; the interlobular stroma was mainly PAS-positive, although sometimes HS positivity remained (but unrelated to menstrual phase). In contiguous normal breast tissue, the cyclical characteristics were manifest.

Thus the breast is a continuously changing organ, both structurally and in its chemical composition, and the levels of acid mucopolysaccharides are increased by oestrogen stimulation.
2.1. CLINICAL PRESENTATION

Cystic disease of the breast occurs in women in the years leading up to, and during, the menopause. It is only very exceptionally found after the menopause. It is clinically the most frequently seen lesion of the breast.

The patient usually presents having discovered a lump in the breast. The lump is invariably symptomless, although occasionally some local tenderness or turgidity is felt, and thus is usually found by palpation. The discovery usually leads to considerable anxiety.

On examination the lump is found to be firm and rounded, and somewhat mobile with no tethering to the skin or adjacent tissue. The size is very variable, from less than one centimetre to about ten centimetres. Cooper (1829) reports examples weighing nine pounds, but such sizes are not found today.

Often more than one cyst is found, sometimes in the opposite breast to the initial discovery. There may also be increased nodularity of one or both breasts, which may be due to numbers of very small cysts, or another related condition such as fibroadenosis. Patients who develop a cyst will often have others, until they pass the menopause.

2.2. MANAGEMENT

If the cyst is palpable the treatment is simple (Bradbeer, 1974). The contents are aspirated using an hypodermic syringe and needle. Local anaesthetic is not really necessary but some surgeons will use it (e.g. Haagensen, 1971).

The needle is passed through the cleaned skin of the breast
and into the cyst, which is immobilised with the free hand. The fluid content is slowly aspirated until no more can be removed. Complete disappearance of the cyst is then checked. If some thickening of the tissue still remains, Haagensen (1971) recommends biopsy of the site to ensure there is no neoplastic lesion. He also suggests the same course if the fluid aspirated is very bloodstained or has the appearance of old blood.

If the patient has multiple cysts, these are all aspirated at the same time.

The cysts will usually disappear completely on aspiration but may refill and in this case biopsy is recommended (Haagensen, 1971). However, distinction of a refilled cyst from a nearby new cyst must be extremely difficult.

2.3. AETIOLOGY

The aetiology of cystic disease of the breast, and especially the so-called 'pink' cell metaplasia (q.v., page 30) which is so closely related to it, have been the source of debate for almost a century.

The first comprehensive studies of mammary cystic disease were made by Reclus in 1883 and Brissaud in 1884. The former stated that such lesions were frequently bilateral, involved most of the gland, and being very small were mainly impalpable. He also regarded them as new formations, unlike Brissaud who thought they arose from existing alveoli by proliferation of the epithelium which then underwent necrosis to form a cyst. This view was supported by Schimmelbusch (1890) who suggested the name "cystadenoma" as being preferable to Reclus' "maladie kystique des mamelles" since there was proliferation of glandular
In 1893, König introduced his inflammatory concept of the origin of breast cysts. He thought that epithelial proliferation and changes in the interstitial tissue were a reaction to an inflammatory irritant.

The idea that the lesion may be related to senile involution was first proposed by Tietze (1900). Bloodgood (1906) also agreed with this view, regarding cysts as the result of hypertrophy during involution. This theory has become very popular since then. McFarland (1922) took the idea one stage further and suggested that all the forms of the disease mentioned by previous workers were "no more than variations in the involutional process in which residual lactational acini appear in various conditions of retrogressive change". Unfortunately he only found cysts in the breasts of women who had lactated and this led him to believe that lactation was a necessary criterion of cystic disease. Ironically, the type of "residual lactational acini" that McFarland described have since been seen in nulliparous women (three out of eleven cases; Bonser et al., 1961).

This confusion serves to highlight a basic problem with the investigation of cystic disease. The condition is a complex of several seemingly different lesions and these may appear singly, or together in various degrees. This results in a variety of different terms, often used to describe the same disease type, many authors inventing their own terminology. Bonser et al. (1961) classify the main components as lobular sclerosis, cystic lobular sclerosis, and cystic lobular degeneration, complicated by hyperplasia and metaplasia. They suggest "abnormal involution" as a name to cover all these lesions since it refers to their probable aetiology. They do stress that these are purely artificial divisions, and do not suggest different
aetiologies. They may possibly even be different stages of the same disease.

2.3.1 Lobular Sclerosis

This is characterised by fibrous tissue proliferation within the lobule before the loss of epithelial cells, and usually in the absence of cystic dilatation. A scarred lobule results, with constricted acini as small clumps of cells, and basement membrane retaining its staining reactions until much later than in normal involution. 'Pink' cell metaplasia may also be found within the sclerosed lobule. This is probably a metaplastic change of the normal breast epithelium to a type of cell, sometimes called an apocrine cell due to its histological resemblance to the cells in the cutaneous apocrine glands. The name merely describes its cytoplasmic colour after haematoxylin and eosin staining (see also page 30).

It is not a common disease. Bonser (1961) found it in 59 patients out of 330 with breast disease, most commonly around the menopause. Karpas (1965) found it in 35% of patients, mostly the 40 - 49 years age group, Cameron (1965) in 54% of patients with 85% in the 35 - 55 age group. The parous: nulliparous ratio of sufferers was higher before the menopause than after (2.5: 1 and 1.1: 1 respectively), suggesting that post-lactational involution may play a part. The disease is frequently found in cystic breasts, which show an even more marked fall in parous: nulliparous ratio after the menopause (3.8: 1 and 1.8: 1) when this factor no longer operates. Thus there may be a common factor in the aetiology of the two conditions.

2.3.2 Cystic Lobular Sclerosis

This is the counterpart of lobular sclerosis where the excessive fibrosis affects the lobule undergoing cystic involution. The loose
intralobular connective tissue is replaced by dense fibrous tissue which fuses with the basement membrane. This leaves a collection of microcysts in dense connective tissue, with no remaining trace of the lobule from which they derived. Thus it is a very persistent condition. 'Pink' cell metaplasia sometimes takes place. This condition parallels lobular sclerosis in incidence, increasing as the menopause is reached. However, it continues beyond the menopause frequently up to 20 or 30 years later, unlike normal cystic involution. The intralobular fibrosis seems to petrify the cystic epithelial component.

2.3.3 Cystic Lobular Degeneration

This is an extension of the process of normal cystic involution. Instead of just the terminal acini, several tubules and acini run together and form a large cyst. The loose intralobular connective tissue is diminished, thus losing vascular and lymphatic access, and the cyst presses out against the dense interlobular connective tissue. When this change occurs, the epithelium usually undergoes 'pink' cell metaplasia. Semb (1928) found the point of origin of 'pink' cells to be the junction of the cyst and its duct, but occasionally it was inside the cyst and both types of epithelium co-exist.

2.4 TENSION CYSTS

The tension cyst is probably the final stage in cyst formation. The fluid content is under considerable pressure and the cysts cannot be collapsed, as the usual type of degeneration cysts can, by pressing on them and forcing the contents into the duct system through the ductule. The neck of tension cysts at the ductule is blocked by the epithelial hyperplasia or kinking of the duct during involution. Consequently,
the majority of tension cysts arise around the menopause during involution.

As the pressure builds up, due to continued fluid secretion, the cyst expands and the epithelium becomes stretched out over the cyst wall. At the neck, expansion is restricted by the more substantial connective tissue. There is little evidence that the cyst, if left, is a decaying structure which will disappear. Cooper (1829) found that secretion continues and the cyst increases in size. He found examples weighing 9 lbs.

This mode of development may have implications for cysts in general. Breast epithelium is capable of secretion and absorption and in 'pink'-epithelial cysts this absorption function is impaired, either due to the different type of epithelium, or the change in the connective tissue removing lymphatic drainage.

In 1928, Semb discounted McFarland's theory that breast cysts were residual lactation acini, and divided breast lesions into three categories in an attempt to unify terminology. The first group corresponded to normal involution, characterised by microscopic cysts, the other two to cystic disease with larger cysts. However, he reverted to the old theory and postulated the cysts as completely new formations, on the basis that they resembled gynaecomastia (male breast development due to hormone irregularities).

Geschickter (1943) subdivided cystic disease into several artificial groups and introduced new terminology. The result is that today people either use confusing terminology which is out of date, or describe the lesion accurately, without using such names.
2.5 HORMONES

The aetiology of cystic disease has often been quoted to be hormonal (Haagensen, 1971). There is little evidence for any specific hormone abnormality in cyst patients, but since the majority of breast cysts occur near the menopause there must obviously be associated endocrine changes. However, this is no indication of a causal relationship. Fechner (1970, 1972) has shown neither oral contraceptives nor oestrogen therapy to have any effect on either the incidence of benign breast disease, including cysts, or the characteristics of such conditions. However, the numbers of patients studied were only small to moderate (43 and 258), but nonetheless must indicate that there is no marked effect of such hormones. England et al. (1974) claim to have shown that serum 17β-oestradiol is increased in the luteal phase of women with cystic disease. They measured the concentrations daily through one cycle in 31 women with cystic disease and 32 normal controls, and found the elevation to be greater in the 40 - 49 years age group than the 30 - 39 years group. The statistical significance for these groups was \( p<0.01 \) and \( p<0.001 \) respectively, but did vary markedly with the method of analysis used.

There is also evidence, mostly in animals, for the effects of hormones on mammary epithelium and particularly 'pink' cell metaplasia (q.v., page 36). This too tends to suggest oestrogen as the hormone mainly involved.

2.6 INCIDENCE

The estimation of incidence of cystic disease is very difficult for a number of reasons, and is subject to many variables. Any hospital-based study is bound to be biased in favour of patients with
breast disease, and efficient diagnosis usually requires a biopsy unless macrocysts are present. As mentioned before, the cysts may be transitory or disappear on pressing, and are in general asymptomatic. Also, many women are too frightened of cancer to present themselves at breast clinics and many will never appear if the cyst should regress of its own accord.

Thus the studies of incidence have relied mainly on examination of material obtained at autopsy or from surgery. As would be expected, there is much variation in the reported results, which are often culled from far too low a number of cases.

The best study is probably that of Frantz et al. (1951). They reviewed material from 225 autopsies, and found gross cysts in 19% and microcysts in 53% of breasts from adult females. Devitt (1972) reviewed 2,281 breast biopsies performed over five years and found that 22% of breasts with carcinoma had an associated diagnosis of "fibrocystic disease". However this value is probably low since he did not re-examine histological material, only the reports, and in carcinoma cases any associated condition may often not be reported. Also, being based on a hospital study the results are obviously biased to abnormal material, unlike those of Frantz et al. (1951) and Sloss et al. (1957) who both found more frequent occurrence of cysts in normal breasts.
In cystic disease of the breast "pink" epithelial cells are an invariable finding. The cells were first clearly noted by Borst in 1904, although they had been mentioned previously. Borst called them "atypische Epithelien", and they have since been accorded a variety of names, such as "blasse Zellen", "helle rosige Epithelien", "eosinophile Epithelien", "oxyphile Epithelien", "cellules claires", "idrosadenoid epithelium", "pale epithelium", "clear cells", and "pink cells". The morphological similarity of these cells to those found in apocrine glands in the skin has led to them also being referred to as "apocrine metaplastic cells", "apocrine cells", or "sweat gland epithelium". In this work the term 'pink' cells will be used since it suggests no aetiology or function (merely referring to the colour of the cytoplasm on staining by haematoxylin and eosin) and avoids confusion between cells in the breast and the cutaneous organs.

3.1. MORPHOLOGY

3.1.1 Microscopic

'Pink' cells are easily distinguishable from normal mammary epithelium. They are cuboidal or columnar in shape with an eosinophilic cytoplasm and a small basally located nucleus (Pier et al., 1970).

In cysts they often appear very flattened due to stretching of the epithelium as the cyst grows. The apical end of the cytoplasm often bulges out into the lumen of the gland, giving a scalloped appearance to the epithelium, which is also very prone to hyperplasia, frequently forming papillary projection. Speert (1942) describes the nuclei as being round, vesicular and hypochromatic, and centrally located, and the cells as two to four times the size of those in
normal mammary epithelium.

The cytoplasm contains numerous granules, first clearly demonstrated by Lendrum (1945) using a carbacid fuchsin stain of his own modification. They are also visible in toluidine blue-stained sections from resin-embedded material (Pier et al., 1970). The granules are mainly located in the supranuclear region and collect near the apical plasma membrane. Lendrum (1945) suggested that they are secretory granules. Higginson and McDonald (1949) noted several types of granules, the majority being eosinophilic, occurring generally; whilst others were found in only a few cells of each acinus and had a yellow or brownish colour and did not stain. Another variety, also not common to all cells, gave a fat reaction with Sudan III, and a fourth type which gave a positive reaction for iron using Berlin blue stain. Whether this last type was identical with any of the others they did not establish, but large numbers of eosinic granules appeared to preclude iron-positive granules, which were found in variable numbers in cells from different acini.

3.1.2 Ultramicroscopic

On electron microscopy, 'pink' cells were found by Pier et al. (1970) to have distinct margins, with infolding of the plasma membrane at the basal end. The cells rest on a basement membrane, except where a myoepithelial cell intervenes. The apical plasma membrane has numerous short microvilli. Junctional complexes are seen between cells and there is little intercellular space (Pier et al., 1970).

The cytoplasm is rich in organelles, and there is an increase in the number of mitochondria compared with normal mammary epithelium (Murad and von Haam, 1968; Pier, et al., 1970; Ahmed, 1975). The mitochondria are mostly at the basal end of the cell and just above
the nucleus. They are variable in size and usually round or oval, but sometimes very irregular in shape. The cristae are few in number, usually long and thin, extend to the centre of the mitochondria and are often incomplete.

The increased numbers of mitochondria led Archer and Omar (1969) to compare 'pink' cells with oncocytes, previously suggested by Fasanotti (1950) as being the origin of 'pink' cells. Oncocytes (Hamperl, 1962) are transformed epithelial cells of various tissues, including salivary and parathyroid glands, characterised histologically by numerous acidophilic granules, which are mitochondria. Tremblay (1968) found low activity of succinate dehydrogenase, a mitochondrial enzyme, by histochemical methods in 'pink' cells, and thus suggested they were not oncocytes. Ahmed (1975) found high levels of this enzyme by histochemistry. However, the electron microscope shows the two cell types are not really similar; oncocytes are packed with mitochondria, seemingly to the exclusion of other cytoplasmic organelles, but this is not true of 'pink' cells. Also, in oncocytes the cristae of mitochondria are increased in number and extend the full length of the organelles (Ahmed, 1975). Thus their resemblance is superficial.

Rough endoplasmic reticulum is present, often parallel to the surface of mitochondria, adjacent to the nucleus and at the base of the cell (Pier et al., 1970), and vesicles of smooth endoplasmic reticulum are scattered throughout the cytoplasm. There is a prominent Golgi complex located above the nucleus. Free ribosomes are seen in the cytoplasm (Ahmed, 1978), and Pier et al. (1970) reported finding glycogen particles in the cytoplasm and endoplasmic reticulum of 'pink' cells from an intracystic papilloma, but not in others.

Two types of rounded osmiophilic granules have been observed by
electron microscopy (Pier et al., 1970). Large granules are found typically grouped at the apex of the cell, with a margin of cytoplasm separating them from the plasma membrane. In some cells there appeared to be transitional forms between these structures and mitochondria. The other type of osmiophilic granule is smaller and found immediately beneath the microvilli. They are also seen closer to the nucleus in the region of the Golgi complex, suggesting they may arise from this organelle.

Hence the structure of 'pink' cells of the breast is very similar to that of apocrine skin glands. They both have extensive infolding of the basal plasma membrane, a prominent Golgi complex, numerous mitochondria, apical microvilli, rough and smooth endoplasmic reticulum, all with a very similar distribution. However, the most distinctive relationship is the type of granules found in both cells, both at the light and electron microscope levels, particularly the large osmiophilic variety which may have an origin from mitochondria and are almost certainly those seen in the pink cells by Lendrum (1945).

3.2 AETIOLOGY

The aetiology of 'pink' cells has been the subject of debate for almost a century. The hypotheses put forward fall into two groups. One is an origin by metaplastic alteration of normal mammary epithelium, the other that the cells are a new growth, or an existing one which becomes prominent.

The mammary glands have long been regarded as modified "sweat" (i.e. apocrine) glands (Benda, 1894). In 1907, von Saar pointed out the similarity of 'pink' epithelium to that of cutaneous apocrine glands and regarded this as further evidence for the phylogenetic development
of mammary glands from apocrine glands. Creighton (1902) had earlier emphasized the relationship of apocrine skin glands to mammary development. He believed that apocrine gland cells were commonly found in normal breasts by accidentally arrested development of the normal epithelium from the "lower type". Krompecher (1916) took the view that 'pink' cells were a reversion and either arose from apocrine glands in the skin which were incorporated into breast tissue, or were due to arrested development of breast tissue at a phylogenetically earlier stage. Like Creighton he believed the latter was most probable.

Such views have since been refuted by many workers who preferred the first hypothesis, mentioned above, that 'pink' cells arise by metaplasia of normal breast tissue. Cheatle and Cutler (1931) stated that whenever apocrine glands resembling mammary structures were found they were always in the subcutaneous tissue and never in the gland itself. These authors never failed to trace a direct anatomical continuity between structures containing 'pink' epithelium and the duct system of the mammary tissue. Dawson (1932) concluded that the 'pink' cells were derived from normal mammary tissue by metaplasia, and found no evidence of inclusion of apocrine skin glands. Her evidence was largely that 'pink' cells and normal epithelium could be observed merging directly into each other in the same acinus, as has also been reported since by Bonser et al. (1961). Krompecher (1924) had earlier described the same phenomenon and sometimes found almost imperceptible transitions between the two types of tissue. He also stated that the similarity of 'pink' cells to those of apocrine skin glands was so striking that they must be identical. However, he revised his previous views and considered 'pink' cells to be derived from normal epithelium.
The cause of a metaplastic alteration of normal tissue to 'pink' epithelium is not known. Hypotheses as to the cause include degeneration, deranged cellular nutrition, and hormonal variations.

3.2.1 Degeneration

The main protagonist of the degeneration theory was Dawson (1932). She attributed the morphological alterations to degenerative changes following stimulation of the epithelium to rapid proliferation. Finding 'pink'-cell-lined cysts predominantly associated with the glandular involution of the menopause, she regarded the metaplasia as evidence of receding epithelial activity.

Berka (1912) had expressed a somewhat similar view, but, unlike Dawson, believed such tissue to result from incomplete post-lactational involution. McFarland (1922) also only found 'pink' epithelial cysts in women who had previously lactated, and thus assumed the cysts to be residual lactation acini. That this is not true was clearly demonstrated by Dawson (1932) and by many others since (Bonser et al., 1961; Higginson & McDonald, 1949).

Considerable doubt has since been cast on this theory. Many authors (e.g. Higginson & McDonald, 1949; Speert 1942; Pier et al., 1970; Bonser et al., 1961) have noted proliferation of 'pink' epithelium, including papillary formation. This is not characteristic of a degenerative cell. In 1971, Izuo et al. found by microdensitometry of breast tissue reacted by the Feulgen method, that 'pink' cells have a tetraploid nuclear DNA population. This they suggest is also not characteristic of degeneration but rather of proliferation. They used apocrine skin glands from vulvectomy specimens as controls and found they had a restricted diploid distribution curve of DNA.

The suggestion by Pier et al. (1970) that the 'pink' cells are secretory, and the work of Lendrum (1945) who suggested high cellular metabolic activity of 'pink' cells, tend to support the view that
degeneration is not occurring. The theory that deranged nutrition is the cause of 'pink' cell metaplasia was put forward by Bonser et al. (1961). They found that as the loose intralobular connective tissue disappears during involution, and is replaced by the denser interlobular type, the associated capillaries and lymphatics are also lost. Thus the access of the epithelium to blood-borne nutrients is decreased by the loss of capillaries and the barrier formed by the less-permeable connective tissue. However, this cannot be the sole cause since 'pink' cells are often found in otherwise normal lobules. Ahmed (1975) found prominent blood vessels in the stroma adjacent to 'pink' epithelium.

3.2.2. Hormonal variation

The possible involvement of hormonal abnormalities in 'pink' cell metaplasia is often quoted. The evidence is largely based on animal studies.

Fifer, in 1934, described cysts with hyperplastic and altered epithelium in the breasts of rabbits undergoing post-lactational involution, but not during functional activity. This would tend to support Dawson's theory of degeneration. However, he also found similar changes in the breasts of males and virgin females. McDonald (1936) reported areas of intracanalicular hyperplasia, some of which resembled 'pink' epithelium, in the mammary glands of castrated rabbits receiving 25 to 200 rat units of oestrogenic hormones daily. In 1937, Herold and Effkeman injected castrated male and female rabbits for periods up to three months with oestrogen in amounts from 150 to 1,000 international units daily. They found similar changes occurring in mammary cysts, but these were rarely produced in similarly treated non-castrated females. Speert (1942) reports that Hartman, Geschickter, and Speert (1941) found a similar result in the mammary glands of rhesus monkeys. The animals were injected weekly with oestrone pellets,
resulting in mammary development. However, only in castrated animals (they used females only) were cells similar to 'pink' cells found, with one exception in the non-castrates. No such change was seen in any of 304 other monkeys, both male and female, under various physiological and experimental conditions. They therefore suggest that the unopposed action of oestrogen (i.e. in castrates), in large quantities, is required for production of 'pink'-type epithelium in animals.

The dangers of applying the results of animal experiments, particularly those using non-physiological amounts of hormones, to humans are many. This is especially true when studying an organ which is affected differently in its normal function by the same hormone in various animals (Cowie & Tindal, 1971). However, there is some slight evidence of a similar effect of oestrogen in human females (England, 1974), those with cystic disease having an elevated serum level of oestrogen, albeit slight. This does not necessarily imply that oestrogen is involved in the causing of 'pink' cell metaplasia. Fechner (1970) found no qualitative difference in the tissue from breasts of women receiving oral contraceptives and that of age-matched controls, and an insignificant increase of 'pink' cell metaplasia in women receiving oestrogen therapy compared with controls (Fechner, 1972).

3.3 incidence

The criticisms of the estimation of cystic disease incidence also apply to 'pink' cell occurrence, with the added difficulty that such cells are only visible microscopically whereas cysts can be seen with the naked eye. Therefore the observations recorded in the literature may well underestimate the frequency with which 'pink' epithelium occurs. Higginson and McDonald (1949) considered this
epithelium to be a normal, rather than abnormal, finding in the breast since the organ is not static but undergoes monthly cyclical changes. Indeed, they go so far as to say "It is our impression that pale epithelium could be found in every breast if diligently sought after.". This they later qualify by adding "...female breasts after the age of puberty.".

Other workers have been somewhat less outspoken. Dawson (1932) said that 'pink'-cell structures are to be expected in practically every pathologic and cystic condition of the breast, malignant or not. She found such structures in 116 of 120 breasts with malignant lesions, and in all of 48 cystic breasts. Pink epithelium was found in 73 (75%) of 97 unselected mastectomy specimens by Higginson and McDonald (1949), and was visible to the naked eye, as pinkish or bluish tissue often with cysts, in 75% of cases when it was present. They saw 'pink' cells in 76% of carcinomatous breasts, in 74% of benign ones, and always in those with chronic cystic mastitis. It must be pointed out that these studies are all of breasts which were abnormal enough to require mastectomy and are therefore a selected group with regard to the whole population.

However, both Berka (1912) and Prym (1928) have reported 'pink' epithelium in otherwise normal breasts, and its occurrence in the mammary glands of children was commented on by Kuru (1909).

4 HISTOCHEMISTRY

The morphological and histological staining similarity of 'pink' cells and those in cutaneous apocrine glands has been pointed out by many authors (e.g. Bonser et al., 1961; Speert, 1942; Higginson & McDonald, 1949). This similarity is also very noticeable at the
ultrastructural level. However, this is not full proof of identity; biochemical evidence is also needed.

Some enzymes have been examined in 'pink' cells and in skin apocrine glands, and a compilation of the results of several workers is in Table 1. All the results were given as visual estimation of the enzyme reaction products and are therefore rather subjective. However general trends can be followed.

Tremblay (1968) considered his finding of a similar distribution of four oxidative enzymes (NADH and NADPH diaphorases, succinate dehydrogenase, and cytochrome oxidase) added further support to the theory that the two cell types are identical. He found the enzyme levels to be different from those of normal breast tissue and eccrine sweat glands. The differences between workers for the activity of some enzymes may reflect variability in the methods used. For example, Tremblay (1968) found much higher levels of succinate dehydrogenase (bringing his results more into line with those of other workers) when using the method of Wattenberg and Leong (1960), which involves adding menadione to the incubation medium.

Harcourt-Webster and Truman (1969) only obtained results for mammary gland and therefore did not compare them with apocrine gland. However, they did suggest that the high levels of dehydrogenases which they found in 'pink' cells were not consistent with the view that they are degenerate. They felt that 'pink' cell characteristics were similar to those of Askanazy cells in the thyroid and oxyphil cells in the parathyroid, and that they arose by metaplasia. Ahmed (1975) obtained similar results to other workers for the enzymes he studied, and supported the view that 'pink' cells arise by metaplasia and show a similarity to skin apocrine gland cells.
Table 1. SOME ENZYMES DEMONSTRATED HISTOCHEMICALLY IN "PINK" CELLS AND APOCRINE SKIN GLAND SECRETORY CELLS

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>&quot;PINK&quot; CELLS</th>
<th>APOCRINE GLAND CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase</td>
<td>low(^2), strong(^1) 3</td>
<td>low(^1), moderate(^5)</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>weak - moderate(^2) 2</td>
<td>weak(^2), strong(^5)</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>-</td>
<td>strong(^5)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>strong (apical)(^3)</td>
<td>moderate (apical)(^5)</td>
</tr>
<tr>
<td>Aminopeptidase</td>
<td></td>
<td>strong(^5)</td>
</tr>
<tr>
<td>Non-specific esterase</td>
<td>-</td>
<td>strong - intense(^5)</td>
</tr>
<tr>
<td>Lipase</td>
<td>-</td>
<td>strong(^5)</td>
</tr>
<tr>
<td>ß-Glucuronidase</td>
<td>-</td>
<td>strong(^5)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>low(^2), strong(^1) 3*</td>
<td>low(^2)</td>
</tr>
<tr>
<td>Phosphohexose isomerase</td>
<td>high(^6)</td>
<td>-</td>
</tr>
<tr>
<td>α-Glycerophosphate dehydrogenase</td>
<td>moderate - strong(^1) 1*</td>
<td>-</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>strong(^1) 4*</td>
<td>-</td>
</tr>
<tr>
<td>ATPase</td>
<td>-</td>
<td>intense(^5)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>absent(^3)</td>
<td>weak (apical)(^5)</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>moderate - strong(^1) 1*</td>
<td>-</td>
</tr>
<tr>
<td>Amylophosphorylase</td>
<td></td>
<td>absent(^5)</td>
</tr>
<tr>
<td>NADH diaphorase</td>
<td>high(^2), intense(^3)</td>
<td>high(^2)</td>
</tr>
</tbody>
</table>

\(^1\)Harcourt-Webster and Truman (1969), \(^2\)Tremblay (1968), \(^3\)Ahmed (1975),
\(^4\)Cohen (1964), \(^5\)Montagna (1974) quoting various authors, \(^6\)Muir et al. (1965)

*enzyme activity reduced in "pink" cells lining cysts (Harcourt-Webster & Truman, 1969)
One finding seems to be consistent when noted. Activities of enzymes are mostly higher in the more proliferative of 'pink' epithelia. The general conclusion is that the results in Table 1 do not disprove the hypothesis that 'pink' and apocrine gland cells are similar.

Of relevance here is the work of Hilf et al., (1970) who measured RNA, DNA, cholesterol, free fatty acids, triglycerides, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, phosphoglucomutase, glutamate dehydrogenase, and hexokinase in homogenates of carcinoma, cystic and normal tissue from human breast. They found only phosphoglucomutase was higher in cystic compared with normal tissue. This would seem to contradict the results of Cohen (1964) who found increased glucose-6-phosphate dehydrogenase in cells from cysts compared with those from normal breast tissue. However, Hilf et al., expressed their results in terms of activity/mg DNA, but Izuo et al. (1971), as previously mentioned, found that 'pink' cells had a tetraploid population of DNA distribution (and thus could have a higher tissue concentration of DNA), whereas true apocrine cells had a restricted diploid population. Unfortunately they did not also measure the DNA of normal epithelium. Also Nass et al. (1965) have claimed that apocrine cells have numerous mitochondria containing DNA.

Thus there may be an apparent lowering of DNA-based enzyme activities in 'pink' cells since they have more DNA than normal breast epithelium. The validity of the results is also questionable because of the difficulty of obtaining relatively homogenous cell samples from breast tissue, which has large amounts of stroma and, in cystic areas, relatively close proximity of different cell types (normal and 'pink').
These glands are found in the skin of many mammals, though they are often somewhat different from those found in man. For example, in the horse they are all over the body and produce a clear sweat as well as a turbid fluid. They are thus thought to be an intermediate between human apocrine and eccrine glands (Hurley & Shelley, 1960).

In man, the apocrine glands are mostly localised in the axilla, pubis, perianal region, and the external auditory meatus, although they are sometimes found on the rest of the body associated with hair follicles. Their fine structure probably varies in the different areas. (Ellis, 1967).

The name apocrine derives from the assumed mode of secretion of the glands, involving the loss of the apical tip of the cell containing secretion products which have gathered there. This contrasts with merocrine (e.g. eccrine sweat glands), where secretion diffuses through the plasma membrane, and holocrine (e.g. sebaceous gland) where the entire cell is secreted.

The glands only develop fully and functionally at, or just before, sexual maturity (Montagna, 1959), and in the female may exhibit changes through the menstrual cycle. However, such changes are hard to see, especially since there are often marked histological differences in adjacent glands in the same patient which mask any minor variations (Hurley & Shelley, 1960). Weiner and Hellman (1960) regarded the glands as accessory sexual organs.

Apocrine glands are often incorrectly referred to as sweat glands. In fact they have nothing to do with sweating in man. This is the function of a different gland, the eccrine type, which produces the characteristic clear watery secretion.
4.1 **EMBRYOLOGY**

The skin apocrine gland develops from the primary epithelial germ, as do sebaceous glands and hair follicles (Hurley & Shelley, 1960). It grows downwards from the upper end of the hair follicle and eventually forms a coil which develops a lumen. By seven months of embryological life, the glandular and ductular parts are differentiated. At birth, only the ear canal (ceruminous) glands are functional, suggesting they are under different endocrine control from other skin apocrine glands. These glands do not become functional until puberty.

4.2 **MORPHOLOGY**

The skin apocrine gland is a simple tubular gland with shunts and diverticuli (Fig. 4). A single duct proceeds from a coiled secretory element and empties usually, but not always, into a hair follicle. The secretory coil is not a simple coiled tube but has small pouch-like projections and is bridged by small shunts. It lies in the deep dermis and subcutaneous tissue and has an average diameter of 1 mm.

4.3 **CELLS**

4.3.1 **Duct**

The epithelium of the duct is a double layer of cuboidal cells. The luminal layers have a poorly developed cuticular border of meshed tonofilaments containing some mitochondria. The basal cells have few mitochondria, unlike eccrine sweat ducts, and do not appear highly specialised, suggesting that apocrine secretion is not modified in the duct as is eccrine sweat (Munger, 1965).

4.3.2 **Secretory cells.**

The secretory coil is lined with a single layer of cuboidal
Fig. 4. Human skin, showing apocrine and sebaceous glands and a hair follicle
or columnar cells, the shape depending on the secretory activity of
the cell (Montes et al., 1960; Montagna, 1962). Between these cells
and the thick hyaline basement membrane are myoepithelial cells. The
coil is surrounded by loose connective tissue which is well supplied with
blood vessels. The diameter of the coil, however, is larger than that
of the duct, and their transition is abrupt.

The secretory cells are eosinophilic, and have a round basal
nucleus with prominent nucleolus. The apical cytoplasm often appears
to be "pinched off", suggesting apocrine secretion. In fact this
appearance is due to the tip of adjacent cells, as shown by serial
sectioning. Under high power the luminal border shows a "fringe" of
small vacuoles. These are also visible in the electron microscope, and
may represent secretory material (Munger, 1965).

The cells contain variable numbers of pigmented and colourless
granules. The pigmented granules are brownish yellow and histochemically
of the lipofuscin family (Hurley & Shelley, 1960). They are responsible
for the colour of the secretion, and are found in large numbers in
the cells of ceruminous glands. They sometimes give a positive
histochemical reaction for iron, but this is never found in the gland
lumen. Sudan black stain produces a stippling of the cytoplasm,
probably due to mitochondria. Many PAS-positive granules are also
found, mainly between the nucleus and the apical end of the cell.
They are not glycogen, but the reaction is abolished by prior incubation
with diastase.

ULTRASTRUCTURE OF APOCRINE GLAND CELLS

The secretory cells are either columnar or cuboidal in shape,
and both types may be found in the same gland. They have a highly
folded villous base, and the folds of adjacent cells intermesh. Between the myoepithelial cells they rest on the basement membrane, and thus allow exchange of metabolites. Biempica and Montes (1965) report a high activity of ATPase at this surface.

At the apical end, adjacent cells are joined by strong junctional complexes, causing the tip of the cell to bulge into the glandular lumen. This surface has abundant microvilli. There is no evidence of apocrine secretion, i.e. pinching off of the cell cap, and it has been suggested that the secretory mechanism is merocrine (Munger, 1965; Biempica & Montes, 1965).

The cytoplasm contains various organelles (Ellis, 1967) including mitochondria, rough and smooth endoplasmic reticulum, a large Golgi complex, fibrils and tubules, and small vesicles - some with dense inclusions. There are also three types of granule, and microvesicles (Ellis, 1967), as listed below.

4.4.1 Abnormal mitochondria

These are found above the nucleus; those below are of normal structure, generally closely surrounded by rough endoplasmic reticulum. Transitional stages are found.

The abnormal types are much larger with a granular matrix and sparse characteristic curved cristae. They stain with the PAS reaction and are identical with the non-pigmented granules seen by light microscopy.

4.4.2 Large dark granules

These are found mainly around the Golgi complex. In the early stages they are bounded by a single membrane. This membrane becomes less obvious, the later stages having a rough outline. The granules contain extremely dense variable-sized globules, fine dense grains of approximately 3 nm diameter, locules containing opaque substance and
surrounded by denser material, large or small pale areas, and a moderately dense ground substance. They appear to be the basophilic granules containing pigment, lipid and iron, mentioned earlier.

4.4.3 Microbodies

These are smaller, spherical vacuoles with a single membrane containing an eccentric dense mass in a matrix of light material. They may be the precursors of the large dark granules (Kurosumi, 1959).

4.4.4 Microvesicles

Small clear vesicular bodies are found throughout the cell, and abundantly around the Golgi complex and in the apical cap of the cytoplasm. Occasionally they contain fuzzy material and are generally around 50 nm in diameter. Kurosumi et al. (1959) did not regard them as secretory, but rather water-carrying vesicles, derived from pinocytotis at the basal infoldings of the plasma membrane. Hibbs (1962), however, concluded that they are the principal secretory vesicles of apocrine cells. This view has since been supported by other workers (Yasuda et al., 1962; Munger, 1965).

4.5. SECRETION AND EXCRETION BY SKIN APOCRINE GLANDS

The term excretion will be used here to denote the passage of material out of the glandular duct, secretion to denote either the cellular process or its product.

In their study of the axillary apocrine glands, Hurley and Shelley (1960) found that fluid was excreted in response to stress - particularly fear and apprehension - pain, manual expression by squeezing the skin and, occasionally, stroking the skin. The volume produced by each gland is very small - about 1 μl per stimulation - and no further response can be elicited during a subsequent 24 to
48 hours. This suggests that on stimulation the myoepithelial cells surrounding the gland contract and force out the contained material. During the refractory period more secretion is produced in readiness for excretion. Montagna (1974) denies this suggestion but offers no alternative explanation.

Little is known about the secretory process in apocrine glands (Montagna, 1974). It is not known which of the granules in the cells are secretory granules or even if any of them are secreted. The process has never been seen to occur in the electron microscopic observations of these cells. The mode of secretion is unknown, though many processes (apocrine, apocrine-merocrine, merocrine, holocrine) have been suggested. As Montagna says (1974), "The whole matter of the mechanism of apocrine secretion has been obfuscated by too many senseless arguments. When all the evidence is in, all the mechanisms of secretion from merocrine to holocrine seem to be present."

4.6. COMPOSITION OF APOCRINE GLAND SECRETION

The lack of knowledge about the mode of secretion extends to the composition of the secretion. This is almost certainly due to the difficulty of obtaining anything other than small amounts of such secretion.

However, Hurley and Shelley (1960) found the fluid to have a pH of between 5 and 6, and to contain protein (using the 2-nitroso β-naphthol test for tyrosine). They also demonstrated, by other spot tests, that carbohydrate (reducing sugars using silver nitrate) and ammonia (manganese nitrate, silver nitrate) were present. Although histochemical studies showed lipids to be present in the gland cells (Sudan black), they were unable to detect such compounds.
in the fluid using spot tests. They did find slight amounts of ferric iron in the secretion, using α, α'-dipyridyl, despite Montagna's (1961) assertion that it is not present histochemically outside the epithelium. Richter (1932) found cholesterol in the secretion, but Montagna (1974) felt this was debatable. In 1954, Rothman suggested the presence of fatty acids but only on the basis of the "caprylic odours" of the areas of skin rich in apocrine glands.

The secretion is typically turbid and may be of a thick creamy consistency, especially in negroes (Hurley & Shelley, 1960). It is usually colourless, but may be pale yellow or, occasionally, even blue, green, or black (Hurley & Shelley, 1960). The ceruminous glands, however, excrete a strongly pigmented secretion (usually yellow to dark orange).

An undoubtedly important feature of the glands is the odour produced by their secretion, especially in the axilla. Hurley and Shelley (1960) found this to be due to bacterial decomposition of the excreted material on the skin surface. They detected no odour in the sterile product. The reasons for, or uses of, this odour production are largely conjectural, but the possibility of its being part of a signalling or pheromone system is not to be dismissed lightly. This view is supported by the release of apocrine secretion under emotional stress.
5 DIRECTION OF RESEARCH

5.1. OBJECTIVES

The problem to be investigated was by what mechanism do cysts arise in the human female breast around the time of the menopause, and what is the nature of 'pink' epithelium in relation to these cysts. It seemed obvious that one way of tackling the problem was to examine the fluid aspirated from breast cysts and to see if its chemical and cellular components revealed any clues as to its origin. For this study, the hypothesis was set up that breast cyst fluid represented, at least partially, a secretion by the 'pink' cells reported to line such cysts. At the time there were no chemical studies of BCF reported in the literature, and the cytopathology books were mainly concerned with the detection of malignancy, and therefore rather offhand about breast cysts.

5.2. MEANS

In our laboratory we had been electrophoresing, on cellulose acetate, serous fluids sent for cytological examination, and thus studying their protein composition. It became apparent that BCF had a completely atypical pattern compared with the other fluids, particularly a lack of \( \gamma \)-globulin, and this stimulated the interest in cystic disease.

The line of research was therefore initially to concentrate on an investigation of the protein content of BCF, and to see how this compared with the composition of milk and plasma. This comparison might give some clue as to the origin of the proteins, and would also be made for other chemical components of the fluid.

The absence of \( \gamma \)-globulin would also be studied and the various
immunoglobulin classes estimated. Again, the levels found would be estimated. Compared with those of milk and plasma, and an attempt made to explain their apparent lack as well as the absence of other serum proteins. An effort was to be made to see whether either secretory IgA or 'secretor piece' was present since this would be supportive evidence for secretion.

It was also intended to raise an antiserum to BCF. This would be useful not only for studies of the protein content, but also to see whether any BCF proteins were localised in cells, particularly 'pink' cells, of the mammary tissue using the technique of immunofluorescence. For this purpose histological as well as cytological material would be needed.

The cells found in breast cyst aspirates would be carefully examined to obtain information on the normal content. This would be correlated with other parameters to see whether any relationships could be found.

In order to undertake these exercises it would be necessary to ensure a high quality of sample material (samples sent for cytology can take a day to arrive). It was therefore considered desirable to attend at least one breast clinic in order to receive the fluids as they were aspirated. This would give the added bonus of allowing a complete patient history to be taken with perhaps some information not normally requested, if the consultant was willing. This bank of data might prove useful and would give some insight into the clinical aspects of cystic disease of the breast.
1 SPECIMEN COLLECTION

1.1 BREAST CYST FLUID

Samples of breast cyst fluid (BCF) were obtained from hospitals in both the St. Helier and the Croydon Groups. They are sent to the laboratory for cytological examination. Over 180 samples were also collected from the Breast Clinic at Mayday Hospital (Croydon). This ensured fresh specimens (others can take over 24 hours to reach the laboratory) and a detailed record of the patient's history.

Any case of a lump in the breast suspected of being a cyst was treated by the consultant surgeon as follows. The adjacent skin was cleaned by swabbing with surgical spirit. Whilst immobilising the lump with the left hand, a needle fitted to a 20 ml hypodermic syringe was pushed into the lump. The syringe plunger was slowly withdrawn, the left hand still holding the lump. The aspiration was continued until no more fluid was obtained and the lump had disappeared. This sometimes required the syringe to be changed during the procedure. If the lump did not completely disappear this was regarded - after checking there was no closely adjacent, but unconnected, cyst - as suspicious. The fluid obtained was placed into sterile Universal bottles for transport to the laboratory. A 10 ml sample of blood was also taken and placed into a sterile Universal bottle. Details of the patient's history were taken and recorded on the form shown in Fig. 5.

On returning to the laboratory, the colour and volume of each BCF was recorded on a form (shown in Fig. 6), and the fluids centrifuged at 1,000 x g for 5 minutes to sediment cells and other matter. The supernatant was poured off the deposit, from which slides were prepared for cytological examination. The fluid was placed into 4 plastic tubes,
**PATIENT INFORMATION**

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**CLINICAL INFORMATION**

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Fig. 5 Form used for recording details of patient history.
**LABORATORY RESULTS**

**BLOOD GROUP:**

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**HAEMOGLOBIN:**

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<td>IONS etc.</td>
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**IONS etc., No:** | Cl: | Urea: |
**K:** | CO2: | Fe: |

**OTHERS:**

| Glucose: |

**CYST FLUID**

<table>
<thead>
<tr>
<th>VOLUME ASPIRATED:</th>
<th>m1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLOUR: Light</td>
<td>Green</td>
</tr>
<tr>
<td>Medium</td>
<td>Brown</td>
</tr>
<tr>
<td>Pale</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

**BLOODSTAINED:**

| Due to aspiration? |

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**Fig. 6**  Form used for recording details of BCF samples.
with 1 ml in each, and any remainder stored in bulk. The tubes were kept in a deep freeze at -20°C. The time from collection to freezing was never more than 3 hours. For investigation of the particulate fraction the specimens were not frozen but stored at 4°C and used rapidly.

1.2 SERUM

The clotted blood samples were centrifuged at 2,000 x g for 10 minutes and the serum carefully removed with a Pasteur pipette and divided equally amongst four plastic tubes. It was stored at -20°C.

From patients who volunteered to provide urine specimens, a 20 ml sample of blood was taken. The serum obtained from this was sent for steroid estimations, except for 2 ml which was retained and stored as usual.

1.3 URINE

Twenty-four hour urine specimens were obtained from breast cyst patients and controls for the estimation of steroids. Patients were asked if they would cooperate and the procedure, and the reason for collecting specimens, explained. None refused to help, and only one defaulted. They were given an instruction sheet (Fig. 7) and a Winchester bottle, together with four standard forms for Chemical Pathology at Mayday Hospital. The samples were collected on a day of their choosing (usually Sunday/Monday) and the bottle returned within six hours to Mayday Hospital, together with one of the forms on which they noted details of menstruation and the date of specimen collection. Another three samples were taken, at weekly intervals, in order to cover one menstrual cycle.
INSTRUCTIONS FOR COLLECTING 24 HOUR URINE SPECIMENS.

All the urine you pass during the 24 hour period must be collected in the bottle provided.

A convenient way of doing this is as follows:-

When you get up in the morning go to the toilet as usual. Then for the rest of that day and the following night, collect all the urine you pass into the bottle provided. Next morning get up at the same time and collect all the first specimen of urine into the bottle. This completes the collection and the bottle and contents should be taken during the morning to:

Fig. 7 Instruction sheet issued to patients who volunteered to provide 24-hour urine specimens.
At the Chemical Pathology laboratory each specimen was mixed well, the total volume noted on the form, and a 100 ml aliquot stored in the deep freeze, the remainder being discarded. This aliquot was stored frozen and later sent for estimation of steroids.

1.4 TISSUE SAMPLES

Breast tissue was obtained from biopsy or mastectomy specimens. The whole specimen was transported, unfixed, from the operating theatre to the Histopathology Department, immediately after its removal. Any cystic areas were excised, and either frozen with liquid carbon dioxide or else fixed in formalin or buffered glutaraldehyde (for light and electron microscopy, respectively).

The formalinised tissue, in sizes up to $1 \times 1 \times 0.5$ cm, was fixed for 24 hours, and then dehydrated and impregnated with wax in a Histokinette automatic processor. After embedding in wax, the blocks were trimmed and sections cut, usually 5 μm thick.

For electron microscopy the tissue was cut into pieces 1 mm square in buffered glutaraldehyde, transferred to fresh fixative for one hour, washed twice in buffer for 30 minutes, post-fixed in 1% osmium tetroxide for one hour and finally washed as before. Following dehydration the tissue was embedded in araldite.

Blocks or sections of breast and other tissues were also obtained retrospectively from the Histopathology Department, if unavailable in fresh form.

For frozen sections the tissue was cut into pieces about 0.5 cm square and up to 0.5 cm thick. They were placed on a small amount of embedding compound (O.C.T., by Lab-Tek Products, obtained from Raymond A. Lamb, London) on a microtome chuck, and then
surrounded with the same material. After fitting into a freezing apparatus, the inside of the metal chuck was blasted with liquid carbon dioxide, initially in short bursts, until the specimen was frozen. The chuck was then placed in a cryostat microtome and sections cut, 5 μm thick. These were picked up on cleaned glass microscope slides.
The methods of electrophoresis used were cellulose acetate, polyacrylamide gel and polyacrylamide gel incorporating sodium dodecyl sulphate (SDS gel).

2.1 CELLULOSE ACETATE

Strips of Cellogel (Chemetron, Milan, obtained from Whatman Ltd.) 15 cm by 6 cm were soaked in 0.04 M sodium barbitone buffer, pH 8.6, for at least ten minutes. The Cellogel was placed so as to allow electrophoresis along the short axis, on wicks of Whatman 3MM paper soaked in the barbitone buffer, in a Shandon electrophoresis tank modified to take the short strips. A further layer of buffer-soaked 3MM paper was laid on top of the wicks, thus holding the strip securely. Samples were applied to the cathodic side of the centre of the strip, using a Cellogel applicator, with one application (1.5 μl) for serum, two (3 μl) for B.C.F. The electrophoresis with 0.04 M sodium barbitone buffer was run at 100 V for about 35 minutes, progress being monitored by adding bromophenol blue, which binds to albumin, to the serum sample on each strip.

The strip was then carefully removed, using forceps, and floated on the staining solution for ten minutes, agitating intermittently. For demonstrating proteins the stain used was 0.5% Amido Black in a mixture of methanol, water and glacial acetic acid (9:9:2). The excess stain was removed by passing the strip through successive baths of a mixture of methanol, water and glacial acetic acid (19:19:1) until the background was clear.

Following destaining the strips were either cleared or whitened. Clearing was achieved by rinsing in fresh methanol for 30 seconds,
placing in the clearing solution (a mixture of methanol, glacial acetic acid and glycerol, 85:14:1) for one minute, then laying face down onto a clean glass sheet and, after removing excess solution, heating at 70°C for 5 minutes. After cooling, the transparent strip was peeled from the glass plate. Whitening was performed by fixing the strips in 40% aqueous formaldehyde for one minute, immersing in 7% aqueous glycerol for three minutes and heating face up on a glass plate at 80°C for about five minutes.

2.1.1. PAS staining

The periodic acid-Schiff technique was used to demonstrate carbohydrate material. For cellulose acetate strips the method used was largely that of Smith (1968).

Following electrophoresis, the proteins were fixed by immersing the strip in a mixture of methanol, water and glacial acetic acid (9:9:2) for ten minutes. After oxidation for ten minutes in 0.02M sodium acetate containing 0.5% periodic acid, the strip was rinsed in 10 mM hydrochloric acid and placed in a bath containing 10% potassium iodide in 2.5 mM hydrochloric acid, freshly prepared. The resultant brown coloration was just removed by adding saturated sodium thiosulphate solution, and the strip agitated in the bath until it was colourless. After rinsing again in 10 mM HCl, the strip was placed into Schiff's reagent for 30 minutes and then washed in three changes of 0.05 M nitric acid, fifteen minutes each.

2.2 ACRYLAMIDE GEL

A variety of systems was used with this technique, in both disc and slab form, in order to attempt better resolution of BCF proteins than was obtained by cellulose acetate electrophoresis.
2.2.1 Disc

Initially the method of Davis (1964) was used, but this was found to be very long and tedious, requiring three different gels of different constitution. Therefore the method of Clarke (1964) was adopted, which he claims to suffer little loss of resolution compared with that of Davis, and is certainly much faster and easier.

Gels were prepared from the following solutions:

(a) Acrylamide 30 g; N,N'-methylene bisacrylamide, 1.0 g; water 123 ml.

(b) N,N,N',N'- tetramethylenediamine (TEMED) 0.28 ml; water to 100 ml.

(c) Glycine, 29 g; tris-hydroxymethylamino methane (TRIS), 6.0 g; water, 980 ml.

(d) Ammonium persulphate, 0.14 g; water to 100 ml.

(e) Glycine, 29 g; TRIS, 6.0 g; 1 M hydrochloric acid, 5.0 ml; water 975 ml.

Gels were cast in precision-bore, glass tubes of internal diameter 5 mm, fitted at the base with a plastic cap, and marked with a scratch 6 cm from the base. A mixture consisting of 6 ml(a), 3 ml(b), 3 ml(c) was prepared in a vacuum flask which was then stoppered, after adding a few glass granules, and connected to a water pump and de-aerated for 2 minutes. To this was added 12 ml freshly prepared solution (d), also de-aerated, and after mixing, pipetted into tubes up to the scratch mark. After filling all the tubes - usually 10 to 12 - the monomer solution was overlaid with water, using a hypodermic syringe barrel with a fine needle attached. The water was allowed to run down the side of the tube to avoid mixing into the monomer solution. The tubes were left for polymerisation.
to occur, and after one hour the overlayering water shaken out, the caps carefully removed and the best eight gels selected (by their regular length and flat surface) and the tubes fitted into the upper electrophoresis chamber. The lower chamber was filled with about 250 ml of a 1:10 dilution of solution (e), the electrode and upper tank holding the gels fitted, and the upper chamber filled with the same buffer.

Samples were prepared in 5% sucrose solution, containing 0.05% bromophenol blue, such that 0.1 ml would contain approximately 300 μg of protein. By means of a pipette 0.1 ml of the solution was carefully layered on top of the gel to form a layer between it and the buffer.

When all eight samples had been applied, the electrode was fitted to the upper chamber, and both electrodes connected to the power supply, with the lower tank as anode. Electrophoresis was continued with a current of about 30 mA until the bromophenol blue "tracking ring" had reached about 1 cm from the bottom of the gel. This usually took about 40 minutes.

The gels were removed from the tubes by "rimming out". An hypodermic needle, attached to a 20 ml syringe filled with water, was gently slid between the gel and the tube. By slowly forcing water from the syringe, whilst rotating the tube and pushing the needle further in, the adhesion of gel to tube was broken and the gel slipped out. The gels were then placed into large test-tubes and 20 ml of stain added. Various stains were used. Initially 0.1% amido black in 7% aqueous acetic acid was used. However, it was felt that since many BCF proteins are glycoproteins, a stronger fixative and more sensitive dye might be better. A solution of 0.1% Coomassie Blue
in 20% aqueous methanol containing 10% trichloracetic acid was therefore tried, with and without heating at 60°C. Both stains were applied for at least one hour.

Following staining the gels were destained electrophoretically after a preliminary rinse for one hour in two changes of 3% acetic acid (amido black), or 5% acetic acid in 10% methanol (Coomassie blue). The gels were placed into 7.5 mm bore glass tubes, the bottom ends of which contained about 1 cm of 7% polyacrylamide gel. To prevent convection during electrophoresis a viscous solution of 7% polyacrylamide without cross-linking agent (N,N'-methylenebisacrylamide) was added to just cover the gels. The tanks were filled with the destaining solution used for the preliminary rinse and a current of 300 mA applied until all the excess dye had migrated into the lower buffer-tank (anode). The gels were removed and stored in plastic, capped tubes in 3% acetic acid.

PAS staining was performed by Clarke's method (1967). The gels were immersed in 1% periodic acid in 3% acetic acid for one hour, and washed in several changes of water over a period of one hour. They were then placed in Schiff reagent for one hour, washed several times in 1% aqueous sodium metabisulphite, and stored in the same solution.

2.2.2 Flat bed

The apparatus for this was constructed according to a design of Akroyd (1968) and consisted basically of two flat sheets of glass 23 x 7.5 cm, onto each of the narrow ends of one being glued a spacer strip of 2 mm thick glass 7.5 x 1.0 cm. Thus with the plain glass sheet resting on the spacer strips a long, narrow glass cell is formed, open at top and bottom (i.e. long) edges.

The plates were cleaned carefully in detergent and a very thin
smear of grease placed on each spacer strip. The plain strip was placed on top and held in place using bulldog clips over the spacer edges. The bottom was sealed using a strip of autoclave tape, which was continued up each side of the cell. Monomer solution was pipetted into the cell, which was held vertical using a burette clamp and stand on a level surface, to 1 cm from the top. After overlaying with water, the gel was left to polymerise, and after 30 minutes the water shaken out and the autoclave tape removed.

Spacers consisting of 5 mm lengths of 2.5 mm O.D. silicon rubber tubing were inserted vertically into the top of the cell at about 8 mm intervals, and pushed down to touch the gel surface. The cell was placed into one of two identical buffer tanks, constructed of 6 mm Perspex, with internal dimensions 24 x 4.5 cm and 3 cm high. By adjusting the position of the bulldog clips attached to the sides of the cell so that they rested on the sides of the tank, the cell could be maintained level and above the floor of the tank. The top of the cell was then filled with distilled water, and samples, mixed with an equal volume of 10% sucrose containing a few drops of bromophenol blue solution, layered onto the gel in the compartments between the spacers. For BCF 50 µl was applied, for serum 10 µl.

Buffer was then added to the tanks, and the upper tank placed behind the cell so that the top of the buffer was about 1 cm above the top of the cell. A wick, consisting of three layers of 3 MM chromatography paper (20 x 10 cm) soaked in buffer and sandwiched between thin polythene sheets, was then placed into the top of the cell with the other end in the upper buffer tank. Platinum electrodes in the tanks were connected to the power supply and a current of 20 mA passed for 10 minutes until the proteins had entered the
gel. The wick was taken out and the spacers removed with a bent dissecting needle. The wick was replaced to 1 mm above the gel and electrophoresis recommenced at a current depending on the buffer system. On completion of electrophoresis the cell was removed, the bulldog clips taken off and the plain glass plate slid off. The slab was removed and stained as for gel rods. Destaining was performed by diffusion, washing the gel in several changes of destaining solution. If PAS staining was to be performed it was found useful to place duplicate samples in each half of the gel. After electrophoresis the gel could be cut and one half stained for proteins, the other by PAS.

A variety of gel and buffer systems were tried with this system, mostly those given by Akroyd (1968). These include Ornstein's discontinuous buffer (TRIS-HCl in the gel, TRIS-glycine in the tank), Poulik's discontinuous buffer (TRIS-citrate in the gel, borate in the tank), and a continuous TRIS-EDTA-borate buffer, as well as Clarke's (1964) system. These were generally used with 7.5% acrylamide gels, but a discontinuous gel was also tried. For the latter, gels 2 cm high were successively cast in the cell of 15%, 10% and finally 5% acrylamide, using Ornstein's discontinuous buffer system.

2.3 SDS GEL

This method was originally used to attempt better resolution of BCF proteins. The sodium dodecyl sulphate (sodium lauryl sulphate, SDS) prevents re-aggregation of protein molecules after chemically breaking any disulphide bonds which link them using 2-mercaptoethanol or dithiothreitol (Cleland's reagent). Initially
the disc method was used, but the flat gel technique was found to be better as it allowed easier comparison of results for different samples, and more samples per run. In each case the simplified method of Clarke (1964) was used for gel and buffer, but incorporating 0.2% SDS in both. It was also found to be necessary to incorporate SDS in the water overlay used in casting the gel to prevent it mixing with the monomer solution.

For preparing the samples a longer procedure was necessary. To 1 ml of a 10 mM Tris-HCl buffer pH 8.0, containing 1 mM EDTA, 1% SDS, 1% dithiothreitol and 10% glycerol was added 0.5 ml BCF. After incubating at 60°C for 15 minutes, 10 μl of the solution was layered onto the gel in the usual way.

After electrophoresis, the gels were stained in a 1% solution of Coomassie Blue, in 25% aqueous isopropanol containing 10% glacial acetic acid and 10% trichloroacetic acid, at 60°C for one hour. The gels were then destained in 10% trichloroacetic acid solution in 10% aqueous methanol.

2.4 MOLECULAR WEIGHT DETERMINATIONS BY SDS GEL

The SDS method was also used to determine molecular weights of the protein sub-units of BCF. The method used was basically that of Weber et al. (1972). The following solutions were prepared.

(a) Dialysis buffer: 0.01 M sodium phosphate (since potassium precipitates SDS) buffer, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol.

(b) Treatment buffer: 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% 2-mercaptoethanol.
(c) Gel buffer (pH 7.2): 8.82 g sodium dihydrogen orthophosphate dihydrate, 20.4 g di-sodium hydrogen orthophosphate, 2.0 g SDS, distilled water to 1 litre.

(d) Acrylamide solution: 22 g acrylamide, 0.6 g N,N'-methylene bisacrylamide, distilled water to 100 ml.

(e) Ammonium persulphate solution: 15 mg/ml freshly prepared.
(This was later changed to 10 mg/ml to increase gelling time).

Gels were prepared by mixing 15 ml gel buffer, 13.5 ml acrylamide solution and adding 45 μl TEMED. After deaerating, 1.5 ml ammonium persulphate solution was added and the solution pipetted into tubes with an internal diameter of 7.5 mm, and 8.5 cm long (Shandon destaining tubes) to a height of 7.0 cm. The monomer solution was carefully overlaid with 1% SDS in distilled water and the tubes left for 1 hour for the gel to form. This gave a 10% acrylamide gel. A 7.5% gel was also used and was prepared in the same way but using acrylamide solution (d) diluted 3:4 with water instead of full strength.

After gelation, the overlay was shaken from the tubes and their caps removed. The tubes were fitted into the Shandon apparatus, but using the destaining tank, as previously described. The tank buffer used was gel buffer (c) diluted 1 + 2 with distilled water. Samples were layered onto the gels and a current of 80 mA passed for 4½ hours by which time the bromophenol blue "tracking ring" was about 1 cm from the bottom of the gels. The gels were "rimmed out" of the tubes, as before, cut off at the centre of the "tracking ring", and placed in 20 ml stain each for 2 hours. The stain used was 0.25% PAGE Blue 83 (a dye similar to Coomassie Blue, from BDH) in a mixture of methanol, water and glacial acetic acid (5:5:1). The
gels were destained first overnight in the dye solvent used for staining, and then electrophoretically in a mixture of water, methanol and glacial acetic acid (35:3:2). It was found that although the gels were originally cast in the larger, destaining tubes (not the usual running tubes) they could still be fitted back into these tubes for destaining since they had shrunk somewhat due to partial dehydration in the stain. Following destaining the gels were stored in water in capped plastic tubes. With transillumination the distance migrated by each band was measured and calculated as mobility (percentage of total length of the gel, i.e. distance travelled by the bromophenol blue tracking ring) to allow for differences between gels.

Samples were prepared for electrophoresis by adding 0.1 ml of BCF to 1 ml treatment buffer (b) at 100°C and incubating at that temperature for 2 minutes. Gel loads were prepared by adding 300 µl of this solution to 4 drops of glycerol, 300 µl of dialysis buffer (a), 20 µl of 2-mercaptoethanol, and 20 µl of saturated aqueous bromophenol blue solution. After thoroughly mixing this solution 100 µl was applied to the gels.

The standards used were BDH molecular weight markers (a series of oligomers of protein cross-linked with diethylpyrocarbonate). These were prepared for electrophoresis by adding 1 ml of treatment buffer to one vial of marker (2 mg), heating at 100°C for 2 minutes and then continuing as for samples. A standard curve was prepared by plotting molecular weight against mobility on semi-log paper (molecular weight on the log scale). The molecular weight of the proteins in the bands of the sample gels was obtained from their mobility using this graph.

Initial trials of the method were conducted using two additional
sample preparation methods on the same specimens. Firstly heating to 100°C was omitted to see if enzymic proteolysis, which should be prevented by heating, was occurring. Secondly the period of heating was extended to 5 minutes to check whether non-enzymatic hydrolysis was occurring during the heat treatment. Also, since glycoproteins are reported to give variable results with this technique (Webber et al., 1972) depending on the gel porosity, identical samples were run on both 7.5% and 10% gels, together with standards.

Some gels were stained for carbohydrate using the PAS technique of Glossman and Neville (1971). Eight gels (four duplicates) were washed overnight suspended in 2 litres of a stirred solution of 40% aqueous methanol containing 7% acetic acid, and then washed in a fresh solution for a further eight hours. One of the duplicates was oxidised in 1% periodic acid in 7% acetic acid in the dark at room temperature for one hour, and all were then washed for 24 hours in 7% acetic acid, changing the solution twice. They were then incubated in Schiff's reagent for one hour in the dark at 4°C, and finally washed in four changes of 1% sodium metabisulphite acidified with a few drops of HCl and stored in the same solution in capped plastic tubes. The tests (treated with periodic acid) and controls (untreated) were compared.

2.5 ISO-ELECTRIC FOCUSING

This was performed in thin-layer polyacrylamide gel to attempt better resolution of BCF proteins.

A pH gradient is formed in the gel by ampholytes in an electric field, which also causes the proteins to migrate to their iso-electric point, where their nett charge is zero and they thus stop. The
apparatus used was the LKB 2117 Multiphor. It consists of a gel casting chamber, a tank with a lid containing two long platinum electrodes, a cooling plate, and a high-output power pack. The gel chamber is two thin glass plates, separated by a rubber gasket running around the periphery, clamped together by small bulldog clips. The gasket is cut at one corner to allow partial removal for introduction of monomer solution.

The gel was prepared from the following solutions.

(a) Acrylamide, 29.1 g; made to 100 ml with distilled water.
(b) N,N'-methylene bisacrylamide, 0.9 g; made to 100 ml with distilled water.
(c) Ammonium persulphate, 1.0 g; made up to 100 ml with distilled water. This was prepared fresh each time.

The glass plates were thoroughly washed using detergent, rinsed in distilled water and then ethanol (IMS) and allowed to dry. The gasket was then fitted and the plates clamped. In a vacuum flask were mixed 10 ml of (a), 10 ml of (b), 7.5 g of sucrose dissolved in 36.4 ml of water, and 2.8 ml of ampholytes (Ampholine, LKB) pH range 3.5 to 10. Following aspiration for two minutes to remove air from the solution, 0.8 ml of (c) was added and mixed and the solution carefully (to avoid bubbles) poured into the gel casting chamber. This was held vertical and with the filling point raised above the rest of the chamber. When the chamber was full the small end of gasket was replaced to seal the chamber, and the last clamp applied. After about one hour the clamps were removed and the chamber stored at 4°C for 15 minutes. This procedure allowed the glass plates to be separated more easily, leaving the gel on one plate. The gasket was peeled off, and the plate placed on the cooling surface.
of the tank, which was wetted to ensure good thermal contact. The cooling plate was connected to a tap, and cold water circulated at a rate which was indicated by the in-line flow meter as being sufficient. Twenty samples were applied, on strips of Whatman 3MM paper 5 x 10 mm. Each strip was wetted with 10 μl of sample and then laid on the gel midway between the electrodes. Two long, narrow strips of thick paper, which had been quickly dipped into electrode solution, were laid on the gel, one at each end, such that after fitting the tank lid the electrodes rested upon them. The electrode solutions were 1 M sodium hydroxide for the cathode and 1 M phosphoric acid for the anode.

After fitting the lid, and connecting the electrodes to the power pack, a voltage of 200 was passed for five minutes, and then increased in stages every ten minutes to a maximum of 1,000 V. After a total time of 2.5 hours the current was switched off, the tank lid and electrode strips removed, and the gel and plate immersed in the stain bath and placed in an incubator at 60°C. The staining solution, used for one gel only, was 0.4 g Coomassie Blue dissolved in 120 ml methanol and added to 250 ml water, followed, after mixing, by 12 g sulphosalicylic acid and 40 g trichloracetic acid. After staining the gel was removed from the bath, using the plate (from which it had now detached) as a support, and placed in the destaining solution, a mixture of water, IMS and glacial acetic acid (65:25:8). This solution was changed at hourly intervals until the gel background was as clear as possible. The gels were photographed, and then placed onto sheets of wetted 3MM paper and dried.
Attempts were made to separate BCF proteins using both gel-permeation and ion-exchange chromatographic techniques. The results were compared with those for milk and serum controls.

3.1 GEL-PERMEATION CHROMATOGRAPHY

This was performed principally on columns of Sephadex G-200 cross-linked dextran gel (Pharmacia Fine Chemicals). The method was also used for de-salting samples to be applied to ion-exchange columns, with Sephadex G-25.

The dried gel was swollen in the eluant to be used for chromatography. For protein separations 0.1M tris-HCl buffer, pH 8.0, containing 0.2M sodium chloride, was used. About 5 g of Sephadex G-200 were weighed into a 500 ml conical flask, 250 ml of buffer added and the flask gently shaken. The flask was then heated on a boiling water bath for 5 hours, occasionally mixing the contents by swirling. After cooling to room temperature, pouring into a 250 ml measuring cylinder and allowing the gel to settle, the volume of buffer above the gel was adjusted to about one-third of the gel volume.

The column was prepared in a glass tube, 2.5 cm in diameter and 45 cm long, fitted with adjustable end-pieces (Whatman). The tube was clamped vertically, with the lower end-piece fitted, and buffer injected from a syringe connected to the outlet tube, so as to cover the bed-support and exclude air. The outlet tube was clamped and the gel slurry gently stirred and then poured in, down a glass stirring-rod, to fill the glass tube. As the gel settled the upper end piece was slowly fitted and pushed down until the inlet tube was filled with buffer, then clamped. The inlet tube was connected to a four-way
valve and the outlet tube to a flow-cell (1 cm path length, 0.1 ml capacity, Hellma) in an SP500 spectrophotometer (Unicam) and thence to a fraction collector (Medical and Biological Instrumentation Ltd.) The layout is illustrated in Fig. 8. Eluant was supplied from a reservoir connected to a peristaltic pump (Watson-Marlow Ltd.) with its outlet running to the four-way valve.

The pump was started and the flow rate measured by collecting eluant for a timed period. The flow rate was gradually increased to that to be used in the experiment, usually about 40 ml/hour. The gel bed was allowed to pack under these conditions and then the end pieces moved to contact the bed and form a completely enclosed column. During this process the outlet tube was clamped and the four-way valve turned to allow the excess buffer from the column to run to waste.

The sample, 1 to 2 ml, was applied in one of two ways. The first simply involved removing the delivery tube from the eluant reservoir and pumping the required volume of sample onto the column. The second method, which avoided running the sample through a long delivery tube and thereby reduced "tailing", was to fit an hypodermic syringe barrel into the top position of the four-way valve, clamp the tube between the valve and the column and then turn the valve to connect syringe and waste. An excess of sample was poured into the syringe barrel and allowed to begin to run into the waste tube, whereupon the valve was switched back to connect syringe and column. This procedure avoided the admission of air into the tubing. The outlet tubing at the fraction collector was then raised to be level with the base of the syringe, and the clamp on the inlet tubing removed. The sample was thus allowed to run onto the column under a very low pressure-head. When the required volume had been applied
Fig. 8 Diagramatic layout of apparatus for column chromatography.
the valve was turned to reconnect the pump to the column, the outlet tube returned to the fraction collector, and the pump re-started.

The effluent from the column was collected in fractions on a volume basis, usually 100 drops. The absorbance of the effluent was measured at 280 nm and recorded using a chart recorder (Vitatron Ltd). The latter was also connected to the fraction collector to allow a reference scale of the fraction number also to be recorded on the chart.

Before running samples the packing of the column was checked, and the void volume estimated, by running 4 ml of a 0.3% solution of Blue Dextran (Pharmacia).

3.1.1 Analysis of fractions

Following completion of the run, the fractions constituting each peak on the absorbance chart were pooled and each pool was concentrated by ultrafiltration. This was performed in an Amicon cell fitted with a UM2 (molecular weight cut-off = 1000) membrane, until the volume of the retentate had been reduced to that of the original sample.

The concentrated pools were then tested by immunoelectrophoresis, and those from two samples were assayed for cholesterol. In one run the fractions were tested, before pooling and concentrating, for the presence of carbohydrate. This was done by adding to 1 ml of each fraction, 0.6 ml of 0.2% carbazole in concentrated sulphuric acid. Carbohydrate material gives a purplish colour.

3.1.2 Desalting for ion-exchange chromatography

The samples of BCF were initially desalted using gel chromatography on Sephadex G-25 fine grade, this grade having been shown to exclude the proteins of BCF. Six grams of dry gel was swollen in 50 ml of
the buffer to be used for the ion-exchange separation and heated on a boiling water-bath for one hour. The suspension was cooled to room temperature and the column prepared, as above, in the 1.5 cm diameter tube, and set up as in Fig. 8. The void volume of the column was estimated using Blue Dextran (Pharmacia), and the elution volume of salts found by running solutions of coloured salts, potassium dichromate and cupric sulphate. This was with a view to minimizing re-introduction of salts into the protein-containing run-off.

The column was then washed with three bed volumes of buffer and the sample, 1.0 ml of BCF, was applied to the column using a syringe, as before. The excluded protein was collected in one fraction, monitoring by means of the absorbance pattern of the effluent at 280 nm. This desalted fraction was then subjected to ion-exchange chromatography without prior concentration, its volume being about 5 ml.

3.2 ION-EXCHANGE CHROMATOGRAPHY

The resin used was QAE Sephadex A-25 (anion exchanger, Pharmacia) in 0.1 M tris-HCl buffer, pH 7.2. This type of ion-exchanger was used in preference to the cellulose type because it was found to be easier to pack and simpler to prepare. The prepared Sephadex bed was also less prone to compression during equilibration. A 1.5 x 20 cm tube was used to prepare the 12 cm column. To 300 ml of buffer was added 15 g of resin which was left to swell for one hour, stirring occasionally. The supernatant was poured off the settled gel and replaced by fresh buffer. The pH of the supernatant was measured before it was discarded. The washing was repeated at ten minute intervals until the resin had almost equilibrated with the buffer. This was shown by the pH of the supernatant becoming close to that of the fresh buffer.
The column was then prepared, as before, and set up as in Fig. 8. Buffer was pumped through the column at a rate of 15 ml/hour, until the pH of the effluent was 7.2 (i.e. the same as the buffer). The desalted sample was then applied by pumping onto the column.

The buffer reservoir was then changed for a gradient mixer. This was a "home-made" device, consisting of two 200 ml plastic beakers with a small plastic pipe cemented into the base of each and connected by a short length of silicon-rubber tubing. A paper clip was found to be satisfactory as a tube-clamp. An outlet tube was cemented into the base of one beaker and this was attached to the tube leading to the pump. This beaker was also fitted with a motor-driven stirrer.

To the stirred beaker was added 150 ml of 0.1M tris buffer pH 7.2, to the other 150 ml of the same buffer containing 1 M sodium chloride. The beakers were placed on a levelled surface, the clip removed from the connecting-tube, and the stirrer started. Thus a linear gradient from 0 to 1 M sodium chloride was produced as the eluant was pumped onto the column. The linearity of the gradient produced by the mixer was checked by using it to prepare a gradient of Bromophenol blue in the tris buffer. This was pumped directly into the flow-cell in the SP500 and the absorbance of the solution, at 580 nm, continuously monitored by recording on the chart recorder.

Fractions were collected at the rate of one every ten minutes, and the column washed through, after the gradient, with the buffered 1 M sodium chloride solution, until no more material absorbing at 280 nm was eluted. As before the fractions were pooled to correspond to peaks on the output absorbance chart, and concentrated by ultrafiltration.

The gel was removed from the tube for re-equilibration with
buffer before making another run. This is much quicker than washing
the column in situ, and also easier since the resin contracts considerably
during the run.
4 IMMUNOLOGICAL METHODS

4.1 PRODUCTION OF ANTISERUM

Antiserum to BCF proteins was raised in two rabbits. Two pools, each of 10 BCF, were prepared. The fluids for each pool were all of "Pure" type, and selected on the basis of the original volume aspirated. One pool was of fluids from cysts greater than 5 ml, the other cysts less than 5 ml. The pools were concentrated four-fold in an Amicon ultra filtration cell fitted with a UM2 membrane (Molecular weight cut off = 1,000).

The concentrated fluids were mixed with Freund's Complete Adjuvant (1 + 3) and injected subcutaneously into two sites, one pool to each rabbit. After one week the animals were re-injected with the same material.

Fortnightly test bleeds were made, the blood allowed to clot, spun down, and the serum removed and stored at -20°C. The titre of each bleed for each rabbit was estimated by rocket immunoelectrophoresis, and maximum titre was obtained 6 months after the original injection. The animals were given booster doses of antigen only, and bled out one month later when the titre had risen again.

4.1.1. Absorption of antiserum

The antiserum was found to contain antibodies to serum proteins and these were removed by absorption with freeze-dried plasma. To 1 ml of antiserum in each of 6 plastic tubes was added 10, 20, 50, 75, 100 and 200 mg of freeze-dried plasma. The tubes were capped, and the contents gently mixed and left at room temperature for 2 hours and then overnight at 4°C. They were then spun in a centrifuge at 2,000 x g for 20 minutes and the precipitates discarded. The lowest concentration of freeze-dried plasma to abolish all reaction of the antiserum with human serum
in immunodiffusion experiments was found to be 100 mg/ml. This was the concentration used to absorb the antiserum.

It was later discovered that a milk-protein antibody was also present in anti-(BCF) and this was absorbed out by adding an equal volume of a pool of five human skimmed-milk samples to the serum-absorbed anti-(BCF).

4.1.2 Purification of immunoglobulins from antiserum

In order to remove unwanted proteins from the absorbed antiserum, the immunoglobulins were precipitated by salting out. To 10 ml of antiserum was added 40 ml of water, and 12.5 g of ammonium sulphate. After mixing to dissolve the salt, the solution was left overnight at room temperature and then centrifuged at 4,000 x g for 30 minutes. The supernatant was discarded and the precipitate washed twice with 10 ml of 1.75 M ammonium sulphate, and then redissolved in 10 ml distilled water and transferred to an Amicon model 52 ultrafiltration cell fitted with an XM 100A membrane (molecular weight cut off = 100,000). The cell was connected to a reservoir which contained the dialysing fluid and, by maintaining a constant pressure in the system, a volume of the fluid was admitted equal to that of filtrate leaving the cell. Thus the effect is the same as dialysis but much faster.

The following solutions were passed through the cell, in each case until 50 ml of filtrate were collected: distilled water; 0.05 M sodium acetate, 0.021 M acetic acid, pH 5.0; distilled water; and the acetate buffer. The solution was then removed from the cell, centrifuged, and passed through a 14 cm high column of DEAE-sephadex A-50, equilibrated with the acetate buffer used for dialysis, in a 1.5 cm diameter chromatography column. The column was eluted with 25 ml of acetate buffer, the eluate collected and returned to the
washed ultrafiltration cell and dialysed, as before, by passing through 100 ml of 0.1 M sodium chloride containing 0.05% sodium azide. Finally the volume of the solution was reduced by ultrafiltration to 10 ml, and the resultant purified immunoglobulin solution stored at -20°C.

4.2. PREPARATION OF AGAROSE GELS

Agarose gels were prepared by a standard technique for all immunological procedures requiring them. To 500 ml of distilled water, rapidly stirred in a conical flask by means of a magnetic stirrer, was added 10 g agarose (Meath). The agarose was dissolved by heating the flask in a boiling water bath, stirring occasionally. When the solution had cleared, 0.5 g sodium azide was added. After mixing, the solution was poured into clean, glass Universal bottles, in aliquots of about 20 ml. The bottles were capped, left to cool, and then stored at 4°C. To prepare gels, as many bottles of 2% agarose as were needed were heated in a boiling water bath until the agarose had melted. Meanwhile double-strength stock buffer was pipetted into glass universal, or test tubes for small volumes, using half the volume of the gel to be prepared. Using a hot pipette an equal volume of molten agarose was added, the container capped and inverted gently to mix the contents, and the solution poured carefully onto the glass plate. These were of various sizes and were cleaned before use in hot Decon, rinsed in distilled water and dried. Before pouring the gel they were placed on a levelling table (Shandon). For 10 x 10 cm plates 18 ml gel was used; for 8 x 8 cm, 12 ml; for 3.7 x 7.5 cm, 5 ml; and 2.6 x 7.5 cm (microscope slides), 3.5 ml.

When the gels had set and cooled they were placed in plastic
boxes lined with damp filter paper, and cooled to 4°C for one hour before cutting wells.

This preparation method had several advantages. Firstly, it was not necessary to store several different buffered agarose solutions. Secondly, a new buffer could be prepared and used quickly. Thirdly, a large bulk of agarose could be stored.

### 4.3 OUCHTERLONY IMMUNODIFFUSION

Samples of BCF, human milk, colostrum, and serum were examined by this procedure using various antisera. These were antisera to human IgG, IgA, IgM, whole serum proteins, albumin, milk, lactoferrin, and BCF.

For immunodiffusion, glass plates size 10 x 10 cm were most frequently used, with 1% agarose gel in 0.15 M phosphate buffer pH 7.6, or 0.05 M tris buffer pH 7.6 containing 0.8% NaCl. Wells were made using a cutter (Wellcome Reagents Ltd), connected to a water-operated vacuum pump in order to remove the unwanted gel. The holes produced were 2 mm in diameter. The usual pattern of wells was an hexagonal array around a central well.

Both test samples and antisera were put up, at least initially, in a series of dilutions to ensure that optimum concentrations of antigen and antibody were subsequently used. Generally, two-fold serial dilutions up to 1:16 were made. This procedure also helped in revealing minor components which would otherwise have been missed if only dilutions appropriate to major constituents had been used.

The plates were then placed in a damp cupboard at room temperature and examined periodically until full development was attained. This usually occurred within 48 hours, and the plates were never incubated
for more than 72 hours.

4.3.1 Washing and Staining

Excess unreacted protein was removed from the gel by one of two methods; washing or pressing. The washing method has two main disadvantages. It is very slow, and the gels are liable to detach from the plates during the process. The latter problem necessitates careful handling of the free-floating gel, and damage usually results. Although a dried coating of agarose on the plates used for preparing the gels ensures good adherence, it was not used here since it also makes difficult the cutting of clean wells and troughs.

The gels were washed in 0.8% saline, containing 0.1% sodium azide, for 3 days, changing the solution twice daily. They were then washed in distilled water for about two hours and dried under a hot-air blower after covering with a filter paper moistened with distilled water.

The pressing method is much faster than washing. The gels were flooded with distilled water to fill all the wells (to prevent their splitting) and a wet filter paper laid over the top ensuring no bubbles were trapped beneath. A pad, 1 cm thick, consisting of squares of absorbent paper slightly larger than the plate, was placed on top and weighted with a thick glass sheet. After 30 minutes the weight and pad were removed, the filter paper wetted and then carefully peeled off the now-flattened gel. The majority of the unreacted protein is thus removed in the absorbent paper pad. The gel was then washed in 0.8% saline for two hours, rinsed in distilled water for 15 minutes and re-pressed, as above, for 30 minutes. The gel, with the filter paper still attached, was then placed under a hot-air blower and dried.

After drying, the filter paper was wetted with distilled water
and peeled off the plate. The gels were then stained, either in the amido black as used for cellulose acetate strips, or else in a mixture of methanol, water and glacial acetic acid (9:9:2) containing 0.5% Coomassie Blue. For the latter method destaining was performed in a mixture of water, methanol and glacial acetic acid (9:5:2).

4.4 RADIAL IMMUNODIFFUSION

This technique was used for the estimation of immunoglobulins (IgG and IgA) in BCF. The agarose was prepared as previously described, using 0.15 M phosphate buffer pH 7.6 containing 0.05% sodium azide. The mixture, in universal bottles containing 12 ml aliquots, was equilibrated in a water bath at 47°C. To each bottle was added 0.2 ml of antiserum (Wellcome) to IgG or IgA, the gel carefully mixed and quickly poured into a 9 cm Petri dish on a levelling table. After cooling the gel, wells 3 mm in diameter were cut, using the vacuum-operated cutter, guided by a perspex template (Wellcome). The wells were examined and any which were not clean-sided and undamaged were marked and not used. The samples were applied, undiluted, using fine pipettes prepared by pulling out Pasteur pipettes. The wells were quickly filled, level to the brim. This method is as accurate as using a measured quantity of sample, and avoids splashing, and theoretically corrects for any variations in thickness of the gel. Standards were prepared from four dilutions of an assayed control serum (Behringwerke AG), and were put up in duplicate.

After replacing the lids on the Petri dishes, they were placed in a damp-cupboard at room temperature for approximately 50 hours to allow completion of reaction. They were then removed and the diameter of each precipitate ring estimated, using a caliper gauge, taking
the mean of two measurements at 90°. A standard curve was prepared by plotting the square of ring diameter for the standards against concentration. From this the values of the unknowns were derived by interpolation.

4.5 IMMUNOELECTROPHORESIS

This technique was used extensively for the examination of BCF proteins using antisera to whole human serum, human milk, immunoglobulins, lactoferrin, and BCF.

Two principal methods were used, macro and micro. The former was performed on 10 x 10 cm slides, the latter on microscope slides, 7.5 x 2.6 cm. For both methods, the agarose was prepared as before, using 0.04 M barbitone-sodium barbitone buffer pH 8.6. The macro-method was preferred for ease of examining the precipitation pattern, but the micro-version was more economical in consumption of antiserum.

For the macro-method, three wells, 3 mm in diameter, were cut as shown in Fig. 9(a). Samples were placed into the wells using calibrated capillary tubes; the volume being determined by pilot experiments and depending upon the antiserum. In general, 5 μl serum and 15 μl BCF were used. A serum control was usually used, and placed in the central well. The electrophoresis was performed either in a Shandon or an MBI tank. The latter, being larger, could accommodate four plates. The buffer used was the same as the gel buffer, and wicks were 2 layers of Whatman 3MM paper. These were moistened in buffer and laid on the ends of the plates, to cover about 7 mm, and a current of 75 mA per plate passed for about 2 hours. The plates were then removed from the tank and 2 mm wide slots for the antiserum cut as shown in Fig. 9(a). For this purpose a gel cutter designed for the micro-method (Shandon)
Fig. 9 Immunoelectrophoresis plates. Patterns of wells and slots used for the macro-method (a), and micro-method (b).
was used. The agarose gel was removed from the slots using a Pasteur pipette attached to a vacuum pump, and the slots filled with antiserum. The gels were placed in a damp-cupboard at room temperature and examined periodically until reaction was judged complete. This usually took 48 to 72 hours. Excess, unreacted protein was removed by washing or pressing (see above). The latter was found to be preferable, since if the gels were detached from the plates the slots made them very fragile. The gels were dried and stained as described previously for immunodiffusion.

For the micro-method, the technique was basically the same. The slides were loaded into a perspex tray (Shandon) and both wells and slots cut using a special cutter (Shandon). This apparatus allows either of the patterns in Fig. 9(b) to be used, the wells being 1 mm in diameter; the slots variable, but 1 mm was used. The agarose plug was removed to form the well which was then filled with sample using, generally, a 1:3 dilution of serum and undiluted BCF.

The tray was placed in a Shandon electrophoresis tank and wicks consisting of two layers of Whatmann 3MM paper laid so as to cover 4 mm of each end of the gels. Following electrophoresis, at 17 mA per slide for approximately 2 hours, the slide tray was removed from the tank and the agar gel removed from the slots (cut before electrophoresis). The slots were filled with antiserum, which was diluted up to 1:8 (depending on the type of antiserum) with gel buffer. Diffusion occurred more rapidly with the small slides and was complete in 48 hours. The gels were washed, or pressed, dried and stained as before.

4.6 CROSSED (LAURELL) IMMUNOELECTROPHORESIS

This technique, together with several modifications, was used
mainly with antiserum to BCF. One such modification was the placing of a gel strip containing serum proteins between the running gel and the antiserum gel. Thus, when the second-dimension electrophoresis was run, the large antigen excess of serum proteins prevented precipitation by any homologous antibodies in the antiserum. In effect, this is absorption in situ.

For this method 8 x 8 cm plates were usually used, but 3.7 x 7.5 cm were later used to conserve antiserum. The technique was similar for both sizes. The plates were poured in the usual way, using the same buffer as for immunoelectrophoresis (0.04 M barbitone, pH 8.6). Wells were then cut using the 3 mm vacuum-operated cutter, as shown in Fig. 10(a). After adding sample, the plates were placed in the Shandon or MBI tank, containing the same buffer as in the gels. After fitting wicks consisting of 2 layers of Whatman 3MM paper, a current of 60 mA per plate (large) or 20 mA per plate (small) was passed for about 2 hours. For BCF and milk 5 μl of sample were used (3 μl for small plates); for serum the same volume was used after diluting 1:2.5. When the sample was not serum, a well was often cut higher up the plate and filled with serum containing bromophenol blue. The dye binds to serum albumin, and thus allows the electrophoretic separation to be monitored during the run. On completion of the electrophoresis, the position attained by the bound dye was marked on the back of the plate in order to provide a reference point.

The gels were removed from the tank and cut as shown in Fig. 10(a), using an old microtome knife. The large portion of the gel was discarded (including the serum control, as above, if included) and the empty part of the plate wiped with a tissue. To 8.8 ml of gel,
Fig. 10 Patterns used for Crossed Immunoelectrophoresis plates, with (b) and without (a) antigen strips.
at 74°C was added 0.2 ml of antiserum and, after mixing, the solution poured onto the plate. For small plates 0.1 ml of antiserum in 3.9 ml of gel was used. Care was taken to ensure it did not run over the top of the gel containing the electrophoresed sample. When the gel had set the plate was returned to the tank and, after replacing the wicks, the second dimension run at 5 mA per plate (2 mA for small plates) for 16 hours, overnight. The plates were then removed from the tank and the gels washed, pressed, and stained, as before.

As mentioned above, some modifications were also used. The main one was incorporation of a gel strip containing excess of antigens, to remove homologous antibodies in the antiserum, which would otherwise interfere. This was accomplished after removing the unwanted gel following the first-dimension electrophoresis. A brass bar of 2 cm square cross-section and 12 cm long, with flat polished surfaces, was placed across the plate 1 cm (2.2 cm for small plates) from the cut edge of the remaining gel (dotted line Fig. 10(b)). To 1 ml of gel at 47°C, 0.5 ml of antigen solution (serum or milk), also at 47°C, was added, mixed, and poured onto the plate between the gel and the brass bar. When the gel had set, the bar was removed and the meniscus at the bar end cut off. For the small plates the end 1.2 cm of gel was cut off to leave an antigen-gel strip of 1 cm. After wiping the plate the antiserum-gel was poured on the remaining blank portion using 0.2 ml of antiserum in 7.3 ml of agarose gel for large plates, 0.1 ml in 3.2 ml of gel for small plates. The second dimension was then run as before.

Another modification used was to run two antigen samples on the same plate. For this purpose, another sample well was cut in front of the usual one (dotted in Fig. 10(a)). The extra antigen was
placed in this well, left to diffuse into the gel for about 5 minutes, and the now-empty well filled with agarose. This is to allow uninterrupted electrophoresis of the sample in the more cathodic well. The remainder of the procedure was completed in the usual way. By comparing two plates, identical in all respects except the extra antigen on one, the identity of components between the two may be established. The method was used here for comparison of BCF with milk. A variation, also used, is to cut a well below the line of, and subsequent to, the first dimension electrophoresis, in an appropriate position along the plate, and place the other antigen therein. Thus this antigen is only electrophoresed in the second dimension, and the technique is used to establish identity of a peak occurring in a known position. It was used here with purified lactoferrin as antigen.

4.7 REVERSED ROCKET IMMUNOELECTROPHORESIS

This technique was used for the estimation of antibody titre in antiserum to BCF. The method is that of Axelsen and Svendsen (1973). By using a buffer of pH 4.9 for electrophoresis, the BCF antigens have zero or slow cathodic mobility, whereas the antibodies in the antiserum have a high cathodic mobility. By electrophoresing antibody into a gel containing antigens, "rockets" are obtained with peak height proportional to titre.

The gels were prepared, as before, using 0.1 M sodium acetate-acetic acid buffer, pH 4.9. To 12 ml of gel at 47°C was added 0.1 ml of BCF (from a pool of equal volumes of 10 fluids) and after mixing poured onto an 8 x 8 cm plate. After cooling the gel, wells 2.5 mm in diameter were cut at 0.5 cm centres along a line 1 cm from the edge of the plate, and this was placed in the electrophoresis tank.
with the wells at the anode. Wicks of 3MM paper soaked in tank buffer (gel buffer diluted 1:2 with water) were fitted and 15 volts applied to the gel. The wells were filled with antiserum samples (diluted 1:5), and after one hour the voltage across the gel was measured and reset to 15, and electrophoresis continued overnight.

The gels were pressed, dried, and stained as before, and the peak heights measured.

4.8 IMMUNOHISTOCHEMISTRY

Two types of labelled antiserum were used for this technique: a fluorescein-isothiocyanate (FITC) conjugate and a peroxidase conjugate. The former was used initially, but the latter was found to have several distinct advantages. Firstly, the peroxidase technique permits of the use of formaldehyde-fixed, paraffin-wax-embedded tissue providing formaldehyde does not affect the antigenicity of the material being examined. Such tissue can also be used for immunofluorescence but extensive washing of the sections is required to remove interfering autofluorescence generated by the fixation. This is time-consuming and sections are often ruined during the process. Extensive washing is not required when using peroxidase conjugates.

Secondly, the staining with immunofluorescent techniques is not permanent, whereas the peroxidase reaction product is. Therefore, if the latter technique is used it is possible to review the results at a later date.

Thirdly, the use of a special microscope is not necessary with the peroxidase method. The slides are also much easier to look at, and clearer.

Fourthly, the easy use of sections from routine histological
blocks enables retrospective studies, especially of rare material, to be made by the peroxidase method.

It was found during this study that even frozen sections of breast tissue contained considerable amounts of autofluorescent material which often masked the specific staining reaction. This also weighted the decision in favour of the peroxidase method.

4.8.1 Immunofluorescence

The method was performed on both frozen sections and wax-embedded material. The techniques for both were essentially the same, but the wax sections required pre-washing.

Frozen sections were fixed in methanol at 4°C for two minutes. Wax sections were warmed to 60°C, placed in xylene for 10 minutes and intermittently agitated, and then rinsed in two successive baths of absolute alcohol and finally in water. The sections were then washed in very gently agitated PBS (phosphate-buffered saline; 0.2 M phosphate buffer, pH 7.2, containing 0.8% w/v sodium chloride and 0.05% sodium azide) for 4 days, changing the PBS twice daily.

The frozen sections were washed briefly in PBS, and the following operations were identical for both types of section. The double-layer, or "sandwich" method was generally used.

The slides, excluding the area covered by tissue, were wiped dry and placed in the lid of a plastic box lined with moistened filter paper (to prevent drying out). Appropriately diluted rabbit antiserum was placed onto the sections, and incubated with the box closed, at room temperature for 30 minutes. The slides were carefully rinsed with PBS and then washed in the same buffer for 30 minutes, changing once. After drying the slides and replacing them in the box, diluted (usually 1:32) FITC-conjugated antiserum to rabbit immunoglobulins (Wellcome) was
placed on the sections. After incubating for a further 30 minutes, the slides were rinsed and washed, as before, for 60 minutes. For the single-layer method, only one application of antiserum was made. This was a labelled antiserum against the appropriate tissue component.

The slides were mounted in a mixture of glycerol and PBS (9:1) and examined without delay in a fluorescence microscope, using a dark-field condenser, with FITC3, BG12 and BG3 excitation filters, and a K500 barrier filter. The light source was a mercury lamp.

The tissues used with this technique were fresh cystic and "normal" breast, and wax-embedded cystic breast. The antisera used were anti-(BCF), and anti-(human IgG) and anti-(human IgA) which were both FITC conjugates used by the single-layer technique. Controls, omitting the first antiserum, and also substituting non-immune rabbit serum, were run with each batch of tests.

4.8.2 Peroxidase

The method used was that of Burns (1975). Formalin-fixed, wax-embedded sections were used generally, but initially the results were compared with those using frozen sections post-fixed in methanol, and glutaraldehyde-fixed, wax-embedded material. The techniques were the same in all cases except that de-waxing was not employed for frozen sections.

The sections (5 \( \mu \text{m} \) thick) were warmed to 60°C and placed into xylene for 10 minutes. They were then washed twice in absolute alcohol for 5 minutes and transferred to 0.5% hydrogen peroxide in methanol for 15 minutes, to block endogenous peroxidase. The slides were then rinsed in tap water and washed for 15 minutes in TBS (Tris-buffered saline; 1 M Tris-HCl buffer, pH 7.6, diluted 1:10 with 0.8% saline). The area around the section was carefully dried and the
slides placed in the lid of a plastic box lined with moistened filter paper. Great care was taken to ensure that at no point did the sections dry out.

The sections were then covered with goat serum, diluted 1:5 with TBS, the box covered, and left for 10 minutes. The serum was drained off and the appropriately diluted (in TBS) rabbit antiserum added to the slides, which were left in the damp-box for a further 30 minutes. The sections were then rinsed in TBS, and washed in stirred TBS for 30 minutes, changing twice. The slides were then removed, wiped dry as before, and returned to the damp box. The sections were covered with a 1:25 dilution of peroxidase-conjugated, goat antiserum to rabbit immunoglobulins (Miles) and left for 30 minutes. They were then rinsed, and washed in stirred TBS for 30 minutes, changing twice.

The sections were then reacted to demonstrate peroxidase. The reagent used was 5 mg of 3,3'-diaminobenzidine tetrahydrochloride in 10 ml of TBS containing 5 μl of hydrogen peroxide, prepared freshly. Five drops were placed on each slide and left for exactly five minutes. They were then rinsed with TBS, washed in running tap water for 15 minutes and finally rinsed in distilled water. The sections were counterstained by immersing the slides in haematoxylin for about 15 seconds, rinsing in distilled water, differentiating in 1% acid alcohol and blueing in running tap water for 15 minutes. The sections were dehydrated by agitating in 3 sequential baths of alcohol (IMS) followed by 2 of xylene, and mounted in DPX.

Various tissue substrates were examined by this method, principally with anti-(BCF) absorbed with serum and milk. These were cystic, normal, and carcinomatous breast; vermiform appendix; skin from male axillae,
and female axillae, nose, breast, and vulva; papillomata; ceruminous adenoma; hidradenoma; apocrine gland cystadenoma; kidney; cervix; rectum; stomach; and colon. Many were from specimens obtained at surgical operation for carcinoma.

As mentioned, the antiserum generally used was anti-(BCF), diluted 1:250. However, others were used, including antisera to whole human serum, and human milk. The direct, one-step method was employed with peroxidase-conjugated antisera to human IgG and IgA (Mercia Brocades) on cystic breast tissue.

Controls were included in each batch of tests. The most frequently used negative control was anti-(BCF) absorbed with BCF. With runs using anti-(BCF), a positive control (cystic breast or axillary skin section) was included. Other controls used were as follows.

(a) Omission of primary antiserum in the two-stage technique, to ensure there was no non-specific binding of the conjugate.

(b) Omission of all antisera, to check for endogenous peroxidase in the section.

(c) Initial incubation with sheep anti-(whole human serum) before the serum-absorbed anti-(BCF), to ensure that no antibodies to serum proteins were remaining in the anti-(BCF).

(d) Blocking after the reaction with rabbit antiserum, by using sheep anti-(rabbit immunoglobulins), before incubation with peroxidase-conjugate, to check for non-specific binding.

4.8.3 Treatment of sections with trypsin

Experiments were made with the use of trypsin to determine whether additional sites of reaction with anti-(BCF) were revealed. After de-waxing and blocking endogenous peroxidase, the sections were placed
into a 0.1% solution of trypsin in 0.1% calcium chloride (adjusted to pH 7.8 with 0.1 M sodium hydroxide) at 37°C. Sections were removed after 5, 10, 15 and 30 minutes, rinsed, and placed for 5 minutes in a 0.1% solution of trypsin inhibitor. After washing in TBS for 15 minutes they were reacted by the peroxidase technique, as usual.
5 CHEMICAL ANALYSIS

5.1 TOTAL PROTEIN

A modification of the method of Lowry (1951) was used to estimate protein. Assays were performed in duplicate in clean glass tubes.

To 0.1 ml of sample, diluted so as to contain up to 100 μg of protein, was added 0.1 ml of water and 1.6 ml of 0.66 M sodium hydroxide solution. The tubes were incubated in a water-bath at 37°C for 30 minutes, to ensure dissolution of any solid proteins, and then cooled to room temperature. After cooling, 1.6 ml of buffer (8.4 g of sodium bicarbonate and 2.5 g of sodium hydroxide in 100 ml of water) was added from a repeating syringe (Zippette), to ensure thorough and rapid mixing, followed by 0.6 ml of copper tartrate solution (0.1% cuprous sulphate solution containing 0.2% sodium tartrate, freshly prepared), also from a Zippette. The use of such devices to ensure rapid mixing had been shown to be essential in pilot experiments whilst setting up the method.

The tubes were left for 15 minutes at room temperature and then 1.2 ml freshly-diluted Folin-Ciocalteu reagent was added, again forcefully. The Folin reagent was diluted with water to make it 1 N in acid. This dilution was determined by titrating each new batch of stock reagent against 1 M sodium hydroxide, with phenolphthalein as indicator, and was usually 1 volume reagent plus 2 of water.

After standing for 30 minutes, the absorbance of the solutions at 750 nm was read against a reagent blank prepared by substituting water for sample. Standards were prepared from bovine serum albumin, using 10, 20, 50, 75, and 100 μg per tube. The protein content of each sample was estimated from the standard curve.
5.2 CHOLESTEROL

The method of assay was a modification by Ness et al. (1964) of the Liebermann-Burchard reaction. This method is unsuitable for fractions from density gradients, since it was found that the reagent charred the sucrose used to prepare the gradient. The resultant carbon interfered with spectrophotometry. Therefore, an extraction procedure, essentially as used by Keenan et al. (1970), was adopted as an initial step for such samples. Free cholesterol was estimated after digitonin precipitation of the lipid extract.

The reagent was prepared by mixing 150 ml of glacial acetic acid and 300 ml of acetic anhydride in a 1 litre conical flask. The flask was placed in a sink full of cold water and the contents swirled to mix, whilst slowly adding 50 ml of concentrated sulphuric acid. After adding and dissolving, 10 g anhydrous sodium sulphate the solution was transferred to a glass bottle and stored at 4°C.

For the direct method, 0.2 ml of sample was pipetted into a clean glass tube, and 5 ml of ice-cold reagent added forcefully, to ensure rapid mixing. Samples containing high levels of cholesterol (e.g. BCF) were either diluted 1:2 with water, or only 0.1 ml was used. The tubes were incubated at 15°C to 16°C for exactly fifteen minutes. The absorbance of the solution was then read at 625 nm against a reagent blank prepared by substituting water for sample. Standards were prepared from assayed control sera and a solution of cholesterol (Biochemical standard, BDH) in chloroform.

For the extraction method, 0.1 or 0.2 ml of sample (as before) was pipetted into a clean glass tube having a ground neck into which a ground glass stopper could be fitted. Water was added to give a total volume of 0.5 ml, followed by 1.85 ml of a mixture of methanol
and chloroform (2:1). The tubes were stoppered, shaken intermittently for 1 hour, and then centrifuged to remove any precipitated protein. The supernatant was poured off into a fresh stoppered tube, 0.65 ml of chloroform and 0.6 ml of water added and the mixture shaken vigorously. The tubes were centrifuged to separate the two phases, and the lower chloroform layer removed to a glass test tube using a Pasteur pipette. The tubes were placed in a water bath at 30°C and evaporated to dryness under a stream of nitrogen. The residue was re-dissolved in 0.2 ml of chloroform and treated as above, adding 5 ml of reagent. Standards were prepared from a 1 mg/ml solution of cholesterol in chloroform, and the values for the samples estimated from the standard curve.

5.2.1 Free and esterified cholesterol

To estimate free and esterified cholesterol the samples were first extracted as above and the dried lipid extract re-dissolved in 1 ml of a mixture of acetone and 95% ethanol (1:1). To this was added 1 ml of a 1% solution of digitonin in 50% aqueous ethanol, the tubes mixed and left at room temperature for 10 minutes before centrifuging at 1500 x g for 5 minutes. The supernatant was discarded and the tubes left upside-down for 5 minutes to drain the precipitate, which was then washed by adding 4 ml of cold acetone, vortex mixing, and centrifuging as before. After draining the precipitate for 5 minutes, 0.2 ml of chloroform was added and cholesterol assayed as above. This gave the value for free cholesterol, and esterified cholesterol was found by subtracting this from the total value.

5.3 PHOSPHOLIPIDS

Total phospholipids in BCF were estimated by the method of Baumann (1924). This involves an extraction of lipid followed by
estimation of phosphate in the extract after digestion to release inorganic phosphate.

To a sample of 1 ml of BCF, in a 25 ml volumetric flask, was added about 15 ml of a mixture of ethanol (IMS) and diethyl ether (3:1). The flask was placed over a boiling water-bath until the solvent boiled, and after cooling, the level in this flask was made up to the mark with ethanol-ether. The flask was shaken and the contents filtered, retaining the extract filtrate.

Into pyrex tubes, 20 mm in diameter and calibrated at 12.5 ml, was pipetted 10 ml of filtrate; two glass beads were added and the tubes placed in a boiling water bath until the solvent had evaporated. Similar tubes were set up containing 1 ml of 30% sulphuric acid, as blank, and 0.5, 1.0, and 1.5 ml phosphate standard solution (containing 40 μg of phosphorous per ml, prepared from dried analytical grade potassium dihydrogen phosphate). A glass bead was added to each tube, and 1.0 ml of 30% sulphuric acid to the unknowns and the standards.

The digestion was performed by heating the tube over a Bunsen burner in a fume-cupboard until white fumes appeared. After cooling the tube for 30 seconds in a rack, 3 drops of 30% hydrogen peroxide were added so as to fall straight into the digest. The tube was then reheated, and digestion continued for two minutes after fumes appeared. The tube was again cooled for 30 seconds and, if the solution was not colourless, 3 more drops of peroxide added, and this process repeated until the solution was clear and colourless. The number of drops of peroxide used was noted and the maximum number used in any batch of assays was added to all tubes in the batch, including standards and blanks.

After digestion, all the tubes were cooled and made up to the
12.5 ml mark with water, and mixed. Duplicate 2 ml aliquots were then pipetted into clean glass tubes and assayed for phosphate. To each tube was added 1.5 ml of 0.6% ammonium molybdate solution in 5% PCA, followed by 0.5 ml of freshly prepared 0.5% ascorbic acid solution. The tubes were mixed and incubated at room temperature for 30 minutes, when the absorbances of the solutions at 720 nm were read against the blank. Phosphate levels for the unknowns were calculated from the standard graph, and from these the amount of phospholipid (as lipid phosphorous) in the samples was derived.

5.4 NUCLEIC ACIDS

Both DNA and RNA were estimated by the method of Schneider (1945), which involves a total extraction of both polynucleotides followed by differential colour reactions to estimate ribose and deoxyribose.

To 1 ml of BCF in a 15 ml stoppered centrifuge tube, maintained at 0°C in an ice-bath, was added 1 ml of water and 10 ml of 5% PCA, both ice-cold. The tubes were vortex-mixed and stood at 0°C for 3 minutes, and then spun in a refrigerated centrifuge at 0°C to produce an acid-insoluble precipitate. The supernatant was discarded and the pellet resuspended in 10 ml of ice-cold 2% PCA, using a glass rod, and then recentrifuged. The supernatant was again rejected and this washing procedure repeated twice more.

The washed pellet was then finely suspended in 3 ml of 5% PCA and extracted at 80°C for 20 minutes, mixing occasionally. After cooling and centrifuging, the resultant pellet was extracted, as before, with a further 3 ml of 5% PCA, and then centrifuged. The two supernatants were combined and used for estimation of DNA and RNA.
Standards were prepared from purified DNA and RNA. Both were obtained from Sigma; the DNA was Type I, the RNA was Type IV. About 6 mg of each was accurately weighed out, dissolved in 5 ml of 5% PCA and heated to 80°C for 20 minutes. To 1 ml of this stock solution was added 9 ml of 5% PCA to produce the working standard. A further 1 ml of stock solution was digested by heating with 0.5 ml of concentrated PCA to hydrolyse the nucleic acids. The phosphate released was then estimated, after diluting the solution to 10 ml, on 2 ml aliquots. This was done to allow estimation of the purity of the standards. The standards were carried through the same extraction procedure as the samples.

For DNA estimation, 1.2 ml of extract was pipetted into each of two clean glass tubes. To this was added 2.4 ml of 1.5% diphenylamine (analytical reagent grade, recrystallised from ethanol), freshly prepared in glacial acetic acid containing 1.5% concentrated sulphuric acid. The solutions were mixed and stored in the dark at room temperature for two days, and the absorbances read at 600 nm. Blanks were prepared, substituting 5% PCA for sample, and standards using from 0.1 to 1.0 ml (equivalent to approximately 10-100 µg of DNA) of the DNA working solution. The specificity of the procedure was checked by running a 100 µg RNA standard in parallel.

For RNA estimations 2.0 ml of extract was used, adding 2 ml of 0.5% orcinol (recrystallised twice from benzene) freshly prepared in concentrated hydrochloric acid containing 0.007% cupric chloride (dihydrate). After mixing, the tubes were heated in a boiling water-bath for 30 minutes, cooled, and the absorbance of the solutions read at 665 nm. Standards were prepared using 0.1 to 1.0 ml RNA working standard solution (equivalent to approximately 10 to 100 µg RNA), and
blanks substituting 5% PCA for sample. A 100 μg DNA standard was
run in parallel.

5.5 LACTOSE

The presence of lactose in BCF was tested for, using Fearon's
methylamine test (Henry, 1964).

To 0.2 to 1.0 ml of sample, made up to 5 ml with distilled water,
was added 1 ml of 0.2% aqueous methylamine hydrochloride solution,
followed by 0.2 ml of 10% aqueous sodium hydroxide solution. The
tubes were vortex-mixed, capped, and placed in a water-bath at 56°C
for 30 minutes. Standards were prepared from 1% lactose solution,
making each tube up to 5 ml with distilled water to give 0, 0.01, 0.05,
0.1, 0.3, 0.5 and 1% lactose solutions, and run as above. Positive
controls were prepared using 0.1 to 0.4 ml of milk.

The tubes were all examined for the characteristic red colour
produced by lactose, and the degree or absence of colour noted.
Tubes with no colour were allowed to stand for 30 minutes at room
temperature, and then re-examined.

5.6 URIC ACID

Uric acid in BCF was estimated using an enzyme method obtained in
kit form from BDH. The uric acid is converted by uricase to allantoin.
The decrease in absorbance of the solution at 293 nm is used to estimate
the amount of uric acid destroyed. The method was used mainly to
assay samples which had levels above the range covered by the AutoAnalyzer.

To 0.2 ml of sample (or less, made up to 0.2 ml with distilled
water, if high levels of uric acid were present) was added 2.8 ml of
glycine buffer containing uricase. A control was prepared for each
specimen in the same way, but using the same glycine buffer without uricase. A standard was prepared using 0.2 ml of standard uric acid solution (0.1 mg/100 ml) and 2.8 ml of buffered uricase, a standard control substituting buffer for uricase, and a uricase blank from 0.2 ml of water and 2.8 ml of buffered uricase. The tubes were all incubated at 37°C in a water-bath for 30 minutes, and then the protein precipitated by adding 1.0 ml of 15% aqueous TCA solution.

The tubes were placed in a boiling water bath for 60 seconds, removed and cooled in cold tap-water, and centrifuged for 15 minutes. The supernatant was poured off and its absorbance measured at 293 nm, in a 1 cm cuvette, reading the samples and standard against the uricase blank, the sample controls and standard control against distilled water. The absorbance of the samples was subtracted from that of the sample controls, and the standard from the standard control and the ratio of the two used to calculate the uric acid concentration.

5.7 AUTOANALYZER

A series of fresh BCFs were run through the Technicon SMA 12/60 AutoAnalyzer, in the Biochemistry Department at St. Helier Hospital. For this, 2.7 ml of BCF were required. The machine assays twelve variables; total protein, albumin, calcium, inorganic phosphate, cholesterol, urea, uric acid, creatinine, bilirubin, alkaline phosphatase, glutamate-pyruvate transaminase (SGPT), and glutamate-oxalacetate transaminase (SGOT).

5.8 INORGANIC IONS

Sodium and potassium levels of BCF were estimated by flame photometry, and calcium was assayed by the SMA 12/60 AutoAnalyzer (Technicon).
Standard solutions of potassium and sodium were prepared from 0.1 M potassium chloride and 1 M sodium chloride respectively. By pipetting 3, 4, 5, 6, and 7 ml of the potassium chloride solution, and 12, 13, 14, 15 and 16 ml of the sodium chloride solution, into 100 ml volumetric flasks and making up with distilled water, standards containing 3, 4, 5, 6 and 7 meq/l of potassium and 120, 130, 140, 150 and 160 meq/l of sodium were produced. Two dilutions of samples and standards were prepared, 1:50 for potassium and 1:1000 for sodium.

The flame photometer was connected to the gas and air supplies, the gas turned on and lit, and then the air turned on. A beaker of distilled water was placed in the sampling position, and after five minutes the burner was checked to ensure the gas control was correctly adjusted. The potassium filter was inserted into the light path, and the galvanometer unclamped and the instrument switched on. The "Sensitivity" control was turned down and then the galvanometer was zeroed using the "Set Zero" control. The distilled water in the sampling position was changed for the highest potassium standard and the sensitivity adjusted until the galvanometer was set to 100. The zero and 100 were then re-adjusted with distilled water and high standard. The remaining standards and the samples were then run, periodically checking the zero with distilled water. Any samples falling out of the standard range were re-diluted accordingly to bring them within range.

The same procedure was repeated for the sodium estimations, using the appropriate filter and zeroing with distilled water and setting the highest standard to 100 on the galvanometer. On completion of the experiment the instrument was flushed through with distilled water until the galvanometer reading returned to zero, then the gas
turned off, followed after one minute by the air supply. The results were interpolated from the standard curves, allowance being made for any variation from the normal dilution.

5.9 CARCINO-EMBRYONIC ANTIGEN (CEA)

A series of twenty BCF samples were assayed for CEA. A radioimmunoassay method was used for the determinations, on 1 ml, randomly-selected samples, and kindly performed by Professor Munro Neville's Department at the Chester Beatty Research Institute, Fulham.

5.10 HORMONAL STEROIDS

Assay of testosterone, 5α-dihydrotestosterone, 5-androstenedione, and 17β-oestradiol, was performed on BCF, and oestrogens and progesterone on sera and 24-hour urines from breast cyst patients and age-matched controls. Several sera from cyst patients were also assayed for prolactin. The estimations were made, using high-pressure liquid chromatography and radioimmunoassay, by Mr. D. Fry at West Park Hospital, Epsom, Surrey.
6.1 PHOSPHATE ASSAYS - VALIDATION

Preliminary work had shown that the estimation of phosphate, as liberated in the assay of phosphate-releasing enzymes in BCF, suffered a major problem. The proteins of BCF are very difficult to precipitate with acid, and addition of sufficient acid made the phosphate assay unreliable or even impossible. A series of experiments was therefore set up to determine optimum conditions for assay with the system being used. These experiments were in two sets; firstly to determine optimum acid concentrations for phosphate assay, and secondly to find a suitable method for precipitation of proteins and particles in BCF.

The standard method being used for phosphate assay was to add 1.5 ml of 0.6% ammonium molybdate in 5% PCA to 2 ml of acidified solution containing the phosphate to be estimated, followed by 0.5 ml of freshly prepared 0.2% ascorbic acid solution. In order to determine the optimum acid concentration in the phosphate solution a series of tubes were set up containing 6 µg of phosphate standard in 0, 1, 2, 3, 4, 5, 10 and 15% TCA, and PCA. The reagents were added, as above, and the absorbances read at 720 nm after 30 minutes, and again after a further 30 minutes. Three of the solutions and a blank were left overnight and their absorbances measured automatically at 30 minute intervals. This gave the optimum concentrations of TCA or PCA required to yield maximal colour development, and the time curve of same. A standard curve was then prepared, using these optima and standard phosphate solutions, in order to check linearity.

Protein precipitation proved more troublesome. Three precipitants were used: TCA, PCA, and trimethylammonium bromide (CAB). To tubes containing 0.1 ml BCF was added water, followed by 20% acid or CAB, to give concentrations of 1, 2, 3, 4, 5 and 10% precipitant in a total volume

* Many of the enzymes were assayed for use as markers of cell membranes and were selected with consideration of the pitfalls inherent in such use. (Hinton & Reid, 1976).
of 3 ml. The tubes were vortex-mixed and centrifuged at 2000 \( \times g \) for 10 minutes, and the degree of precipitation estimated. This was done by eye, judging the degree of turbidity of the supernatant and the amount of precipitate at the bottom of the tube. Thus the optimum concentration range of each precipitant was found. The supernatant from these tubes was used in phosphate assay as above. However, since the optimum concentrations of precipitants were found to vary with different BCF specimens, and further precipitation occurred on adding molybdate reagent, it was decided to look for other methods.

Such methods tried included filtration through Millipore filters of small pore size, addition of methanol and ethanol to the precipitant in concentrations up to 20%, and mixtures of TCA and PCA, rather than the individual acids.

Eventually it was found that heating the centrifuged, acidified solution caused further precipitate to appear in the supernatant. Tubes containing 0.1 ml of BCF in 2.9 ml of 4% TCA were heated to 60, 80 and 100°C for 0.5, 1, 2, 3, 5 and 10 minutes, rapidly cooled in iced water and centrifuged as before. The supernatants were assayed for phosphate as above.

From these results it was found that using optimum conditions of acid and heat the proteins and particles could be removed from incubation mixtures containing BCF. The subsequent reaction for phosphate was found to give a linear response. For some substrates which are hydrolysed by hot acid, the alternative of freezing was found to be effective in aiding complete precipitation. The method was first tested with the 5'-nucleotidase assay (see below) the chosen substrate for which is stable in hot acid.
6.2 PHOSPHATASE ENZYME ASSAYS

When more than one such enzyme was to be assayed, particularly with fractions from density-gradient centrifugal separations, only one set of blanks was prepared. This saved valuable material. The same volume of sample was therefore used for all assays and the set of blanks, which consist of sample made up with water to the volume of the incubation mixtures. These were then precipitated and assayed for phosphate in the same manner as the assay solutions. Thus, by running a reagent blank in parallel for each enzyme estimation, the phosphate released by the enzyme was found by subtracting from the test absorbance those due to the appropriate sample and reagent blanks. Two reagent blanks were usually used; one heat-treated or frozen as for the assay tubes, the other untreated. Thus any effect of such treatment could be observed.

For one density-gradient centrifuge run, the assay of phosphate was performed on a Technicon AutoAnalyzer at the Wolfson Bioanalytical Centre, at the University of Surrey. The machine was set up to use the same ammonium molybdate assay method as was used manually.

6.3 5'-NUCLEOTIDASE

This enzyme was assayed by two methods. Initially a kit version (BDH) of the method of Persijn and van der Slik (1968) was used. This specific and highly sensitive assay is based on the estimation of ammonia produced by an excess of adenosine deaminase from the adenosine released by the test enzyme from adenosine-5'-monophosphate (AMP). Non-specific action of alkaline phosphatase is selectively inhibited by phenyl disodium orthophosphate. Unfortunately the method was found not to work with samples containing sucrose, and
therefore the phosphate-assay approach was used with density-gradient centrifuge-separated material.

6.3.1 Persijn and van der Slik method

Into each of two tubes was pipetted 0.1 ml of sample, and into one of each pair (test) was added 1 ml of substrate reagent (barbitone buffer, containing magnesium, adenosine deaminase and AMP), into the other (test blank), 1 ml of the buffered adenosine deaminase. A substrate blank was prepared using 1 ml of substrate reagent and 0.1 ml of water, and a standard and standard blank using 1 ml of buffered adenosine deaminase solution containing 0.1 ml of 3.7 mM adenosine, and 0.1 ml of water, respectively. The tubes were all capped and incubated at 37°C for 60 minutes. Reaction was stopped by addition of the two, freshly diluted, colour reagents. The first was a 1:5 dilution of concentrated phenol reagent (50 g of phenol and 0.25 g of sodium nitroprusside made up to 1 litre with distilled water), containing EDTA to prevent precipitation of magnesium salts. The second was a 1:5 dilution of concentrated alkaline hypochlorite reagent (25 g of sodium hydroxide in 250 ml of water, with 72 ml of 1 M sodium hypochlorite in 0.1 M sodium hydroxide, made up to 1 litre with water). The phenol reagent (5 ml) was added first, followed by 5 ml of hypochlorite as quickly as possible, both forcefully. The tubes were left in the water bath at 37°C for a further 30 minutes, and then their absorbances at 625 nm measured, reading the tests against their corresponding test blank, and the substrate blank and standard against the standard blank. The enzyme activity was calculated from the absorbance of the test solution after subtracting that of the substrate blank.
6.3.2 Method entailing phosphate assay

Into each of two clean glass tubes was pipetted 0.4 ml of tris buffer pH 7.7, containing 12.5 mM magnesium chloride and 25 mM sodium tartrate (to inhibit acid phosphatase), followed by 0.3 ml of water and 0.2 ml of sample. The tubes were placed in a water bath at 37°C and 0.1 ml of 0.05 M AMP solution added and vortex mixed. A substrate blank was prepared, substituting water for sample, and a series of test blanks omitting substrate. As mentioned previously, only one set of test blanks was prepared if several phosphate-releasing enzymes were being assayed together. After incubating at 37°C for 60 minutes the reaction was stopped by forcefully adding 1.5 ml of 5% TCA. The tubes were placed in a boiling water-bath for one minute and rapidly cooled in ice-water. After centrifuging for 5 minutes, the entire supernatant was carefully poured off and assayed for phosphate as described previously.

6.3.3 Validation of assay

As mentioned previously the validation of this assay was combined with that of the phosphate assay. The aim was also to discover the longest permissible incubation time, in order to increase the sensitivity of the assay.

Three BCFs and one serum were used for the experiment, which was run twice on different days. As a check on comparability of the two assay methods, the samples were also checked by the Persijn and van der Slik method in the usual way.

Into a clean glass tube was pipetted 6 volumes of the reagents and samples given above. Immediately after adding the AMP solution, and vortex mixing, 1 ml of the reaction mixture was pipetted into a tube containing 1.5 ml of 5% aqueous TCA. This was used as the
zero-time blank. The tubes were incubated at 37°C and further 1.0 ml aliquots removed into TCA at 30 minute intervals up to 2 hours. The tubes were then heated in a boiling water bath for one minute, cooled, centrifuged and the entire supernatant used for phosphate assay as before. Two reagent blanks were also run, substituting water for sample, and one heated to 100°C with the other tubes to check the stability of AMP in hot acid. All the assay tubes were read against the unheated reagent blank. After subtracting the absorbance of the zero-time blank from the absorbances of the tests a velocity curve was plotted for each sample, and the results compared with those from the Persijn and van der Slik method.

6. 3.4 Stability of enzyme

The stability of the enzyme was also estimated, to check that losses on storage enforced by the density-gradient centrifugation were not too serious. Two BCF were used, one with high, the other with low, levels of the enzyme.

The two BCF were obtained on successive weeks and each was halved. One half was stored at -20°C, the other at 4°C. The assays were performed 8 and 15 days after collection for the first fluid, 1 and 8 days after collection for the second. Part of the sample stored at -20°C was frozen and thawed six times by placing the tube alternately in ice-salt mixture and cold tap-water. This was done to check stability on repeated freezing, and to see if any activation occurred. The assays were performed by the method of Persijn and van der Slik, this being the easier technique and correlating well with the other method.

6. 4 Na⁺, K⁺-DEPENDENT ADENOSINETRIPHOSPHATASE

ATPase was estimated by assay of phosphate released from
ATP substrate (Post & Sen, 1967). In this case the test-blanks were identical to the tests, but with the addition of ouabain. This inhibits the Na\(^+\), K\(^+\)-dependent enzyme and thus allows correction for non-specific hydrolysis of ATP, and hydrolysis by other ATPases.

Into tubes was pipetted 0.5 ml of buffer-substrate solution, consisting of 30 mM histidine buffer pH 7.5 containing 3 mM magnesium chloride, 130 mM sodium chloride, 20 mM potassium chloride, and 3 mM ATP. The ATP was added freshly to the required volume of before use. An identical series of tubes was set up with 1 mM ouabain added to the buffer. After adding 0.3 ml of water and 0.2 ml of sample, and mixing, the tubes were incubated in a water-bath at 37\(^\circ\)C for 60 minutes. The reaction was then stopped by adding 1.5 ml of ice-cold 5% TCA. The tubes were placed in a deep freeze until ready for phosphate assay, in order to assist protein precipitation. The heating method could not be used since ATP is hydrolysed under such conditions. On thawing, the tubes were centrifuged and the supernatant assayed for phosphate, as before.

6.5 GLUCOSE-6-PHOSPHATASE

The method for this enzyme was that of Nordlie and Arion (1966), again estimating phosphate released. Since glucose-6-phosphate is also somewhat unstable in hot acid, freezing was used to assist protein precipitation.

Into tubes were pipetted 0.6 ml of 0.1 M cacodylate buffer pH 6.5, 0.2 ml of 0.15 M glucose-6-phosphate, 0.2 ml of water, and 0.2 ml of sample. Blanks were prepared using 0.2 ml of sample and 1.0 ml of water, and a substrate blank substituting water for sample. The tubes were vortex-mixed and incubated in a water-bath at 30\(^\circ\)C.
for one hour. The reaction was stopped by adding 1.5 ml of ice-cold 5% TCA. After freezing and thawing, the tubes were centrifuged, and the entire supernatant carefully decanted and assayed for phosphate, as before.

6.6 ACID PHOSPHATASE

Total acid phosphatase was estimated by the p-nitrophenol-releasing method of Bessey, Lowry and Brock (1946). The substrate, 4-nitrophenyl phosphate, is hydrolysed to 4-nitrophenol, and this is converted to a yellow coloured complex on adding sodium hydroxide. An assay set (BDH) containing pre-weighed ingredients was used.

Into duplicate tubes was pipetted 1 ml of buffered 4-nitrophenyl phosphate, followed by 0.2 ml of sample. A sample blank was prepared by immediately adding 5.0 ml of 0.05 M sodium hydroxide solution to one of each pair of tubes, and one substrate blank for each batch, substituting water for sample. After mixing well, and incubating in a water-bath at 37°C for 30 minutes, 5.0 ml of 0.05 M sodium hydroxide solution was added to the tests and substrate blank. The absorbances were then measured at 400 nm, reading against the substrate blank.

A calibration curve was prepared from a standard solution of 100 mM 4-nitrophenol. Six tubes, in duplicate, containing 0.5, 1.0, 1.5, 2.5, 3.5 and 4.5 ml of standard solution were made up to 6.2 ml with 0.05 M sodium hydroxide solution and their absorbances measured at 400 nm against a distilled water blank. The calibration graph was drawn, plotting absorbance directly against enzyme activity of the sample; 1 ml of standard solution, made up to 6.2 ml, is equivalent here to 16.6 mIU/ml of sample.
6.7 **ALKALINE PHOSPHATASE**

This was estimated by essentially the same technique as for acid phosphatase, but using an alkaline buffer.

In duplicate tubes for each sample, 1.0 ml of glycine-magnesium buffer containing 4-nitrophenyl disodium orthophosphate was pre-incubated in a water-bath at 37°C for 5 minutes. To one of each duplicate pair was added 10 ml of 0.2 M sodium hydroxide solution, and then 0.1 ml of sample pipetted into each. After mixing and incubating at 37°C for 30 minutes, 10 ml of 0.02 M sodium hydroxide was added to the tests, and the absorbances read at 400 nm against a substrate blank. The absorbances of the test blanks were subtracted from those of the corresponding tests, and activities interpolated from a calibration curve constructed as above, but making the standard solutions up to 11.1 ml with 0.02 M sodium hydroxide. In this case 1 ml of standard is equivalent to 33.3 mIU/ml of sample.

6.8 **ALKALINE PHOSPHODIESTERASE**

This enzyme was also estimated by a p-nitrophenol releasing method, but in this case the substrate was bis-(4-nitrophenyl) phosphate.

Into tubes was pipetted 1.0 ml of 0.25 M tris buffer, pH 8.8, containing 10 mM magnesium chloride, followed by 0.5 ml of distilled water, 0.3 ml of 10 mM bis-(4-nitrophenyl) phosphate, and finally 0.2 ml of sample. Tissue blanks were prepared by adding 0.2 ml of sample to 1.8 ml water, and a substrate blank by substituting water for sample. The tubes were mixed and then incubated at 37°C for one hour. Reaction was stopped, and colour developed, by adding 2 ml of 1 M sodium hydroxide containing 0.15 M EDTA, and the absorbances measured at 400 nm against a water blank. The absorbances of the standard and appropriate test blanks were subtracted from those for
the test, and the enzyme activity calculated from a standard curve prepared from 4-nitrophenol, as above.

6.9 ACID PHOSPHODIESTERASE

This was estimated by the same method as was used for alkaline phosphodiesterase, but substituting 0.3 M acetate buffer, pH 5.0, for the tris buffer, pH 8.8, and omitting EDTA from the sodium hydroxide solution used to stop the reaction. When the two enzymes were assayed together, only one set of tissue blanks was prepared and used for both estimations.

6.10 CATHEPSIN D

The method used to assay this enzyme was that of Barrett (1967). The substrate used is haemoglobin, from which the enzyme produces peptides that are soluble in TCA. These may be estimated by their absorbance at 280 nm.

The substrate was prepared from bovine haemoglobin. A 10% w/v solution in water was prepared, and dialysed in Visking tubing against 3 changes of distilled water at 4°C, for 24 hours. The solution was then made up with distilled water to give a final concentration of 8%, and stored frozen in 5 ml aliquots.

To 0.2 ml of sample, in triplicate, the following reagents were added: 0.25 ml of 1 M formate buffer, pH 3.0, 0.3 ml of water, and 0.25 ml of 8% haemoglobin substrate. One of the triplicates was precipitated immediately by adding 5 ml of 5% TCA, the remaining tubes were incubated in a water-bath at 45°C for 2 hours. Two substrate blanks were prepared, substituting water for sample, and one precipitated immediately with TCA, the other incubated with the tests.
Following incubation, 5 ml of 5% TCA was added to each tube, and all the tubes heated in a boiling water-bath for 5 minutes, then centrifuged to remove precipitated protein. The supernatants were poured off and their absorbance measured at 280 nm. The tests were read against the incubated substrate blank, and the test blanks against the unincubated substrate blank.

The assay was performed mainly on whole BCF. A few rough separations of particle- and fluid-fractions were run to see whether the enzyme was localised in one or the other.

6.10.1 Validation of assay

As with 5'-nucleotidase, great trouble was experienced initially with this assay due to difficulty in precipitating all the BCF proteins. The use of other precipitants was tried, with no success, and eventually filtration was attempted. Millipore filters (0.22 μm pore size) were used in a Swinnex filter. The supernatant from the assays was drawn into a 10 ml hypodermic syringe, to which the filter was then attached, and the fluid ejected through the filter into a clean tube. However, this method although satisfactory was very time-consuming, and the heating method used for 5'-nucleotidase was tried. Even with heating at 100°C for 10 minutes, complete precipitation was not obtained.

Raising the concentration of TCA in the precipitant from 3% to 5% was tried and found to give protein precipitation after heating for five minutes at 100°C.

A series of experiments was then undertaken to determine the effects of raising acid concentration and heating. Using two BCF specimens, and setting up the assay medium as given above, eight tubes were run for each specimen. Two were precipitated immediately with 5 ml of 3% TCA, another two with 5 ml of 5% TCA, and the remainder
incubated at 45°C for one hour. These were then also precipitated with 3% and 5% TCA as for the blanks. The tubes to which 5% TCA had been added were then heated in a boiling water-bath for 5 minutes and all the tubes centrifuged. The supernatants from the unheated tubes were filtered through a Millipore membrane. The absorbances were read as before at 280 nm. Substrate blanks were prepared, substituting water for sample, then half were incubated, half immediately precipitated. One for each pair was precipitated with 3% TCA, the other with 5% TCA and the former centrifuged and filtered, the latter heated to 100°C for 5 minutes, cooled and centrifuged. Thus the effect of hot acid on the substrate could be determined.

A time-curve for the assay was then prepared. To 1.6 ml of sample (a threefold concentrate of a pool of 3 BCFs) was added 2.0 ml of 1 M formate buffer pH 3.0, 2.4 ml of water, and 2.0 ml of 8% haemoglobin substrate, and after mixing, 1 ml of the fluid removed into a tube containing 5 ml of 5% TCA solution. The tubes were incubated at 45°C and 1 ml aliquots removed into 5 ml of 5% TCA solution after 30, 60, 90, 120, 180 and 240 minutes. The tubes were placed in a boiling water-bath for 5 minutes, cooled in ice-water, centrifuged, and the absorbance of the supernatants measured at 280 nm.

6.10.2 Activation and stability of the enzyme

The stability of the enzyme was investigated to determine whether assays could be made on stored samples, and also whether storage at 4°C would allow continuing proteolysis of BCF constituents. Experiments were also performed to see if the enzyme could be activated by freeze-thawing, as in the classical experiments on lysosomes by de Duve et al. (1955).
The two BCF samples used were the same as those used for the experiments on stability and activation of 5'-nucleotidase. They were assayed following storage at 4°C and -20°C, after 1 and 8 days for one, and 8 and 15 days for the other. A part of the frozen specimen was freeze-thawed six times. The assays were performed as described above.

6.11 SUCINATE DEHYDROGENASE

This enzyme was assayed by the method of Pennington (1961). To 0.3 ml of 0.3 M phosphate buffer, pH 7.4, containing 1.25 mg of INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) per ml, was added 0.3 ml of water, 0.2 ml of 0.3 M succinate (brought to pH 7.4 with potassium dihydrogen orthophosphate), and 0.2 ml of sample. Blanks were prepared for each specimen in the same way, but substituting 0.3 M malonate for succinate, and reagent blanks substituting water for sample. The tubes were incubated in a water-bath at 37°C for 30 minutes, and reaction then stopped by adding 1.5 ml of ice-cold 6% TCA solution, followed by 4 ml of ethyl acetate. The ethyl acetate layer was removed with a Pasteur pipette, and its absorbance measured at 490 nm in a 1 cm cuvette. After subtracting the absorbance of the blanks from the tests, the enzyme activity was calculated from the molar extinction coefficient of reduced INT (20.1 x 10^3).

6.12 AMYLASE

The enzyme was assayed by the method of Somogyi (1938) as modified by Henry and Chiamori (1960). Starch is hydrolysed to glucose by amylase, and this is measured by the method of Folin and Wu (1920), as modified by Lauber and Mattice (1944) and Tonks (1952).

To 0.3 ml of sample was added 2.1 ml of 1% soluble starch solution
in 0.1 M phosphate buffer pH 7.0, and the tubes incubated in a water-bath at 40°C for 30 minutes. Reaction was stopped by adding 1.5 ml of 0.33 M sulphuric acid and, after mixing, 0.5 ml of 10% sodium tungstate solution. The tubes were centrifuged, and the supernatant poured off and assayed for glucose. Controls were prepared by adding the precipitants immediately to duplicates of the reaction mixture.

For the glucose assay, a copper reagent was prepared by adding to 40 ml of distilled water, dissolving after each addition, 4 g of anhydrous sodium carbonate, 0.75 g of tartaric acid, and 0.45 g of cupric sulphate (pentahydrate). The solution was made up to 100 ml with water. Phosphomolybdic acid was prepared by mixing 7.0 g of molybdic acid, 1.0 g of sodium tungstate, 40 ml of 10% sodium hydroxide solution, and 400 ml of water. The solution was boiled for 20 minutes, cooled, diluted to 70 ml with water; 25 ml of orthophosphoric acid (85%) was added, and finally the solution made up to 100 ml with water.

To duplicate 0.5 ml aliquots of supernatant from the amylase reaction was added 0.5 ml of copper reagent, and the tubes placed in a boiling water-bath for 6 minutes. The tubes were removed from the water-bath, 0.5 ml of phosphomolybdic acid reagent added, and then reheated at 100°C for 2 minutes. After cooling and adding 3.5 ml of water, the absorbance of the solutions was measured at 420 nm. A reagent blank was prepared by substituting water for sample, and standards by running 0.1, 0.2, 0.3, 0.4 and 0.5 ml of standard glucose solution (freshly prepared by diluting 1 ml of a 10 mg/ml stock solution of glucose, saturated with benzoic acid, to 100 ml), each made up to 0.5 ml with water.

6.13 XANTHINE OXIDASE

This enzyme was assayed by the method of de Lamirande et al. (1958).
urate production by the enzyme is monitored by its absorbance at 293 nm.

To 0.2 ml of sample was added 0.6 ml of distilled water, 1.0 ml of 0.1 M phosphate buffer pH 7.5, and 0.3 ml of 2 mM potassium cyanide. After incubating in a water-bath at 37°C for 5 minutes, the solution was poured into a 1 cm cuvette which was then fitted into a heating-block in an SP500 spectrophotometer (Pye Unicam). Water at 37°C was circulated through the heating-block, from a water-bath. After adding 1.3 ml of 0.55 mM xanthine solution at 37°C, mixing, and equilibrating for 1 minute, the absorbance of the solution at 293 nm was measured, at 5 minute intervals for 1 hour, against a control consisting of 0.2 ml of sample in 3.2 ml of water. Standards were prepared from 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of a 1 mM uric acid solution, made up to 3.4 ml with water, to cover the range corresponding to 0 to 41.7 mIU enzyme/ml of sample, for a one hour incubation.

6.14 LACTOPEROXIDASE

This was assayed by measuring the oxidation of o-dianisidine (3,3'-dimethoxybenzidine dihydrochloride) by hydrogen peroxide, according to the method of Oram and Reiter (1966).

To 0.2 ml of sample was added 3.8 ml of 0.1 M sodium acetate buffer pH 5.7, and the tubes incubated at 37°C for 5 minutes. The solution was poured into a 1 cm cuvette, which was placed in a heating-block in an SP500 spectrophotometer, and 1.0 ml of 0.04 mM o-dianisidine solution, containing 1 mM hydrogen peroxide, added. The solution was mixed, and the absorbance at 520 nm measured at 5 minute intervals for 30 minutes, against a blank consisting of 0.2 ml of sample in 4.8 ml of water.

6.15 AUTOANALYZER

As mentioned previously, three enzymes were assayed on the Technicon
SMA 12/60 AutoAnalyzer. These were alkaline phosphatase, SGOT and SGPT.
In order to separate out the particle-fraction of BCF, various methods of centrifugation were used. These were: high-speed centrifugation of whole BCF, flotation methods involving the use of materials to raise the density of the sample, and isopycnic density-gradient centrifugation.

7.1 HIGH-SPEED CENTRIFUGATION WITHOUT A GRADIENT

This technique was the first one used. Initially, the purpose was to "clear" the BCF samples, since this was not achieved by the low-speed centrifugation used to remove cells.

Samples were placed in 15 ml capacity polypropylene tubes (M.S.E. Ltd.) and pairs of tubes balanced by adding more sample to the lighter one. The tubes were capped and fitted into an M.S.E. 16 x 15 ml angle-head rotor in an M.S.E. refrigerated centrifuge, then spun at 18000 r.p.m. (30,000 x g) for periods up to 4 hours, at 4°C.

It was found that if only small volumes were being centrifuged they could be placed in M.S.E. 5 ml capacity polycarbonate tubes. These could then be gently inserted into the 15 ml tubes, which they fitted snugly. Following centrifugation, the smaller tubes were removed using a dissection needle with the tip bent over at right-angles. This method also had the advantage that any separation of particles could be seen easily through the clear polycarbonate tubes, but not the white, polypropylene type.

7.2 FLOTATION METHOD

This method was used to improve the separation of the particle-fraction of BCF. The density of the samples was raised by adding sodium bromide.
To 5 ml of BCF was added 2.0 g of sodium bromide, which was dissolved by gently mixing, to give a density of 1.3. The solution was transferred to a 15 ml capacity polycarbonate tube, and then carefully overlayed with 1 ml of sodium bromide solution, of density 1.22, in 0.05 M tris buffer, pH 7.5. After balancing by adding overlay, the tubes were centrifuged in the angle-head rotor at 18,000 r.p.m. for 2 hours.

The particles formed a layer on the surface of the overlay, and were carefully removed, with the overlay, using a Pasteur pipette. This fraction and the clear fluid-fraction were each dialysed against 0.01 M tris buffer, pH 7.5, in an Amicon ultrafiltration cell with a UM2 membrane (Amicon), and finally made up to 5 ml with the same buffer, before analysis.

7.3 DENSITY GRADIENT CENTRIFUGATION

This method was used to separate the particle-fraction in seven samples of BCF, and, as controls, two milk specimens and one plasma sample. Sucrose was adopted as a more satisfactory density-producing medium than sodium bromide, despite the viscosity of concentrated solutions.

7.3.1 Gradient Formation

Gradients were prepared from two solutions: 0.01 M tris-HCl buffer, pH 7.4, and 2 M sucrose in the same buffer solution. An M.S.E. gradient-mixer was used. This consists of two cylindrical vessels of identical dimensions, joined at the base by a narrow-bore tube fitted with a tap. One vessel is fitted with a three-way outlet tube at the base, and a motor-driven stirrer. With the tap off, to disconnect the vessels, 24 ml of buffer was poured into the unstirred vessel and the tap opened slightly to allow the solution
to fill the connecting tube and exclude air from it. The tap was
turned off, and the other vessel filled with 24 ml of the buffered
2 M sucrose solution. The three outlet tubes were connected to three
silicon tubes fitted into a peristaltic pump, and which led into three
polycarbonate tubes (25 ml capacity, M.S.E. Ltd.), supported in a
vessel containing crushed ice. The outlet tubes were arranged to
touch the inside of the tops of the centrifuge tubes, and held there
with adhesive tape. The stirrer was started, the tap opened, and
the pump switched on. The gradient solution, becoming gradually
less dense as light solution was mixed in in the gradient-former,
was thus pumped into the centrifuge tubes, and ran down their sides,
the progressively lighter fluid smoothly overlaying the more dense.
When all the solution had left the gradient-mixer, the apparatus was
switched off.

7.3.2 Sample preparation and addition

The BCF samples used were six different specimens, centrifuged
in two batches of three, and one large specimen, concentrated from
50 ml to 12 ml by ultrafiltration, using nitrogen at 75 p.s.i. (5.2 kgf/cm²),
in an Amicon cell fitted with a UM2 membrane (molecular weight cut off =
1000), and run in all three tubes of the centrifuge. The samples were
all fresh, unfrozen, and were stored briefly at 4°C before centrifuging.
The large sample was maintained at below 4°C during ultrafiltration,
by surrounding the cell with crushed ice. Two human skimmed milks
and a plasma sample were also centrifuged by the same techniques used
for BCF. The fresh milk samples were centrifuged at room temperature
for 15 minutes at 2000 x g, and then cooled to 4°C and the skimmed
milk removed from under the solidified fat-layer. The plasma sample
was a 10 ml blood sample obtained from a 45 year old woman (i.e. in
the cystic disease age range) selected at random in the Haematology Outpatients Department at St. Helier Hospital. The blood was placed into a tube containing EDTA anticoagulant. After centrifuging, to sediment the cells, the plasma was removed using a siliconised Pasteur pipette. EDTA was chosen as it prevents oxidative changes to lipoproteins.

The three samples were brought to a density greater than that of the gradient by adding 4 ml of sample to 6 g of sucrose in a plastic tube, capping the tubes, and gently mixing by inversion on a rotator in the cold-room. When the sucrose had completely dissolved, the tubes were left standing upright until all air bubbles had risen. The prepared samples were then layered under the gradients. This was achieved by aspirating an aliquot into a 5 ml hypodermic syringe, to which was then fitted a short blunted needle attached to a 10 cm length of narrow-bore, plastic tubing. A small amount of sample was expelled to fill the tube, which was then inserted through the gradient solution to the base of the centrifuge tube. Four ml of sample was gently forced from the syringe, taking great care to ensure that no air bubbles were released into the gradient, and the syringe tube then slowly withdrawn. When all three tubes had been charged with sample they were weighed, and the two of least weight made up to that of the greatest by carefully overlaying 0.01 M tris buffer, pH 7.4, onto the gradients.

7.3.3 Centrifugation and fractionation

The tubes containing the gradients were fitted into an M.S.E. 3 x 23 ml swing-out rotor which had been cooled to 4°C. The rotor was then fitted into a Super Speed 65 ultracentrifuge (M.S.E.Ltd.) and spun at 29,000 r.p.m. overnight. The temperature was maintained at 4°C. The centrifuge was accelerated slowly, using manual control,
up to 5000 rpm, in about 5 minutes. Thereafter it was allowed to accelerate freely. At the end of the run, usually 16 hours, the machine was switched off, and the rotor allowed to decelerate without using the brake.

The rotor was removed from the centrifuge, and the tubes were taken out of the buckets. The gradient was pumped out by upward displacement. The tube was placed in a stand and a perspex cap, fitted with a rubber gasket, screwed down to seal the mouth of the tube. The inside of the cap was ground to a conical shape and had a small, horizontal outlet tube running from near the top of this cone. A hollow needle was fitted vertically through the centre of a vertical bolt in the apex of the cone. This needle was gently pushed to the base of the tube and the bolt tightened, compressing a small rubber ring fitted below it, which hermetically sealed the needle. The needle was joined at its upper end to a silicon-rubber tube connected to a syringe pump. The syringe, tube, and needle were all filled with aqueous 2.1 M sucrose, and care was taken to ensure that this contained no air bubbles. The pump was set to deliver 1 ml per minute, and then started. The dense sucrose solution underlaid the gradient and pushed it up the centrifuge tube and through the outlet tube. Here it was collected in fractions of 1 ml, in small plastic tubes, by moving the outlet tube from one container to the next at one minute intervals. For one set of three BCFs the absorbance of the effluent at 600 nm was monitored during displacement, using a Unicam SP500 spectrophotometer fitted with a Hellma flow cell of 1 cm light path and 1 ml capacity.

7.3.4 Analysis of fractions

The twenty or so fractions collected from each sample, and the
remainder of that sample, were maintained at 4°C, and enzyme assays
performed as soon as possible. The refractive index of each fraction
was measured in an Abbé refractometer. Protein and cholesterol assays
were made on all fractions from all samples. In addition 5'-nucleotidase
was estimated in the BCF fractions. The refractive indices of the
fractions from the large BCF sample were measured to check that they
matched in corresponding fractions from the three tubes. Each set of
three corresponding fractions was then pooled. The bulked fractions
and starting material were assayed for protein, cholesterol, 5'-
nucleotidase, Na⁺-K⁺-ATPase, acid and alkaline phosphodiesterases,
glucose-6-phosphatase, and succinate dehydrogenase. Some of the
particle-containing fractions were also examined in the electron
microscope as negatively stained preparations.
8 CYTOLOGY

Cells from BCF specimens and histological sections of cystic breast, were examined using light microscopy, after staining by a variety of methods.

8.1 CELLS FROM BCF

The cell deposit from centrifuged BCF (see "Specimen collection", p. 52) was drained well, by inverting the centrifuge tube on absorbent paper for 3 minutes. Three air-dried and 3 wet-fixed smears were made on glass slides, using a small wire loop.

Air-dried smears were prepared first, by taking a small portion of the drained deposit on the loop and smearing this material down the long axis of the slide. A series of short strokes were then made with the loop, starting from the original streak, and running at right angles to it. The object was to produce a thin smear which dried rapidly. When dried, two smears were fixed in methanol, one by briefly flaming, using a Bunsen burner.

Wet-fixed smears were prepared after re-inverting the tube containing the cell deposit, thus allowing the film of BCF on the walls of the tube to run back. The deposit was mixed with this fluid, using the loop, and one loopful of material streaked down the slide, which was then quickly transferred to fixative. Initially Schaudinn's fixative had been used, but this was later substituted with IMS to avoid the dangers involved in using mercuric chloride. The fixed smears were stored in IMS before staining.

After preparing the slides, the remainder of the cell deposit was suspended in fixative (formol saline, IMS, glacial acetic acid, 45:50:5) for the preparation of a filter slide. Fixation was continued for 20 minutes, and the suspension was then filtered through a Millipore
membrane (type SM, 5 μm pore size), supported in a Millipore holder connected to a vacuum pump. The membrane was removed and clipped to a microscope slide, with the cells facing outwards.

This filter was stained by haematoxylin and eosin (HE), the wet-fixed smears by HE, Papanicolaou's (Pap.) and Perls' methods; the air dried smears were stained by May-Grünwald-Giemsa (MGG), PAS, and Gram's stain. The techniques used are given below.

8.2 HISTOLOGICAL SECTIONS

Sections of formalin-fixed, paraffin-wax-embedded tissue were cut at 5μm, and stained by a variety of methods. Sections were initially examined, using HE stain, to select tissue which contained 'pink' cells and microcysts. Such blocks were then used for preparing slides reacted by special methods, and for immunohistochemistry. The special methods used were PAS, diastase-PAS, Alcian Blue in solutions containing various concentrations of magnesium chloride, phosphotungstic acid-haematoxylin, Heidenhain's method, chrome alum-haematoxylin, Schmorl's technique, Perls' method, and Turnbull's method.

Wax sections were first brought to water by dewaxing the slides, after heating to 60°C, in two 10-minute changes of xylene, agitating intermittently, followed by two changes of IMS, and then one bath each of 90, 70 and 50% aqueous IMS, and finally water.

Frozen sections were fixed by placing the slides either in formol saline or methanol.

8.3 STAINING METHODS

8.3.1 Haematoxylin and eosin

The stain was used for wet-fixed and filter preparations of BCF
cells, and histological sections.

1) Place in Harris' Haematoxylin (Ortho Chemicals Ltd), 5 minutes (filters, 3 minutes).
2) Rinse in deionised water.
3) Differentiate in acid alcohol (1% HCl in 70% aqueous IMS), 10 to 15 seconds.
4) Rinse, and blue in tap-water, 15 minutes (filters, 30 minutes)
5) Rinse in IMS.
6) Stain in 1% alcoholic eosin, 30 seconds (filters, 15 seconds)
7) Rinse in three changes of IMS.
8) Clear in two changes of xylene (for filters, 30 minutes in second bath).
9) Mount in DPX.

For filter preparations of BCF cells, the membranes were unclipped from the slides, the bare edges trimmed away using a razor blade, and, after blotting off excess xylene, the membrane mounted between thin layers of DPX beneath a coverslip.

8.3.2 May-Grunwald-Giemsa

The stains were prepared from stock solutions (Raymond A. Lamb), diluting the May-Grunwald stain 1:2.5, and the Giemsa 1:5, with Sorensen's buffer, pH 6.8. Air-dried smears, fixed in methanol, were used.

1) Place in May-Grunwald stain, 10 minutes.
2) Rinse in deionised water.
3) Place in Giemsa stain, 30 minutes.
4) Rinse in tap-water.
5) Place in Sorensen's buffer, pH 6.8, 15 minutes.
6) Dry in air, mount in DPX.

8.3.3 Gram's Stain

Air-dried, flame-fixed smears were used.
1) Place in Lillie's Crystal Violet, 1 minute.

2) Wash off under tap.

3) Place in Lugol's Iodine, 1 minute.

4) Wash off under tap.

5) Decolourise by running acetone over the slide.

6) Wash off under tap.

7) Counterstain with Safranin, 30 seconds.

8) Wash off under tap.

9) Air dry, and mount in DPX.

8.3.4 Papanicolaou's stain

Wet-fixed smears were used, and the staining performed in a Shandon automatic stainer. The staining solutions were purchased in bulk from Ortho Chemicals Ltd. The slides were continuously agitated in the following solutions.

1) 66 O.P. IMS, 30 seconds (66 overproof alcohol is an approximately 95% aqueous solution).

2) Distilled water, 90 seconds.

3) Haematoxylin, 5 minutes.

4) Distilled water, 2 x 90 seconds.

5) Acid alcohol, 1 minute.

6) 66 O.P. IMS, 2 x 1 minute.

7) Ammoniacal alcohol, 1 minute.

8) 66 O.P. IMS, 3 x 1 minute.

9) Orange G., 90 seconds.

10) 66 O.P. IMS, 3 x 1 minute.

11) EA50, 90 seconds.

12) 66 O.P. IMS, 2 minutes.

13) Absolute IMS, 3 x 1 minute.

14) Xylene, 3 x 90 seconds.

The slides were left in xylene before mounting in DPX.
8.3.5 PAS stain

Smears were air-dried, methanol-fixed.

1) Place in 1% aqueous periodic acid, 5 minutes.
2) Wash in tap water and rinse in deionised water.
3) Place in Schiff reagent (Raymond A. Lamb), 15 minutes.
4) Rinse in deionised water.
5) Wash in tap water, 10 minutes.
6) Counterstain in Harris' Haematoxylin, 2 minutes.
7) Blue in tap water, 15 minutes.
8) Dehydrate, clear, and mount in DPX.

8.3.6 Diastase - PAS

Saliva was used as the source of diastase. It was obtained by rinsing the mouth out thoroughly with distilled water, and then spitting into a clean glass tube. The saliva was well mixed with an equal volume of distilled water.

1) Rinse in distilled water.
2) Treat with saliva solution, 30 minutes.
3) Wash well in running tap water.
4) Stain by PAS, as above, together with an untreated control of identical material.

8.3.7 Perls' Prussian blue stain (for smears)

Wet-fixed smears were used. (The method for sections is given below.) The reagent was prepared by mixing equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid.

1) Rinse well in deionised water.
2) Place in freshly prepared reagent, 30 minutes.
3) Wash well in tap water.
4) Counterstain in Safranin, 15 seconds.
5) Wash off in tap water.
6) Dehydrate, clear, and mount in DPX.

8.3.8 Alcian Blue stain

This stain was used with various electrolyte concentrations by the method of Scott and Dorling (1965). They report that this allows differentiation of types of acid muco-substances.

A stock solution of 250 mg of Alcian Blue in 500 ml of distilled water was prepared. To 50 ml aliquots was added sufficient magnesium chloride to produce 0.06, 0.3, 0.5, 0.7 and 0.9 M solutions.

1) Stain serial sections in the various Alcian Blue solutions, overnight.
2) Wash in tap water.
3) Counterstain in neutral red, 3 minutes.
4) Wash in tap water.
5) Dehydrate, clear, and mount in DPX.

8.3.9 Phosphotungstic acid haematoxylin (PTAH)

The PTAH solution was prepared by dissolving 0.5 g of haematnin in 100 ml of water, and 5 g of phosphotungstic acid in 400 ml of water. The two solutions were mixed and stored overnight in an airtight bottle. Post-chromate solution was prepared by mixing 12 ml of 10% hydrochloric acid in IMS and 36 ml of 3% aqueous potassium dichromate. Acidified potassium permanganate was produced by mixing 50 ml of 0.5% aqueous potassium permanganate and 2.5 ml of 3% sulphuric acid.

1) Place solution in post-chromate solution, 30 minutes.
2) Wash in tap water.
3) Treat with acid permanganate solution, 1 minute.
4) Wash in tap water.
5) Differentiate in 2.5% aqueous ferric ammonium sulphate.

The slide was dipped into the solution, rinsed with tap
water, and examined microscopically. This process was repeated until mitochondria were visible.

6) Wash in running tap water, 10 minutes.
7) Dehydrate, clear, and mount in DPX.

8.3.10 Schmorl's Method

This stain (Schmorl, 1934) was used to detect lipofuscin in sections. Ferrous iron interferes with the reaction, but controls to detect this (Turnbull's method) were also prepared. The sections were examined microscopically after staining for 30 seconds, to observe the reaction, and staining terminated when judged to be adequate, in order to prevent background staining.

The staining solution was freshly prepared by mixing 37.5 ml of 1% aqueous ferric chloride, 5 ml of 1% aqueous potassium ferricyanide, and 7.5 ml of distilled water.

1) Immerse sections in staining solution until reaction is judged satisfactory. This took about 2 minutes.
2) Rinse in tap water.
3) Place in 1% acetic acid, 2 minutes.
4) Wash well in tap water.
5) Counterstain in Neutral Red, 2 minutes.
6) Wash in tap water.
7) Dehydrate, clear, and mount in DPX.

8.3.11 Chrome Alum Haematoxylin

This method (Pearse, 1954, after Gomori) was used to detect lipofuscin. The stain was prepared by mixing 50 ml each of 1% aqueous haematoxylin and 3% chrome alum, then adding 2 ml of 5% potassium dichromate and 1 ml of 5% sulphuric acid, and storing for 3 days.

1) Place sections in 0.5% potassium permanganate solution
2) Bleach in 1% aqueous oxalic acid solution, 1 minute.
3) Wash in tap water.
4) Stain in chrome alum haematoxylin, 10 minutes.
5) Differentiate in 1% alcoholic hydrochloric acid.
6) Counterstain in 1% aqueous eosin, 5 minutes.
7) Wash in running tap water.
8) Dehydrate rapidly, clear, and mount in DPX.

8.3.12 Perls' Method (for Sections)

This technique (Perls, 1867) demonstrates ferric iron, usually found in the form of haemosiderin.

1) Rinse in distilled water.
2) Place in 1% potassium ferrocyanide in 1% hydrochloric acid (both analytical grade reagents), 20 minutes.
3) Rinse in distilled water.
4) Counterstain in 1% Neutral Red, 1 minute.
5) Rinse in tap water.
6) Dehydrate, clear, and mount in DPX.

8.3.13 Turnbull's Method

This technique demonstrates ferrous iron, and was used principally as a control for the tissues stained by Schmorl's method.

1) Rinse in distilled water.
2) Place in 10% potassium ferricyanide solution in 1% aqueous hydrochloric acid (analytical grade reagents, freshly prepared), 15 minutes.
3) Transfer to 1% aqueous hydrochloric acid, 15 minutes.
4) Rinse in distilled water.
5) Counterstain in 1% Neutral Red, 1 minute.
6) Dehydrate, clear, and mount in DPX.

8.4 TRANSMISSION ELECTRON MICROSCOPY

Both whole tissue, and cells from BCF, were examined by transmission electron microscopy; the particles in BCF were examined using negative staining techniques. Scanning electron microscopy was employed to reveal the internal structure of small cysts.

8.4.1 Fixation

Small pieces of cystic breast tissue were prepared by cutting into 1 mm cubes; cells were obtained by centrifuging BCF, as described previously. Both were pre-fixed in 3% glutaraldehyde, and post-fixed in 1% osmium tetroxide.

Glutaraldehyde buffer was prepared by mixing 12 ml of a stock solution of 25% glutaraldehyde and 88 ml of 0.1 M cacodylate-HCl buffer, pH 7.2. Osmium tetroxide solution was prepared by adding 0.5 g of the salt to 50 ml of the same buffer.

The cells were fixed in glutaraldehyde for 30 minutes at 4°C, and then centrifuged. The fixative was poured off, and the cells washed in cacodylate buffer for 30 minutes. Following a rinse in the same buffer, they were post-fixed for 2 hours in osmium tetroxide at 4°C, and rinsed in cacodylate buffer.

Tissue specimens were treated in the same way, except that centrifugation was unnecessary, the solutions being merely poured off. Also, fixation times were increased to 90 minutes in glutaraldehyde, and 7 hours in osmium tetroxide.

8.4.2 Embedding

The tissues and cells were firstly dehydrated by washing in the following solutions.
(a) 50% aqueous alcohol (IMS), 10 minutes.
(b) 70% " " 15 minutes.
(c) 90% " " 15 minutes.
(d) Absolute alcohol, 30 minutes.
(e) Absolute alcohol, 30 minutes.

They were then embedded in EMix resin (Emscope Laboratories Ltd), an Araldite medium, of unspecified type, supplied in pre-measured quantities for mixing.

The procedure for clearing and embedding the dehydrated specimens was as follows.

(a) Propylene oxide, alcohol (1:3), 15 minutes.
(b) " " " (1:1) "
(c) " " " (3:1) "
(d) Absolute propylene oxide, 15 minutes.
(e) " " " "
(f) Specimen transferred to a small glass bottle, and a small amount of fresh propylene oxide added, followed by an equal volume of resin. The vessel was capped and placed on a rotator for 30 minutes, at 37°C in an incubator.

(g) The propylene oxide-resin mixture was removed and replaced by resin alone. The vessels were replaced on the rotator, uncapped, for 60 minutes, at 37°C.

(h) The specimens and resin were poured into plastic moulds, topped up with more resin, and polymerised overnight at 60°C.

8.4.3. Sectioning

Sections of the blocks were made on a Huxley ultramicrotome (Cambridge Instruments Ltd), using glass knives produced in the LKB
knife-maker.

Blocks were cut free from the mould and fitted into the microtome chuck. They were trimmed - around, and down to, the specimen - with a razor blade, to produce a small cutting face with the top and bottom edges parallel. A knife was selected, and a trough formed, using fabric sticking-plaster (Elastoplast), in front of the cutting edge, and the knife fitted to the microtome. The trough was filled with filtered double-distilled water. The knife was then advanced until it was almost touching the block face, and the motor drive started.

Sections were first cut at 2 μm thickness, using manual advance, until the wholeblock face was being cut, and then at 0.5 μm. Unwanted sections were removed from the water's surface with a small artist's brush, and the water in the trough topped-up as necessary. Two or three 0.5 μm sections were picked up on the brush, and transferred thus to a drop of water on a glass, microscope slide. The slide was left on a hot-plate at 70°C until dry, and a drop of 1% toluidine blue in 1% borax solution placed on the sections. When the stain began to steam, the slide was washed under the tap, dried, and examined with a light microscope. By this means the specimen was checked to ensure it was the type required before cutting thin sections. If not so, another block was selected, or else the block trimmed further into the tissue.

Thin sections were then cut using the automatic feed system of the microtome. The sections varied in colour when illuminated obliquely, due to interference phenomena, depending on their thickness. Only pale gold or silver sections (120 - 60nm) were selected for use, the others discarded after removing them from the trough, with a brush. The sections to be used were mounted on copper grids (usually 300 mesh).
The grid, held with fine forceps, was pushed below the water in the trough, the sections (usually about three) positioned near it using a mounted eye-lash, and the grid withdrawn obliquely with the sections adhering. The grid was dried by touching onto filter paper, and stored in a special box.

8.4.4 Staining

The sections were stained, generally with alcoholic uranyl acetate and aqueous lead citrate, in batches. Double-distilled and filtered water was used for preparing stains and wash baths. All stain solutions were filtered immediately before use through Whatman No.50 filter paper, and the uranyl acetate was prepared freshly.

Alcoholic uranyl acetate solution was prepared by adding 1.5 g of uranyl acetate to 50 ml of IMS and shaking until dissolved. Reynolds' (1963) lead citrate solution was prepared as a stock solution and stored at room temperature. To a mixture of 13.3 g of lead citrate and 17.6 g of sodium citrate (dihydrate) in a 500 ml volumetric flask, was added 300 ml of water. After shaking the flask intermittently for 30 minutes, 80 ml of 1 M sodium hydroxide solution was added, mixed, and the flask filled to the mark with water.

The staining was performed in grid holders made from perforated, plastic, block moulds, in which grids were transferred from one solution to the next. These were stored in 5% acetic acid, and washed twice in water before use. The stains were placed in small, capped, plastic pots. The staining routine was as follows.

(a) The grid-holder was placed in water and one grid placed in each bucket, ensuring that they sank.

(b) Wash in 70% aqueous IMS.

(c) Incubate in 3% alcoholic uranyl acetate at 37°C for 60 minutes.
(d) Rinse in 70% aqueous IMS.
(e) Rinse in three changes of water.
(f) Stain in Reynolds’ lead citrate for 5 minutes.
(g) Wash in two changes of 0.02 M NaOH solution.
(h) Rinse in two changes of water.
(i) Remove grids, dry by touching on filter paper, and store in grid-boxes.

8.4.5 Carbohydrate stain

For the demonstration of carbohydrate material in the electron microscope a variation of the PAS technique was used. The method was that of Vye and Fischman (1971), entailing staining by periodic acid-thiosemicarbazide-silver protein, which is a modification of the method of Thiéry (1967).

Sections were cut from blocks prepared as before, but with tissue fixed for 12 hours in buffered glutaraldehyde, and for only one hour in osmium tetroxide. The method was only used for breast tissue, not BCF cells. The sections were mounted on gold grids since copper reacts with the stain. The method used was as follows.

(a) Oxidise in 1% periodic acid for 30 minutes.
(b) Rinse in double-distilled water.
(c) Incubate in a 1% solution of thiosemicarbazide in 25% acetic acid for 60 minutes.
(d) Rinse in double-distilled water.
(e) Incubate in 1% aqueous silver protein (EMscope Laboratories Ltd.) at room temperature for 30 minutes, in the dark. The reagent was prepared fresh and filtered through Whatman No.50 filter paper, also in the dark.
(f) Wash for 60 seconds in running double-distilled water, dry, and store in grid-box.
Serial sections of the same block were used as controls, stained as above but, with one, omitting the periodic acid incubation and, with a second, omitting the silver protein reagent. These indicate any non-specific binding of silver protein, or any staining already in the section, respectively.

8.4.6 Negative stain

This technique was used for examination of the particles of BCF. Two stains - sodium tungstate and ammonium molybdate, both as 1% aqueous solutions - were used, and the samples were placed on collodion-coated grids.

The coated grids were prepared by the following method.

(a) A Petri dish containing a wire basket was filled with double-distilled water.

(b) Two drops of 2% collodion in amyl acetate were placed on the water surface from a height of 3 inches. After about 30 seconds the film produced had dried, and it was removed with forceps. This procedure cleans the surface of the water.

(c) About twenty copper grids (100 mesh) were placed on the basket under the cleaned water.

(d) A collodion film was made and dried on the water, as in (b), and the basket lifted out of the dish and placed on filter paper to dry.

(e) The film was cut by running a dissecting needle round each grid, and the grids then removed from the wire basket, using fine forceps, and transferred to a clean Petri dish for storage.

The samples, either whole BCF or fractions obtained from
density-gradient centrifugation, were carefully placed onto coated grids and after 30 seconds washed off with stain solution. Another method used was to mix equal parts of the sample and stain, and apply a drop to the grid. In both cases the grids were left for 60 seconds, and any remaining moisture removed with filter paper. The dried grids were stored in a grid-box.

8.4.7 Examination and photography

The grids were examined in a Phillips EM100B electron microscope. The machine was pumped down according to the manufacturer's instructions. A grid was placed into the holder which was then inserted into the microscope. The high tension, lenses, and filament current were turned on, the sections found on the grid using low magnification, and the specimen examined at appropriate magnification.

Photographs were taken on imperforate 35 mm Kodak Fine Grain Safety Positive film. Exposure was estimated by eye; the usual practice was to turn down the filament until the screen was barely illuminated, and then open the camera shutter for 5 seconds. The magnification was noted by the position of the coarse control and the reading of a meter connected to the fine control. From this the actual magnification could be determined from a calibration table prepared by measuring photographs, made in the microscope, of a replica grid of known dimensions.

The film was developed in Ilford Contrast FF developer (diluted 1:20 with water) at 20°C for 5 minutes. Development was stopped with a 1% acetic acid stop-bath, and the film fixed in Ilford IF23 fixer (diluted 1:3 with water, then 5% Ilford Acid Hardener added). After washing in running tap water for 30 minutes, the film was rinsed in distilled water and hung up to dry. When printing, the magnification of the print was also noted; which for the sake of convenience was
8.5 SCANNING ELECTRON MICROSCOPY

Two cysts excised from a mastectomy specimen, and about 4 mm in diameter, were carefully cut across to expose the interior, and placed into cacodylate-buffered 3% glutaraldehyde for 3 hours. After rinsing in cacodylate buffer they were placed in small, screw-capped bottles and posted to Dr. M. Johnson at the University of North Wales (Aberystwyth).

Preparations of the particle fraction were also made for scanning electron-microscopy. Samples of particles from a density gradient separation were pooled to give a total volume of 1 ml, and fixed by adding 10 ml of buffered glutaraldehyde solution. A Millipore filter (type GSWP, 0.22 µm pore size, 25 mm in diameter) was fitted into an Amicon ultrafiltration cell, the fixed particle solution added, and the cell pressurised to 10 p.s.i. (0.7 kgf/cm²) with nitrogen. When the liquid level had barely reached the filter, the pressure was slowly released, the filter removed and placed in a screw capped glass vial containing cacodylate buffer, and posted with the other specimen.

In Dr. Johnson's laboratory the specimens were dehydrated and dried as follows.

(a) 30% aqueous ethanol, 15 minutes.
(b) 50% aqueous ethanol "
(c) 70% aqueous ethanol "
(d) 100% ethanol "
(e) Flurosol, ethanol (1:3), 10 minutes.
(f) " " (1:1) "
(g) " " (3:1) "
(h) absolute Flurosol, 15 minutes.
(i) " " "

145
The specimens were then dried in a E3000 critical point drying apparatus and mounted onto stubs using Kodaflat. After coating with gold-palladium in a diode sputtering unit, they were examined in a Cambridge scanning electron microscope, and photographs taken on 35 mm Ilford FP4 film.

8.6 MICROBIOLOGY

In order to establish the sterility of BCF, and exclude the presence of any infection, attempts were made to culture organisms from BCF. A series of 20 BCFs was carefully collected aseptically. Following aspiration of the fluid, the syringe needle was removed and the contents of the syringe gently expelled into a sterilised, Universal bottle. The cap of the bottle was only removed enough to allow access to the syringe, and was rapidly replaced afterwards.

On returning to the laboratory the fluids were each streaked onto four plates; two blood agar, one McConkey, and one Sabouraud plate (for yeasts). One of the blood agar plates was incubated anaerobically, the others aerobically, at 37°C. They were examined after 16, 24, and 48 hours.
9 PHOTOGRAPHIC METHODS

9.1 PHOTOGRAPHY

Monochrome photographs were produced using Ilford PAN F film at the normal rating of 50 ASA. Light micrographs were taken in a Leitz photo-microscope fitted with Leitz flat-field objectives. Slides and microscope lenses were carefully cleaned, and the microscope adjusted by the Köhler method. Exposure was automatic, being estimated by the built-in, integrating, exposure meter.

Photographs of electrophoresis strips and immunoelectrophoresis plates were taken using a Nikon F camera, fitted with a Photomic exposure meter (Nikon). The lens used was a 50 mm, f3.5 Macro-Nikkor (Nikon), with an extension ring for close-up work. The specimens were transilluminated using a Shandon daylight lamp on which was rested a sheet of opal glass, to act as a diffuser. The specimen was placed on this and photographed from above, with the camera fitted to a Nikon copying stand. Exposure was estimated using the built-in exposure meter. Three exposures were made, one at the indicated value and one at one stop above, another one stop below. For lightly stained subjects, an appropriate filter was used to increase contrast.

Photography in the electron microscope has already been described (see "Electron Microscopy", above).

9.2 PROCESSING

The films were developed in a Patterson tank, using ID11 developer (Ilford). The solution was brought to 20°C, and poured into the tank. The film was agitated continuously for the first 20 seconds of development, and thereafter by twice inverting the tank every minute. After 6½ minutes, the developer was poured out and fixer (Ilford IF23, diluted
1:3 with water and then adding 1 ml of Ilford Liquid Acid Hardener for each 20 ml), also at 20°C, rapidly poured in. The film was agitated continuously for 1 minute, then the tank lid was removed, and the time taken for undeveloped emulsion to clear was noted. Fixation was continued for twice this clearing time, and the film then washed in running tap water at approximately 20°C for 30 minutes. After rinsing twice in distilled water, and once in distilled water with a drop of liquid detergent added, the film was hung up to dry. When dry, it was cut up into six-frame lengths and stored in glassine envelopes.

9.3 PRINTING

Prints were made on Ilford YRP glossy paper of a suitable hardness, usually grade 2 (normal), using a Durst enlarger (model 606), fitted with a Durst Companar, 75 mm, f4.5 lens. The prints were developed and stabilised in an Ilford automatic processor (type 1502) with IA11 activator and IS21 stabiliser. Satisfactory prints were then fixed (The stabiliser allows the prints to be examined in artificial light, but the results are not permanent) for 5 minutes in Ilford IF23 fixer (diluted 1:6 with water and adding 5% v/v Ilford Liquid Acid Hardener). For prints requiring extra permanence, a further 5 minutes in a fresh bath of fixer was given.

The fixed prints were washed in running tap water for one hour, and then dried and glazed using a rotary glazer.

Some prints were also made on other types of paper and developed in Ilford PQ Universal developer (diluted 1:10 with water) for 2 minutes. Fixation and washing were carried out as before.

Colour photography was carried out using Kodak Vericolour II, Professional, Type S negative film. This was mainly used for photo-
micrography using the Leitz photomicroscope, with a blue filter to correct the colour temperature of the light from the tungsten filament.

In the camera the film was exposed in daylight, and rated at 100 ASA. Developing and printing were carried out by a commercial laboratory. Some colour prints were prepared on reversal-type paper, from colour slides. This was also carried out by a commercial laboratory.
RESULTS

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1.1 **PATIENT DATA**

1.1.1 **Age**

The ages of 1043 breast cyst patients from all hospitals in the area are shown graphically in Fig. 11. Those of the smaller sample of patients, from Mayday Hospital Breast Clinic, are shown graphically in Fig. 12, together with their menstrual status.

The great majority of the patients were of menopausal age, and the age distribution of the small sample, from which extensive data was obtained, is shown to be the same as that of the large group. In the younger patients, a history of recent lactation was a frequent finding.

1.1.2 **Parity**

The parity of the patients in the small group is shown in Fig. 13. This shows that all parity groups up to 6 are represented, and suggests that there is no major influence of this factor on breast cyst formation.

1.2 **CYST PHYSICAL DATA**

1.2.1 **Volume**

The volumes of BCF obtained at aspiration (presumably representing cyst volume), and their depth of colour, are shown in Fig. 14. The median volume was 2 to 4 ml, and the majority of cysts were smaller than 10 ml.

1.2.2 **BCF colour**

The colour of cyst fluid was found to be very variable, ranging from a pale straw, through various shades of yellow, brown, and green, to a very dark greenish-brown (see Fig. 15).

The fluids also had a characteristic "milkiness" which varied from a slight opacity to greater than that of human milk.
Fig. 11  Age distribution of breast cyst patients (1043 patients).

Fig. 12  Age distribution of patients of the Mayday Hospital series, showing their menstrual status.
Fig. 13  Parity of patients of the Mayday Hospital series.
Fig. 14. Volume and colour of BCF samples from the Mayday Hospital series.
Fig. 15  Appearance of breast cyst fluid. Note the range of colour, and the characteristic "milkiness".
1.2.3 Site

The quarters of the breasts in which cysts of the Mayday sample were located are shown diagramatically in Fig. 16. Of these cysts the majority (56.2%) were found in the left breast. In both breasts the commonest site was the upper outer quadrant, and over 80% of cysts were in the upper half of the breast.
Fig. 16 Site of breast cysts in patients of the Mayday Hospital series.
The cellular content of BCF was examined in order to gain information about the types of cell present, and to relate such information to the presence of other components in the fluid. Histological sections of breast tissue were used to show the structure of 'pink' cells, and to test their reaction to various cytochemical methods. The ultrastructure of 'pink' cells was observed, by electron microscopy, in order to see whether information on secretion by such cells could be obtained.

2.1 CELLS FROM BREAST CYST FLUID

The free cells in BCF are a mixed population, with various numbers of each type present in different fluids. They are sometimes rather degenerate in appearance, and usually accompanied by what appears to be fine granular debris, presumably of cellular origin.

2.1.1 Epithelial cells

The most frequently found type is the 'pink' cell (see page 30). In cytological preparations these invariably appear in groups or large clusters, and sometimes appear degenerate. The cells (see Fig. 17) have a central to polar nucleus, with evenly dispersed chromatin and a large nucleolus, and abundant granular cytoplasm. In large groups, the periphery shows the characteristic lobulated appearance also seen in histological sections of 'pink' cells. The frequent finding of papillary formations suggests that this type of epithelium is proliferative in nature.

The degenerate 'pink' cells are of similar appearance, but often smaller, with a pyknotic nucleus. They sometimes have an enlarged nucleus showing margination of chromatin, and a very prominent nucleolus. The cytoplasm is also often vacuolated.
Fig. 17 The appearance of 'pink' cells in a smear of the cell deposit obtained by centrifuging BCF. The 'pink' cells are on the left of the picture. M.G.G. stain. (x400)
The 'pink' cells contain PAS-positive granules (see Fig 18), and these are often concentrated at one side of the cytoplasm. If the cells are in large groups, this side is seen to be the apical end. The reaction with Perls' stain was usually negative, but occasional very weak positives showed an overall reaction in the cytoplasm. A granular pattern was never seen.

Normal duct cells were sometimes seen. These were somewhat smaller than 'pink' cells and had a smaller, denser nucleus, and much less cytoplasm.

2.1.2 Other cell-types

The other cell-type which was frequently found is the polymorphonuclear leucocyte. These were found to be present, in small numbers, in 60% of BCF specimens, in large numbers in 25%, and to be absent in 15%. In the fluids which had large numbers, other leucocytes were also present, including lymphocytes and plasma cells (see Fig. 19).

Erythrocytes were found in 40% of BCF specimens, usually in small numbers. They are presumably picked up by the aspirating needle as it travels through the breast tissue surrounding the cyst.

2.1.3 Relationship of cell type to BCF electrophoresis pattern

In a series of 227 BCFs, the presence of lymphocytes and plasma cells was correlated with the cellulose-acetate-electrophoretic pattern (See p.176). The results (see Table 2) show that there is a strong association between the presence of many leucocytes (including lymphocytes and plasma cells) in the fluid, and the serum-type electrophoretic pattern. The cytological patterns are illustrated in Fig. 19, the electrophoretic types in Fig. 32.
Fig. 18 'Pink' cells stained by the PAS technique. Note the strongly stained granules. (×400)
Fig. 19  (a) BCF cell smear, showing pattern with large numbers of leucocytes, including lymphocytes. HE stain. (x400)
(b) BCF cell preparation, showing the pattern with very few leucocytes, and a clump of epithelial cells. Filter preparation, HE stain. (x400)
<table>
<thead>
<tr>
<th>Cytology</th>
<th>Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocytes (including lymphocytes)</td>
<td>Serum-type</td>
</tr>
<tr>
<td></td>
<td>41 (18.1%)</td>
</tr>
<tr>
<td>No leucocytes, or a few polymorphs</td>
<td>15 (6.6%)</td>
</tr>
</tbody>
</table>

TABLE 2  Correlation of cellulose acetate electrophoretic patterns and cell types in breast cyst fluid
2.2 HISTOLOGICAL SECTIONS

2.2.1 Cystic breast tissue

In histological sections, the cysts were found to be lined by 'pink' epithelium (Fig. 20). The cells are generally in one layer, but papillae are often found projecting into the lumen. The cell shape varies with the intracystic pressure, but in microcysts the cells are moderate to tall columnar. In larger cysts, with higher pressure, the epithelium tends to become flattened. The epithelium rests on a basement membrane, with occasional myoepithelial cells.

Schmorl's stain gave only a slight over-all background reaction, showing absence of lipofuscin. This was borne out by the chrome-alum haematoxylin stain. Alcian blue staining varied with the salt concentration; the results are given in Table 3. They show that 'pink' cells contain only carboxylated or weakly sulphated mucins. The material in the cyst lumen showed traces of strongly sulphated mucins. The connective tissue reacted at all electrolyte concentrations, and showed a trace of keratan sulphate.

Turnbull's reaction was negative in 'pink' cells, as was Perls' stain, showing the absence of ferrous and ferric iron.

2.2.2 Cytoplasmic granules

Granules which are PAS-positive were found in the histological sections of 'pink' cells (Fig. 21), as well as in the cytological preparations mentioned above. They are located mainly at the apical end of the cells. Staining was not abolished by prior incubation with diastase, and the granules are therefore not glycogen.

Mitochondria were demonstrated in 'pink' cells by the PTAH stain (Fig. 22). The same type of reaction was given by Haidenhain's method, although showing fewer granules.
Fig. 20  Histological section of 'pink' cells lining a microcyst. The Golgi complex may be seen as an understained area in the supranuclear region.  (c.f. Fig. 24)  HE stain.  (x 315)

Fig. 21  Histological section of 'pink' cells lining a micro-cyst, stained by the PAS technique. The PAS-positive granules are seen to be at the apical end of the cells.  (x 400).
TABLE 3  Staining of Cystic breast sections by Alcian Blue at various concentrations of electrolyte

<table>
<thead>
<tr>
<th>Electrolyte concentration</th>
<th>'Pink' cells</th>
<th>Lumen material</th>
<th>Connective Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06 M</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>0.3 M</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>0.5 M</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>0.7 M</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>0.9 M</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+++ strong reaction
++ moderate  
+ weak  
- Negative

Components stained at various electrolyte (MgCl₂) concentrations (Scott and Dorling, 1965) are as follows.

0.06 M : carboxylated and sulphated mucins.
0.3 M : weakly and strongly sulphated mucins.
0.5 M : strongly sulphated mucins.
0.7 M : highly sulphated connective-tissue mucins.
0.9 M : keratan sulphate only.
Fig. 22 Histological section of 'pink' cells lining a microcyst, stained by the PTAH method. (x 315).

Fig. 23 Histological section of axillary skin, showing secretory cells of an apocrine gland. The Golgi complex is seen as an understained area in the supranuclear region (c.f. Fig. 20). HE stain (x 315)
2.2.3 Golgi complex

No staining methods were used to show positively this organelle. However, it is clearly seen in HE-stained sections (Fig. 20) as an understained area in the supranuclear region. It is also shown in the PTAH-stained sections by a reduction in density of mitochondria in the same area.

The organelle can also be seen in the skin apocrine gland cells (see Fig. 23), in the same position as that in 'pink' cells.

2.3 ELECTRON MICROSCOPY

2.3.1 'Pink' cells

The ultrastructural appearance of 'pink' cells is shown in Fig. 24. They have many characteristics of a secretory cell, and strongly resemble apocrine skin gland cells, ultrastructurally (e.g. Ellis, 1967). The shape is variable, depending on the distension of the cyst, but is usually taller than its width. The nucleus is basally situated and has one or two nucleoli.

The apical surface is covered by microvilli, and below these are usually many small vesicles, not unlike those found in apocrine skin gland cells (Ellis, 1967).

Mitochondria are present in moderate to large numbers, and frequently have few, or altered, cristae. They are seen more clearly at higher power (Fig. 25). Endoplasmic reticulum, both rough and smooth, is present, and sometimes abundant.

The plasma membrane at the base of the cell is very invaginated, (see Fig. 26), presumably to give greater surface area for the intake of nutrients.

A well developed Golgi complex is seen in the supranuclear
Fig. 24  Electron microscopic appearance of 'pink' cells. Microvilli, mitochondria (M), endoplasmic reticulum (both rough and smooth) and Golgi complex (G) may be seen. The dense osmiophilic bodies in the supranuclear region in (a) are seen in an understained section (b) to contain small dense granules. Lipoprotein vesicles are also seen (arrowed) in (b). Fixed in glutaraldehyde and OsO₄, stained uranyl acetate and lead citrate.
Fig. 25  High power electron micrograph of a section of a 'pink' cell, showing mitochondria surrounded by rough endoplasmic reticulum (arrow). The cristae of the mitochondria are sparse and somewhat deformed. Fixation and Stain as in legend to Fig. 24. (x 24,500).

Fig. 26  Electron micrograph of a section of 'pink' cells, showing the invaginations of the basal plasma membrane. The epithelial basement membrane and nucleus of a myoepithelial cell are also visible. Fixation and Stain as in legend to Fig. 24. (x 15,000).
region (Fig. 24). It is of the same size, and in the same position, as the body seen using the light microscope, as mentioned above. Around this are a number of large osmiophilic bodies which, in an understained section, can be seen to contain small dense granules (Fig. 24). These probably correspond to the PAS-positive granules seen by light microscopy, and are identical to those in apocrine skin gland cells (Ellis, 1967). It is possible that they are secretory granules, but their secretion has not been seen in any sections, and they may be altered mitochondria. The attempts to stain these granules by the electron-microscope equivalent of the PAS stain (the Periodic acid-thiosemicarbazide-silver proteinate reaction) were not very successful. Some reaction was obtained with the osmiophilic bodies, but there was insufficient contrast to prepare usable photographs. The controls were negative.

2.3.2 Particle-fraction of breast cyst fluid

The appearance of the particles separated by centrifugation (see "Centrifugation Methods" in Results) from a BCF sample is shown by the negative-stained preparation in Fig. 27. They have a very variable shape and size, but the average diameter is 0.2 μm. Their appearance suggests a membranous nature. Attempts were made to prepare sections of the material for electron microscopy, but they were not successful. However, a section of the material in the lumen of a cyst is shown in Fig. 28. It should be emphasised that this is the material from a microcyst, whereas the negative-stained preparation is of unsectioned material separated from an aspirated macrocyst fluid. However, the particles seen are of a similar appearance.

2.3.3 Scanning electron microscopy

The interior of a breast cyst, as revealed by scanning electron microscopy, is illustrated in Fig. 29; a high-power view of the
Fig. 27  Electron-microscopic appearance of the particle-fraction from two BCF specimens, in negative-stained preparations. The particles have a variable shape and size, with an average diameter of 0.2 μm, and have a similar appearance in the two specimens. Negative stain using sodium tungstate. (Both x 19,440).

Both preparations are of particles separated from whole BCF by sucrose density gradient centrifugation.
Fig. 28  Electron micrograph of a section of a microcyst which was excised and fixed whole. The contents of the lumen of the cyst are shown, and they have a similar appearance to the particles in Fig. 27. Fixation and stain as in legend to Fig. 24. (x 19,400).

Fig. 29  Scanning electron micrograph of the interior of a bisected microcyst. Several papillae are seen inside the cyst. Fixed in glutaraldehyde and coated with gold-palladium.
epithelium is in Fig. 30. In Fig. 29, several papillae may be seen
inside the cyst.

The microscopic appearance of the particles by this method is
shown in Fig. 31. The exercise was not very successful, but some idea
of particle shape is obtained.

2.4 MICROBIOLOGY

No growth was obtained on any of the plates which were inoculated
with the 20 aseptically collected BCF specimens; this demonstrates that
the fluids do not contain bacteria or yeasts. The possible presence of
viruses was not investigated.
Fig. 30 Higher power scanning electron micrograph of the cyst illustrated in Fig. 29. The epithelial cells appear irregular in size, and have a rough surface presumably due to the microvilli. In (b) the epithelium is folded back, thus revealing the under-side and the stroma beneath. Preparation as in legend to Fig. 29.
Fig. 31. Scanning electron micrograph of the particles of BCF. The glutaraldehyde-fixed particles are on a Millipore filter-membrane, and coated with gold-palladium.
The proteins of BCF were shown to be generally unlike those of serum and much of the work was concentrated upon them. A variety of methods was used to attempt to separate them; their identity was investigated by immunological techniques. The necessity to raise an antiserum to BCF became obvious after this work, in order to characterise the proteins, and to see whether any of them could be shown to occur in the epithelial cells lining breast cysts.

Estimations of molecular weight of the proteins were made by SDS-acrylamide-gel electrophoresis, since the method is simple and can be applied to mixtures of proteins.

3.1 ELECTROPHORESIS

3.1.1 Cellulose acetate

Typical examples of the results of cellulose acetate electrophoresis of BCF are illustrated in Fig. 32, together with serum and human milk controls. The patterns for BCF can be divided into two major groups. These are designated "pure-type" and "serum type", and represented roughly 80% and 20% of BCFs, respectively. Pure-types had a long streak stretching from the application point up to, or beyond, the position of albumin in the serum control; no discrete bands, with the possible exception of albumin; and no cathodically migrating material (γ-globulin). Serum-types were so classified on the basis of their possessing at least a trace of γ-globulin. They ranged from those having a trace of γ-globulin and perhaps one discrete, anodically migrating band, to those having a pattern identical to that of serum. In this study, no attempt was made to subdivide the two patterns, since the number of such smaller groups would have been very large.
Cellulose acetate electrophoresis of BCF, human mature skimmed milk, and serum. Identical specimens are stained to demonstrate protein (upper strip) and carbohydrate (by PAS, lower strip). The electrophoretic pattern of pure-type BCF (P) is shown, together with a range of serum-type BCFs (S), and milk (M) and serum (Se). The anode is at the top end of each strip.
Electrophoresis of patient's serum revealed no abnormalities.

Use of a PAS stain following cellulose acetate electrophoresis, showed a strong reaction of pure-type BCF proteins (Fig. 32), indicating that much of the protein is glycoprotein.

Since poor resolution of the proteins in pure-type fluids was obtained using this method, other techniques, such as polyacrylamide-gel electrophoresis and isoelectric focussing were tried.

3.1.2 Acrylamide gel

No improvement in resolution of pure-type BCF proteins was obtained using this technique, and the use of various buffer systems and gel concentrations had no effect. The pattern obtained was very similar to that on cellulose acetate electrophoresis, with a long, weakly staining band stretching almost the full length of the gel.

The serum-type fluids again gave a pattern resembling that of serum. However, for some, the pattern had an appearance of being combined with that of pure-type fluids, suggesting that both pure-type and serum-type proteins were present.

3.1.3 SDS - gel

The incorporation of SDS into the gels and samples had a great influence on resolution of pure-type BCF proteins. The technique was used with both disc and flat-bed methods, and showed, in the pure-type fluids, at least four bands in disc gels, and up to ten in some flat-bed runs. The presence of glycoprotein was again shown by PAS staining.

The technique was also used solubilise the particle-fraction of BCF and thus allow electrophoresis of its protein. The results were rather inconclusive, but seemed to show the presence of at least one protein not found in the fluid-fraction of BCF. Whether the other proteins were simply contaminants from the fluid fraction of BCF is not known.
3.1.4 Molecular weight determinations

The results of the SDS gel electrophoresis of pure-type BCF, by the method of Weber (1972), in order to estimate protein molecular weights, are illustrated in Fig. 33. A number of results are also shown diagramatically in Fig. 34.

The majority of the sub-unit bands occurred in all pure-type fluid samples, but their relative concentrations were variable. There were four major bands, representing molecular weights of 16,000, 29,000, 43,000 and 70,000. One of these always predominated (M.W. 29,000), the others appeared more variable. The molecular weights of all pure-type BCF proteins ranged from 15,000 to 100,000.

Omission of heating the sample at 100°C, in order to check for any enzymic hydrolysis, produced a pattern with fewer bands. This pattern was consistent, even if the samples were left for 24 hours before running, and it was concluded that the variation was due to incomplete reaction with SDS or mercaptoethanol, rather than enzymic breakdown of protein. The extension of sample heating produced no changes in the normal pattern.

The use of 7.5% gels, instead of the normal 10%, gave some variation in the calculated molecular weights, but for most bands this was no more than 10%.

The PAS staining method used for these gels showed several non-reacting components to be present. The major bands stained strongly, and, although some showed a very weak positive reaction in the controls, the presence of glycoproteins is demonstrated.

3.1.5 Isoelectric focussing

This gave by far the best resolution of BCF proteins. With pure-type fluids, from 14 to 23 components were demonstrated (Fig. 35),
Fig. 33  SDS-polyacrylamide-gel electrophoresis of pure-type BCF specimens. The anode is at the bottom of the photograph, and thus the molecular weight of the proteins decreases down the gel (see also Fig. 34).
Fig. 34 Diagram of pure-type BCF proteins and lactoferrin (L), separated by SDS-polyacrylamide-gel electrophoresis. The molecular weight scale (in thousands) is derived from the results for standards run coincidentally. The major BCF-protein bands are indicated by open arrows. Lactoferrin showed four trace contaminants; the main band is indicated by the solid arrow.
Isoelectric focussing of BCF. Five pure-type fluids are shown, with two serum-type specimens on the right of the photograph. The picture covers the range from pH 3 (at the top) to pH 6 (at the bottom).
and over 50 were seen in serum-type fluids.

In the pure-type BCF, the proteins were all shown to have a low pI. The range was from pH 3 to pH 6 or less. In the serum-type fluids, the range covered almost the entire gel, from pH 3.5 to pH 10.

The technique was also used to analyse the fractions obtained from column chromatography of BCF and milk (see below).

3.2 COLUMN CHROMATOGRAPHY

A typical separation of Sephadex G-200, as shown by the effluent absorbance at 280 nm, of pure-type BCF is presented in Fig. 36, and of human skimmed-milk in Fig. 37.

The milk gave four well-separated peaks, the BCF samples gave three main peaks (1,3 and 5) with two minor ones (2 and 4). In both cases, peak 1 was the fraction excluded from the gel. Peaks 2, 3 and 4 in milk corresponded approximately with 3, 4 and 5 in BCF.

The peaks were analysed, after concentration, for serum proteins, using immunoelectrophoresis. The milk had IgG and IgA in peak 1, a complete milk pattern, but with reduced immunoglobulins, in peak 2, a weak arc in the β-globulin position in peak 3, and no serum protein in peak 4. The BCF samples had albumin in peak 3, and a β-globulin arc in peak 1. Peaks 2, 4, and 5 had no serum protein.

The carbohydrate analysis showed all such material to be present in the excluded fraction (peak 1) of the BCF separations. This peak was also found to contain all the cholesterol of BCF. It contained most of the protein, and had a very milky appearance, due to the BCF particle-fraction. The milk cholesterol was also mainly in peak 1, but with a trace in peak 2.

The proteins in each peak from BCF and skimmed milk were also
Fig. 36 Absorbance at 280 nm of the effluent from a chromatographic separation of pure-type BCF on a column of Sephadex G-200. Five peaks were obtained. (For details of the method, see "Column Chromatography" in Methods.)
Fig. 37 Absorbance at 280 nm of the effluent from a chromatographic separation of human skimmed milk on a column of Sephadex G-200. Four well-separated peaks were obtained. (For details of the method, see "Column Chromatography" in Methods.)
separated by isoelectric focussing. The results for the whole milk and peaks 1, 2 and 3 are shown in Fig. 38, and for the whole BCF and peaks 1 to 4 in Fig. 39. The majority of the milk protein is seen to be in peak 2, and that of BCF protein to be in peak 3. These peaks were eluted at approximately corresponding volumes, suggesting the majority of BCF and milk proteins have molecular weights of a similar order of magnitude.

The proteins of BCF in peak 1, which contained all the carbohydrate material reacting with the carbazole reagent, were all of very low pI, with one exception. This is a protein seen around the centre of the gel, with a pI of about 6.8. Since the particle-fraction is in the gel-excluded peak (peak 1), this may be a protein which is bound to, or forms a part of, the particles. Peaks 4 and 5 contain no protein material demonstrable by isoelectric focussing, neither does peak 4 of milk. Presumably they represent either non-protein components of low molecular weight which absorb at 280 nm, or small peptides which cannot be fixed and are therefore leached from the gel during staining. This may be the same material that is found in BCF ultrafiltrates (see p. 195)

During the fractionation, the colour of the BCF was found to separate into two components. The dark-coloured fluids produced a brown and a green band, which appeared in peak 3 and 5 respectively. With paler fluids, these components appeared in the same fractions, but were brown and yellow in colour.

3.2.2 Ion-exchange

A typical elution pattern, again measured by absorbance at 280 nm, is shown in Fig. 40. Six, mostly poorly separated, peaks were obtained. These were analysed by immunological methods using absorbed
Fig. 38 Isoelectric focussing of a human skimmed milk specimen and the fractions corresponding to the peaks obtained after it was separated by chromatography on a Sephadex G-200 column (see Fig. 37). Reading from left to right the specimens are: whole skimmed milk; fractions 1, 2, and 3.
Fig. 39 Isoelectric focusing of a pure-type BCF and the fractions obtained after it was separated on a Sephadex G-200 column (see Fig. 36). Reading from left to right the specimens are: whole BCF; fraction 1, 2, 3 and 4.
Fig. 40 Absorbance at 280 nm of the effluent from a chromatographic separation, on a column of QAE Sephadex A-25 anion exchanger, of a pure-type BCF specimen (previously de-salted on Sephadex G-25). The molarity of sodium chloride in the buffer is shown at top. Six mostly poorly separated peaks were obtained. The BCF-specific proteins were in peak No. 2. (For details of the method, see "Ion-exchange Chromatography" in Methods.)
anti-(BCF). Both the BCF-specific proteins (see below, Section 3.3.5) were found in peak 2. Since the aim of the experiment was to separate the two proteins from each other, different conditions, such as using a shallower gradient and a different buffer pH, were tried. No improvement in separation was obtained.

The gradient-mixer used was found, when tested, to prepare reproducibly linear gradients of bromophenol blue.

3.3 IMMUNOLOGICAL TECHNIQUES

These techniques were used for the detection of specific proteins of serum and milk in BCF. The object was to see whether BCF resembled either of these fluids; this would then throw some light upon its source. The anti-(BCF) was also used to show the presence of milk and serum proteins in BCF, and to demonstrate the existence of BCF-specific proteins.

3.3.1 Serum proteins

The reaction obtained between BCF and anti-(whole human serum) in immunoelectrophoresis depended on the type of fluid. Pure-type BCF gave very little reaction, while serum-types gave more arcs, the number increasing the more the cellulose-acetate-electrophoretic pattern resembled that of serum.

The pure-types all gave one arc, in a position corresponding to that of albumin, although of a slightly higher electrophoretic mobility in many cases (Fig. 41). In many fluids, this arc was very indistinct and tailed back towards the application well, occasionally reaching it. In 30% of pure-type fluids, there was a small arc around the application well. In 10% there were one or two additional arcs in the \( \alpha_2 \) and \( \beta \) regions.

Albumin was shown to be present in pure-type BCF, using anti-
Fig. 41 Immunoelectrophoresis of BCF against antiserum to whole-human-serum proteins. A pure-type BCF is on the left, a serum-type BCF on the right, and a control human serum in the centre. The serum-type fluid has a pattern similar to that of serum, but with fewer arcs; the pure-type fluid shows very little reaction with the antiserum.
(human albumin) in immunodiffusion experiments. A reaction of complete identity was obtained with albumin of serum controls. Originally, attempts were made to demonstrate this identity in immunoelectrophoresis using anti-(whole human serum), and allowing the BCF arc and that of a serum control to form close together. However fusion of the arcs, and a reaction of identity, were not obtained.

Serum-type fluids showed more reaction, with up to 12 arcs, including IgG, IgM, and IgA (Fig. 41). They never showed as many arcs as serum controls, and therefore had fewer proteins than serum. A most noticeable feature in some of these fluids was a tailing of the albumin arc, similar to that seen in pure-types but less marked. This almost seemed to be a mixture of pure-type pattern and serum.

The serum obtained from breast cyst patients of the Mayday sample showed no gross abnormalities in immunoelectrophoretic examination using anti-(whole human serum).

3.3.2 Immunoglobulins

Although cellulose acetate electrophoresis revealed no γ-globulin in pure-type BCF, immunodiffusion of un-diluted fluid against suitably diluted specific antisera showed IgG and IgA to be present in low concentrations; IgM was not found.

The results of the assay of IgG and IgA are given in Table 4. They show IgG to be low relative to serum values, but within the range for human milk, and IgA to be low relative to both serum and milk.

Only 30% of the fluids had levels of IgA greater than IgG. In milk and other secreted fluids IgA predominates. Also, neither secretory IgA nor secretor piece could be found in BCF, by immunodiffusion against specific antisera.
<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF</td>
<td>10.5 (10.8)</td>
<td>5.4 (4.0)</td>
</tr>
<tr>
<td>*Serum</td>
<td>720–1680</td>
<td>80–480</td>
</tr>
<tr>
<td>*Milk</td>
<td>4–43</td>
<td>150–1735</td>
</tr>
</tbody>
</table>

Values are all mg/100 ml. Standard deviations in parentheses.

*Normal ranges for serum and milk from Documenta Geigy.
3.3.3 Lactoferrin

The presence of lactoferrin in BCF was shown by immunodiffusion against antiserum to human lactoferrin. The precipitate gave a reaction of identity with that of human milk controls. The concentration was roughly 20% of that in milk.

Confirmation of this observation was obtained by the presence of antibody to lactoferrin in the BCF antiserum. This was demonstrated by its reaction with purified lactoferrin in immunodiffusion, and Laurell immunoelectrophoresis.

3.3.4 Milk proteins

Immunodiffusion of pure-type BCF against anti-(whole human milk) revealed several reacting proteins. These were shown to be, with one exception, due to low concentrations in pure-type BCF of serum proteins also present in milk. This was demonstrated using Laurell immunoelectrophoresis with antiserum to milk and incorporating a serum strip. The antigen excess of serum proteins removed all the peaks from the gel, except the one previously mentioned. By running purified lactoferrin in the same system, from a second well on the plate, two fused peaks were obtained giving a reaction of identity. Therefore the one non-serum protein was lactoferrin.

3.3.5 BCF-specific proteins

BCF was shown to contain at least two specific proteins relative to milk and serum. Despite the poor reaction of pure-type BCF with anti-(whole human serum), there was a sufficient quantity of serum proteins present to produce antibodies in the rabbits.

With anti-(BCF), the pure-type fluids showed the presence of 8 to 10 proteins in immunodiffusion and immunoelectrophoresis. The antiserum also reacted with human serum, and therefore some of the reacting proteins
must be serum proteins. After absorption of the antiserum with 50 mg of freeze dried plasma per ml, no further reaction was obtained with serum in immunodiffusion. With BCF, the absorbed antiserum gave 3 zones of precipitation in immunodiffusion, and 3 arcs on immunoelectrophoresis.

The same effect was shown using Laurell immunoelectrophoresis. With pure-type BCF against unabsorbed anti-(BCF), 7 peaks were obtained (Fig. 42). Introduction of a serum-containing strip, as before, effectively absorbing the antiserum in situ, reduced the number of peaks to 3. These peaks all had a similar electrophoretic mobility, close to that of albumin. Addition of human skimmed-milk to the serum-containing strip caused the smallest of the peaks to disappear (Fig. 42), showing it to be a milk protein. This peak was again identified as lactoferrin (see section on "Milk Proteins", above), since it gave a reaction of identity with purified lactoferrin electrophoresed coincidentally with BCF, from a separate well. The pure lactoferrin, milk, and BCF all gave one line of precipitate, with a reaction of identity, when diffused against absorbed anti-(BCF).

When the anti-(BCF) absorbed with freeze dried plasma was further absorbed with human skimmed-milk, only two lines of reaction were obtained against BCF. This, therefore, was the antiserum used for immuno-histochemical experiments.

3.3.6 Titre of antiserum to BCF

This was estimated by rocket electrophoresis, and was found to reach a maximum after 6 months. Pool number one was found to yield the higher titre of both BCF and serum protein antibodies, and this one was therefore used for most experiments.

3.3.7 Ultrafiltrate of BCF

The filtrate produced during concentration of BCF, for various
Fig. 42 (a) Laurell immunoelectrophoresis of pure-type BCF against unabsorbed antiserum to BCF. Compare with Fig. 42 (b), overleaf.
Fig. 42 (b) Laurell immunoelectrophoresis of pure-type BCF against antiserum to BCF. A strip of gel, containing human milk and serum, was placed between the running and antiserum gels. This effectively absorbs out in situ any antibodies to milk and serum proteins, leaving the two peaks due to BCF-specific proteins (arrows). The two reaction lines half-way up the antiserum gel are due to there being insufficient antigen in the strip to create an antigen excess, and thereby push them right off the plate.
purposes, using a UM2 membrane, was found to contain materials absorbing
strongly in the ultra-violet region. A typical absorption spectrum of
a 1:100 dilution of the filtrate is shown in Fig. 43. The strength of
this absorbance is thus shown to be high, since the original filtrate
will contain only the BCF components with a molecular weight of less
than 1000. The absorption at 280 nm is very strong, and is probably
due to small peptides, or even amino acids. There is also a small
peak at just above 290 nm, and this could be due to uric acid, which is
often present in high levels in BCF, and has a peak of absorption at
293 nm.

3.4 IMMUNOHISTOCHEMISTRY

Having shown the presence of two BCF-specific proteins, it was
necessary to attempt to discover whether they were present in the cells
of the cyst epithelium. Such a location of specific protein would be
strong evidence for secretion. The investigation was made using the
immunohistochemical technique. Tissues other than breast were used
as controls, with anti-(BCF); and other antisera were used on cyst
sections to locate any milk or serum proteins, particularly immuno-
oglobulins. The latter was done to try to explain the virtual lack
of immunoglobulin in pure-type BCF.

3.4.1 Breast cyst sections - anti-(BCF)

For anti-(BCF), absorbed with milk and plasma, the optimum
dilution using the "sandwich" technique was found to be 1:250. This
gave a strong specific reaction, but almost no non-specific background
reaction. Immunofluorescence was initially used, but the peroxidase-
conjugate technique gave better results. Brief formalin fixation
did not affect the results, but one glutaraldehyde-fixed, wax-embedded
Fig. 43 Ultraviolet-absorption spectrum of a 1:100 dilution of an ultrafiltrate of pure-type BCF. This represents the components of BCF not retained by an Amicon UM2 membrane (i.e. molecular weight less than 1000).
The specimen was found to have no reaction.

The antiserum was found to react with both the contents, and the epithelial cells, of cysts. Typical results are illustrated in Fig. 44. The material in the cyst, and in some closely adjacent ducts (presumably connected to the cyst), was strongly positive, but there was no staining of nearby areas of normal glandular tissue.

Strong and characteristic reaction with the epithelial cells lining the cysts -'pink'cells - was obtained. Vacuoles in some cells had a rim of staining round their periphery; and the cell 'cap', and possibly plasma membrane, reacted strongly. There was no nuclear staining, but very strong reaction was obtained with a supra-nuclear body, which had a tortuous appearance and is suggested (with supportive evidence in the "Cytology" section in the Results) to be the Golgi complex (see also Fig. 20). The cytoplasm also had a weak overall stain which varied in intensity in different sections, and was sometimes stronger at the apical end of the cell. The same reactions were noted in 'pink' cells which were not lining cysts.

Of the controls, no reaction was obtained if the anti-(BCF) had been absorbed with BCF, if all or primary antiserum were omitted, or if the reaction with peroxidase conjugate were blocked with unlabelled sheep anti-(rabbit immunoglobulins). Initial incubation of sections with sheep anti-(whole human serum) had no effect on the subsequent reaction with the absorbed anti-(BCF), showing that no antibodies to serum proteins remained in the absorbed anti-(BCF).

Treatment of the sections with trypsin also had no effect on their reaction with absorbed anti-(BCF).

3.4.2 Breast cyst sections - other antisera

With anti-(whole human serum) some weak reaction was obtained.
'Pink' epithelium lining a breast microcyst, stained by the immunohistochemical peroxidase technique using milk and plasma absorbed antiserum to BCF. This is seen to react with the cyst contents (top) and with the 'pink' cells. Note the strong reaction with the cell 'cap' and the Golgi complex (arrow), and slight reaction of the apical cytoplasm. Stained by diaminobenzidine (to demonstrate peroxidase) and haematoxylin.
with the material in the lumen of cysts and adjacent ducts, and stronger reaction with connective tissue and blood vessels. However, there was no staining of "pink" epithelial cells.

A very similar result was obtained with anti-(human milk), with the exception that there was weak, generalised staining of the cytoplasm of 'pink' cells.

Antiserum to IgG and IgA also showed a weak overall reaction of the whole section, with stronger reaction in blood vessels. However, no immunoglobulin-producing cells were revealed around, or in, the cysts.

3.4.3 Axillary skin sections - anti-(BCF)

Axillary skin sections were examined for the presence of BCF-specific proteins. It was felt that this may help to resolve the problem of the similarity of apocrine gland cells and 'pink' cells which has often been shown structurally, but never functionally (see also p. 30).

In sections of both male and female axillary skin, absorbed anti-(BCF) is seen to react with the cells lining the apocrine glands, in an identical manner to 'pink' cells in the breast. No reaction is obtained with sebaceous glands or eccrine sweat glands.

Typical results are seen in Fig. 45. The anti-(BCF) reacts with the supra-nuclear body in the secretory cells of apocrine glands (i.e. Golgi complex, c.f. Fig. 23), and with the cell 'cap', but not with the duct cells. There is also staining of the secreted material within the glands, and this can be traced up through the ducts and out onto the skin surface.

3.4.4 Other tissues - anti-(BCF)

The reaction of various tissues with absorbed anti-(BCF) is shown in detail in Table 5. Positive reaction is only obtained in cells which are, or are derived from, apocrine-type cells. In no
Fig. 45 (a) Secretory cells of apocrine gland in a section of axillary skin, reacted with anti-BCF using the peroxidase technique (as in legend to Fig. 44). Note the similarity of the reaction to that in 'pink' cells (Fig. 44). The material in the gland lumen also reacts, and can be traced through ducts (D) and out onto the skin surface, as seen in the low-power photograph of the same section (b). (a x 400; b x 40).
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cellular</th>
<th>Secretion</th>
<th>Apocrine cells present †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vulval hidradenoma (1)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Nodular hidradenoma (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ceruminal adenoma (2)</td>
<td>+ (occasional cells)</td>
<td>+</td>
<td>† (slight resemblance only)</td>
</tr>
<tr>
<td>Apocrine gland cystadenoma</td>
<td>-</td>
<td>+</td>
<td>†  &quot;  &quot;</td>
</tr>
<tr>
<td>&quot;   &quot;   &quot;</td>
<td>†  &quot;  &quot;</td>
<td>+</td>
<td>†  &quot;  &quot;</td>
</tr>
<tr>
<td>Skin from nose</td>
<td>-</td>
<td>+ (hair follicle, apocrine duct)</td>
<td>-</td>
</tr>
<tr>
<td>Vulvectomy specimen</td>
<td>++ (apocrine gland)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stomach</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Colon</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rectum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Appendix</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cervix &amp; Cervical brush biopsy</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

++ Strong reaction † presence or absence of cells bearing a strong resemblance to "pink" or skin apocrine gland cells.
+ Moderate reaction † Weak or scattered reaction
case was the intracellular staining pattern the same as that found in 'pink' or skin apocrine gland cells. In the vulval hidradenoma, some cells had an appearance very like 'pink' cells, and showed staining of a supra-nuclear body. The reaction was different from that in 'pink' cells, being more diffuse and granular.

Staining of secretion was more frequently found. In all cases this was presumably derived from apocrine-type glands.

3.4.5 Breast carcinoma - anti-(BCF)

Ten carcinomas of the breast were examined. Of these, six were negative, three were weakly positive, and one strongly positive. The strong positive was a so-called "sweat-gland", or apocrine, carcinoma. The neoplastic areas in this specimen all showed a positive reaction, with some cells in each area being very strongly stained. The pattern, again, was unlike that of 'pink' and skin apocrine gland cells, being granular, and evenly distributed throughout the cytoplasm.
The objective of the chemical assays of BCF was threefold. Firstly, to establish some basic knowledge of the levels of various parameters in BCF. Secondly, to compare the chemical composition of BCF with that of milk and serum, and thus to see if it resembled either. Thirdly, to obtain evidence for, or against, BCF being a secretion of 'pink' epithelium.

4.1 AUTOANALYZER PROFILE

Table 6 shows the assay results of 12 variables in 120 BCFs, assayed on the SMA 12/60 AutoAnalyzer. The samples were 98 pure-type (no discrete bands, or γ-globulin, on cellulose acetate electrophoresis) and 22 serum-type (serum-like electrophoretic pattern) fluids (see also p.176). The mean, standard deviation, and range of each constituent is given for each type, together with the normal ranges for human milk and serum. Of the twelve parameters, seven showed a statistically significant (p<0.05) difference between the pure- and serum-type fluids using the Paired t-Test. With two exceptions, the serum-type had levels of these seven parameters closer to, but not necessarily within, the normal serum range, than the levels in the pure-type. The exceptions are SGOT, which is within normal serum range in both types, and bilirubin, which is above the normal range in serum-types. It must be emphasised that these are comparisons of the means for each variable to a normal range. The range of values of most of these variables in BCF is very large.

Total protein values are low relative to serum, but comparable with milk, as are the levels of albumin, especially in the pure-type fluids.

Calcium is found in the serum-types to be generally within the
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Pure Type (N=98)</th>
<th>Serum Type (N=22)</th>
<th>Range</th>
<th>Normal Ranges†</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Total Protein (g/dl)</td>
<td>2.06 (0.80)</td>
<td>3.39 (2.66)</td>
<td>0.9 - 6.7</td>
<td>6.0 - 8.0</td>
</tr>
<tr>
<td>*Albumin (g/dl)</td>
<td>0.36 (0.51)</td>
<td>1.33 (1.31)</td>
<td>0 - 4.1</td>
<td>3.5 - 5.0</td>
</tr>
<tr>
<td>*Calcium (mg/dl)</td>
<td>7.86 (2.57)</td>
<td>9.50 (2.90)</td>
<td>4.0 - 16.0</td>
<td>8.5 - 10.5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>&quot;</td>
<td>3.57 (2.28)</td>
<td>0 - 15.0</td>
<td>2.5 - 4.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&quot;</td>
<td>589 (271)</td>
<td>701 (313)</td>
<td>110 - 1384</td>
</tr>
<tr>
<td>BUN</td>
<td>&quot;</td>
<td>15.8 (6.62)</td>
<td>15.0 (2.95)</td>
<td>9.0 - 69</td>
</tr>
<tr>
<td>*Urate</td>
<td>&quot;</td>
<td>15.8 (8.23)</td>
<td>8.15 (7.28)</td>
<td>2.2 - 25</td>
</tr>
<tr>
<td>Creatinine</td>
<td>&quot;</td>
<td>1.35 (0.55)</td>
<td>1.28 (0.52)</td>
<td>0.7 - 4.2</td>
</tr>
<tr>
<td>*Bilirubin</td>
<td>&quot;</td>
<td>0.48 (0.48)</td>
<td>1.02 (2.19)</td>
<td>0 - 4.2</td>
</tr>
<tr>
<td>*Alkaline Phosphatase (K.A units/dl)</td>
<td>19.8 (16.8)</td>
<td>8.59 (13.8)</td>
<td>0 - 60</td>
<td>2.5 - 14</td>
</tr>
<tr>
<td>SGPT (mIU/ml)</td>
<td>4.4 (7.7)</td>
<td>1.90 (3.4)</td>
<td>0 - 48</td>
<td>0 - 13</td>
</tr>
<tr>
<td>SGOT</td>
<td>&quot;</td>
<td>33.2 (14.3)</td>
<td>43.1 (31.4)</td>
<td>8.0 - 115</td>
</tr>
</tbody>
</table>

* Statistically significant difference between Pure and Serum types (p<0.05)

† Normal ranges for serum from Technicon chart, for milk from Documenta Geigy
normal serum range, whereas in pure-types the level is lower. Both types have very low calcium levels relative to milk. The same applies to inorganic phosphate.

Urea is low in both groups relative to serum, but within the range for milk; with creatinine the reverse is the case. For both types bilirubin is within normal serum range, although it is higher in the serum-types.

Alkaline phosphatase is high relative to serum in the pure-types, but within serum range for serum-types.

SGPT is at the lower end, and SGOT at the upper end, of normal serum range in both types.

Cholesterol and uric acid both had levels well above the serum range in most fluids - especially cholesterol, with levels up to 1900 mg/dl.

4.1.1 Correlation of variables

Coefficients of correlation were calculated between all pairs from the 12 variables in each fluid, and these are shown in Table 7. They are all correlation coefficients on a linear regression; various transformations (e.g. logarithmic) made no improvement to the results. Despite the low values of most of the coefficients, many are statistically significant, several at p<0.001.

The twelve variables were also compared with values assigned to each fluid from a visual assessment of its darkness of colour and degree of turbidity ("milkiness"). Attempts were made to measure the colour spectrophotometrically, but this was subject to interference due to the turbidity. This latter was measured in ten fluids using a nephelometer. Results were very variable and greatly increased if the fluid had been frozen and thawed (although this increase was remarkably constant at...
<table>
<thead>
<tr>
<th></th>
<th>Alb.</th>
<th>Calcium</th>
<th>Phosphate</th>
<th>Chol.</th>
<th>BUN</th>
<th>Urate</th>
<th>Creatine</th>
<th>Bili.</th>
<th>A.P.</th>
<th>SGPT</th>
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<tr>
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<td>0.01</td>
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<td>0.06</td>
<td>0.82</td>
<td>-0.07</td>
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<td>0.28</td>
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<td>Albumin</td>
<td>0.25</td>
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<td>0.05</td>
<td>0.33</td>
<td>-0.25</td>
<td>0.05</td>
<td>0.75</td>
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<td>-0.09</td>
<td>0.26</td>
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<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td>-0.22</td>
<td>-0.01</td>
<td>-0.10</td>
<td>0.03</td>
<td>0.09</td>
<td>0.20</td>
<td>-0.09</td>
<td>0.02</td>
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<td>Phosphate</td>
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<td></td>
<td></td>
<td>-0.05</td>
<td>-0.05</td>
<td>0.11</td>
<td>0.26</td>
<td>0</td>
<td>0.21</td>
<td>0.10</td>
<td>0.11</td>
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<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.23</td>
<td>-0.15</td>
<td>-0.17</td>
<td>-0.12</td>
<td>-0.17</td>
<td>-0.26</td>
<td>0.04</td>
</tr>
<tr>
<td>BUN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.39</td>
<td>-0.37</td>
<td>-0.03</td>
<td>0.07</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Urate</td>
<td></td>
<td></td>
<td></td>
<td>0.26</td>
<td>-0.10</td>
<td>0.38</td>
<td>0.23</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
<td>0.18</td>
<td>-0.03</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.01</td>
<td>0.06</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.21</td>
<td>-0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGPT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance

1 p< 0.001
2 " 0.01
3 " 0.05

The remainder are not significant.
60 to 65% of the original value). Therefore the visual estimation, being in addition much easier, was used. The correlation coefficients are given in Table 8.

They show that as colour depth increases, BUN, water and bilirubin levels rise. Cholesterol is strongly and positively associated with "milkiness", but all other variables are negatively related.

Serum from breast cyst patients was found to have no deviation of any of the 12 variables from their normal serum ranges. It was noticed that the levels of cholesterol tended to be at the upper end of the normal range.

4.2 TOTAL PROTEIN

The manual estimates of protein were found to agree well with those from the AutoAnalyzer. However, they were consistently 5 to 10% higher, for serum as well as BCF, probably reflecting the difference in the two techniques used.

4.3 CHOLESTEROL

Cholesterol estimations also gave results which agreed well with those from the AutoAnalyzer for BCF and serum. In most cases, the BCF levels were above the range for the AutoAnalyzer, and the manual method was used for these fluids.

The cholesterol in BCF was found to be mainly in the particle-fraction (see Section on "Centrifugation" in Results). In some samples of BCF, small crystals were seen floating on the surface of the fluid. When examined by light microscopy, they had the characteristic form of cholesterol crystals (Documenta Geigy), and are illustrated in Fig. 46.
### TABLE 8

**Correlations of cyst fluid 12/60 results with colour and milkiness**

<table>
<thead>
<tr>
<th></th>
<th>Colour</th>
<th>Milkiness</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.P.</td>
<td>0.25²</td>
<td>-0.12</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.19</td>
<td>-0.08</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.20³</td>
<td>0.02</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.04</td>
<td>-0.11</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-0.15</td>
<td>0.49¹</td>
</tr>
<tr>
<td>BUN</td>
<td>0.33¹</td>
<td>-0.22³</td>
</tr>
<tr>
<td>Urate</td>
<td>0.24²</td>
<td>-0.19</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.12</td>
<td>-0.27²</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.24²</td>
<td>-0.19</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>0.21³</td>
<td>-0.37¹</td>
</tr>
<tr>
<td>SGPT</td>
<td>0.28²</td>
<td>-0.45¹</td>
</tr>
<tr>
<td>SGOT</td>
<td>-0.06</td>
<td>-0.12</td>
</tr>
</tbody>
</table>

Statistical significance ¹ $p < 0.001$

² " 0.01

³ " 0.05

The remainder are not significant.
Fig. 46  Unstained smear of BCF, showing crystals with the characteristic appearance of cholesterol crystals.
4.4 TRIGLYCERIDES

No triglyceride was detectable in the twenty samples of BCF on which the estimation was performed. If the fluid was centrifuged, no fat layer was formed at the surface, as happens with milk.

4.5 PHOSPHOLIPIDS

The results of phospholipid estimations on BCF, and serum controls, are given in Table 9. The values for BCF were very variable, with a range from 4.0 to 16.1 mg P/dl.

Attempts were made to separate the phospholipids by the method of Parsons and Patton (1967), using two-dimensional thin-layer chromatography on silica gel HR. The results were not very good, giving only two phosphate-containing components, and the assay was abandoned.

4.6 NUCLEIC ACIDS

Neither DNA nor RNA were detected in the assay on BCF. Two series of assays were run, with ten fluids in each. That the method was working adequately was shown by the results of the standards, and a control using homogenised leucocytes.

4.7 LACTOSE

Lactose was not detected in twenty samples of BCF. Human skimmed-milk was found to contain roughly 2.5 g/dl. The test was performed in order to determine whether any synthetic activity, comparable to that in milk production, was occurring in breast cysts.

4.8 URIC ACID

A manual method for the assay of uric acid was used to verify the AutoAnalyzer results and to obtain values for the levels in those
TABLE 9  Phospholipid content of BCF and serum

<table>
<thead>
<tr>
<th></th>
<th>Phospholipid (as mgP/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF</td>
<td>7.20 (4.0)</td>
</tr>
<tr>
<td>Serum</td>
<td>10.25 (1.6)</td>
</tr>
</tbody>
</table>

Values are means, with standard deviation in parentheses.

TABLE 10  Ion levels in BCF

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>95.0</td>
<td>(32.0)</td>
</tr>
<tr>
<td>Potassium</td>
<td>16.0</td>
<td>(14.2)</td>
</tr>
</tbody>
</table>
BCF samples which were off-scale on the instrument.

The heating step modification to the method (see p.105) was validated using sera. It was found to have no effect on the results. In addition, two UM2 membrane ultrafiltrates of BCF were assayed, since they have the interfering proteins removed, and could therefore be assayed without the heating step. No effect on the results was observed in the heated specimens, and the levels found were very near to those in the corresponding BCF (without ultrafiltration) by the heating method.

The assay was therefore used to determine uric acid levels in those fluids which were off-scale in the AutoAnalyzer, and the results are amongst those in Table 3.

4.9 INORGANIC IONS

The mean, standard deviation, and range of values obtained for sodium and potassium levels in BCF are given in Table 10, together with the normal values for human milk, serum, and whole blood.

The values for potassium were frequently very high relative to serum and milk, and of the order of levels in whole blood. Sodium is low relative to serum, but generally higher than in milk. Neither ion could be correlated with other factors, but they are negatively related (p<0.01) to each other (correlation coefficient = -0.67). The serum-type fluids tended to have potassium levels in the normal serum range, but still had low sodium.

The values for calcium are in Table 6.

4.10 CARCINO-EMBRYONIC ANTIGEN

The assay was made, following a report of the presence of
CEA-like material in BCF (Fleisher et al., 1974), to confirm the finding in the BCF material used in this study.

The results of twenty assays on BCF are presented in Table 11. The levels are high in all the samples, compared with serum. Two cysts, aspirated on different occasions from the same patient, showed similar values.

4.11 HORMONAL STEROIDS

The levels of four steroids assayed in BCF are shown in Table 12. They are mostly around the range found in serum, with two notable exceptions. These are both very high levels of 5α-dihydrotestosterone and are from cysts of patients who both subsequently had mastectomies for carcinoma of the breast. Unfortunately, serum was not obtained in either case at the time of aspiration of the cysts, but a blood sample obtained 3 months post-operatively from one patient, showed no elevation of DHT.

The oestrogen and progesterone assays of serum and 24 hour urine samples were made in an attempt to check the frequently suggested hypothesis that hormonal irregularities were involved in the aetiology of cystic disease (see p. 28). They showed the same patterns in both the breast cyst patients and the controls. Both groups fell into the normal range of serum levels and excretion rates for women of the appropriate menstrual status.

The prolactin levels in BCF were very low, and in the sera were within the normal range.
TABLE 11  
Levels of carcinoembryonic antigen in BCF.

<table>
<thead>
<tr>
<th>Levels of CEA in BCF (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
</tr>
<tr>
<td>38</td>
</tr>
<tr>
<td>57</td>
</tr>
<tr>
<td>61*</td>
</tr>
<tr>
<td>79*</td>
</tr>
<tr>
<td>86</td>
</tr>
<tr>
<td>88</td>
</tr>
<tr>
<td>122</td>
</tr>
<tr>
<td>128</td>
</tr>
<tr>
<td>140</td>
</tr>
<tr>
<td>190</td>
</tr>
<tr>
<td>192</td>
</tr>
<tr>
<td>280</td>
</tr>
<tr>
<td>339</td>
</tr>
<tr>
<td>510</td>
</tr>
<tr>
<td>560</td>
</tr>
<tr>
<td>570</td>
</tr>
<tr>
<td>630</td>
</tr>
<tr>
<td>720</td>
</tr>
</tbody>
</table>

If a normal distribution,

Mean = 254

Population standard deviation = 228

Pooled plasma level = 12 μg/ml

*Same patient; the two were cysts aspirated on different occasions.
<table>
<thead>
<tr>
<th>5 Androstenedione</th>
<th>Dihydrotestosterone</th>
<th>Testosterone</th>
<th>Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.81</td>
<td>178</td>
<td>-†</td>
<td>35</td>
</tr>
<tr>
<td>2.89</td>
<td>193</td>
<td>-†</td>
<td>94</td>
</tr>
<tr>
<td>1.10</td>
<td>90</td>
<td>86</td>
<td>57</td>
</tr>
<tr>
<td>0.83</td>
<td>106</td>
<td>116</td>
<td>13</td>
</tr>
<tr>
<td>0.69</td>
<td>123</td>
<td>83</td>
<td>19</td>
</tr>
<tr>
<td>1.26</td>
<td>332</td>
<td>386</td>
<td>25</td>
</tr>
<tr>
<td>0.64</td>
<td>259</td>
<td>146</td>
<td>7</td>
</tr>
<tr>
<td>1.03</td>
<td>82</td>
<td>89</td>
<td>46</td>
</tr>
<tr>
<td>3.76</td>
<td>158</td>
<td>239</td>
<td>26</td>
</tr>
<tr>
<td>1.79</td>
<td>130</td>
<td>162</td>
<td>10</td>
</tr>
<tr>
<td>* 0.40</td>
<td>1354</td>
<td>289</td>
<td>49</td>
</tr>
<tr>
<td>0.70</td>
<td>170</td>
<td>194</td>
<td>0</td>
</tr>
<tr>
<td>* -</td>
<td>1680</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* patient subsequently presented with carcinoma of the breast.

† no assay performed.
The objectives of the enzyme assays were two-fold. They were, firstly to obtain data on the levels of some enzymes in BCF, especially in relation to milk and blood serum, and secondly to use results of marker-enzyme assays in the examination of the particle-fraction of BCF (see "Centrifugation Methods", in Results).

In the estimations of phosphate-releasing enzymes, considerable problems were experienced in the assay of phosphate. A novel method of protein precipitation was developed in order to overcome the problem.

5.1 VALIDATION OF PHOSPHATE ASSAY

The maximum absorbance of a standard phosphate solution in the phosphate assay was found to be achieved with acid concentrations, in the solution to be assayed, of between 2 and 10% for TCA, and 1 and 5% for PCA. Outside these ranges the colour production fell off markedly, mostly with PCA above 5%. The absorbance increased considerably if left for a further 30 minutes; on average by 65%.

The time curve of absorbance, using optimum acid concentrations, showed that colour development continued beyond 60 minutes, but at a lower rate and reaching a maximum after 2.5 to 3 hours. Although a further rise in absorbance was found after 3 hours, this was matched by an increase in absorbance of the blank. With no acid in the solution to be assayed, the absorbance continued to rise rapidly beyond 3 hours. It was decided to continue to use the normal 30 minutes incubation time for the assay, but to take extreme care to perform the absorbance measurement after exactly 30 minutes.

The best precipitation of BCF protein was found to be given by 1 to 2% CAB, 4% TCA, or 1 to 3% PCA. However, the addition of assay
reagents resulted in further protein precipitation. The precipitate also absorbed the blue-coloured reaction product and thus spoiled the assays. The CAB was also precipitated by the phosphate reagent, and was therefore discarded as a protein precipitant.

Further tests showed that different BCF samples had different optimum acid concentrations for protein precipitation. Mixtures of TCA and PCA were not effective; neither were ethanol or methanol, or a mixture of the two (1:1), in concentrations up to 20% with 4% TCA. Filtration was moderately effective, but time-consuming, expensive, and frustrating, since the Millipore filters were inclined to block halfway through a filtration.

Heating of the acidified solutions was found to be the answer. With 4% TCA, heating in a boiling water bath gave the best results. The minimum time was 1 minute. When the solutions were cooled, centrifuged, and the supernatants assayed for phosphate, repeatable and accurate results were obtained.

5.2 5'-NUCLEOTIDASE

The results obtained for the assays of 5'-nucleotidase are given in Table 13. A wide variation of levels was found in BCF. The highest was 98.5 mIU/ml, the lowest 1.0 mIU/ml. Values for serum from the cyst patients were found to lie mostly in the upper region of the normal serum range of 3.5 to 11 mIU/ml, with a mean, from 20 assays, of 9.5 mIU/ml (standard deviation, 1.2). The values for BCF did not correlate with other variables. The enzyme is a marker for plasma membrane, and was used as such in the studies of the particle-fraction of BCF (see "Centrifugation Methods", in Results).
### TABLE 13

<table>
<thead>
<tr>
<th></th>
<th>Activity (mIU/ml)</th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF</td>
<td></td>
<td>27.6</td>
<td>29.5</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>9.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

### TABLE 14

<table>
<thead>
<tr>
<th></th>
<th>Validation of 5'-nucleotidase assay - effect of incubation time</th>
<th>Phosphate-assay Method</th>
<th>Adenosine deaminase method (60 mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 mins</td>
<td>60 mins</td>
</tr>
<tr>
<td>BCF 1</td>
<td></td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.6</td>
<td>4.5</td>
</tr>
<tr>
<td>BCF 2</td>
<td></td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCF 3</td>
<td></td>
<td>5.8</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

All values are mIU/ml, and each specimen (except BCF2) was assayed in duplicate on two occasions.

221
The two enzyme assay methods were found to give excellent agreement of results. In solutions containing sucrose, the Persijn and van der Slik (1968) method failed to work. Tests, adding varying amount of sucrose to 1 ml of ammonia standard (2 μl of conc. ammonia solution in 100 ml of water), followed by the colour reagents, showed sucrose to have no effect on colour development. Since 5'-nucleotidase is not complete inhibited by sucrose, the effect must be on adenosine deaminase.

5.2.1 5'-Nucleotidase assay - validation

The results of the assay validation experiment, using the phosphate-estimating method, are given in Table 14, together with values obtained by the adenosine deaminase method. After 60 minutes the activity of the enzyme fell, but at 30 and 60 minutes the same results for activity were obtained. It was noticed that the fall in activity was less marked with serum samples. The assay also gave good repeatability when re-run, and good agreement with the adenosine deaminase method. The heating step did not affect the substrate, as the heated and unheated reagent blanks both had the same absorbance.

5.2.2 Stability of 5'-nucleotidase

The levels of 5'-nucleotidase in 2 BCFs after storage for 8 or 15 days at 4°C and -20°C, and freeze-thawing, are given in Table 15. These show that the enzyme is very stable when stored at 4°C, but slightly less so at -20°C. The freezing and thawing resulted in little loss of activity in both fluids tested.

5.3 ACID PHOSPHATASE

The activity of acid phosphatase in BCF is given in Table 16. The assay results for the p-nitrophenol-releasing method are not very
### TABLE 15  
**Effect of time and method of storage on 5'-nucleotidase**

<table>
<thead>
<tr>
<th>Storage</th>
<th>1 day</th>
<th>8 days</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF 1</td>
<td>4°C</td>
<td>5.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>-</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Freeze-thawed</td>
<td>-</td>
<td>5.3</td>
</tr>
<tr>
<td>BCF 2</td>
<td>4°C</td>
<td>51.0</td>
<td>51.4</td>
</tr>
<tr>
<td></td>
<td>-20°C</td>
<td>45.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Freeze-thawed</td>
<td>49.8</td>
<td>-</td>
</tr>
</tbody>
</table>

All activities are in mIU/ml

### TABLE 16  
**Levels of acid phosphatase in BCF and sera from cyst patients**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Serum</td>
<td>7.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

All activities are in mIU/ml
revealing as to enzyme source, as discussed by Jacques (1974), since several phosphatases will break down the substrate under acid conditions. The assay was therefore discontinued, but the results show generally low activity - mostly within the serum range - with occasional fluids having somewhat higher levels. The sera from cyst patients all had levels at the upper end of normal range.

5.4 ALKALINE PHOSPHATASE

The activity of this enzyme in BCF, estimated by AutoAnalyzer, is given in Table 6. The results of the manual assay, using p-nitrophenyl phosphate as substrate, gave good agreement with the AutoAnalyzer results. The levels in sera from cyst patients were within normal serum range.

5.5 CATHEPSIN D

This enzyme was assayed in order to determine whether lysosomal enzymes were present, and also to see if proteolysis could be occurring in BCF in vitro.

The enzyme was found to be present in BCF. Activity was measured as increase in absorbance obtained in one hour by 1 ml of sample. The levels in BCF were found to be low, with values from 0.05 to 0.70 units for most fluids, one exception being a value of 1.33 units.

5.5.1 Validation of assay

The heating method (see p.118) was found to give identical results to that using Millipore filtration, and was therefore adopted for all assays. The heating method gave slightly lower absorbance values for all the solutions, but the differences between tests and
blanks were unaffected.

The results of the time curve assay are given in Table 17. The reaction was found to be linear only up to one hour. For the three-fold concentrated pool of 3 BCFs, the activity was 1.45 units.

5.5.2 Stability and activation of cathepsin D

The results for the assay of cathepsin D on BCF stored at 4°C or -20°C and for freeze-thawed BCF are given in Table 18. The activity was largely unaffected by storage up to two weeks at either 4°C or -20°C. Freeze-thawing had no activating effect on the enzyme activity, suggesting that it was not present in intact lysosomes in the fluid. The process caused a slight reduction in activity, presumably due to denaturation of the enzyme. The apparent rise in activity of one sample over a period of one week is difficult to account for, but is probably experimental error rather than real.

5.6 AMYLASE

No activity of amylase was detected in twenty BCF samples. The method was working, as shown by the activity which was obtained in saliva samples used as positive controls. This result contradicts the report of Bonser et al. (1961) that BCF is rich in amylase. The assay was made in order to confirm this unsubstantiated claim.

5.7 XANTHINE OXIDASE

It seemed unlikely that the high levels of uric acid in BCF (see p. 208) could be derived from serum. However, if it was formed in the cyst, xanthine oxidase would be required; the fluid was therefore tested for the presence of the enzyme.

No activity was demonstrated in ten BCFs which were assayed for
### TABLE 17  Time curve for cathepsin D assay

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Absorbance</th>
<th>Activity (ΔE/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.73</td>
<td>1.46</td>
</tr>
<tr>
<td>60</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>90</td>
<td>1.64</td>
<td>1.09</td>
</tr>
<tr>
<td>120</td>
<td>1.78</td>
<td>0.90</td>
</tr>
<tr>
<td>180</td>
<td>1.89</td>
<td>0.63</td>
</tr>
<tr>
<td>240</td>
<td>2.01</td>
<td>0.50</td>
</tr>
</tbody>
</table>

### TABLE 18  Effects of storage and freeze-thawing on activity of cathepsin D

<table>
<thead>
<tr>
<th></th>
<th>1 day</th>
<th>8 days</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stored 4°C</td>
<td></td>
<td>0.49</td>
<td>0.94</td>
</tr>
<tr>
<td>stored -20°C</td>
<td></td>
<td>0.55</td>
<td>0.94</td>
</tr>
<tr>
<td>freeze-thawed</td>
<td></td>
<td>0.55</td>
<td>0.80</td>
</tr>
<tr>
<td>BCF 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stored 4°C</td>
<td>1.33</td>
<td>1.33</td>
<td>-</td>
</tr>
<tr>
<td>stored -20°C</td>
<td>1.31</td>
<td>1.33</td>
<td>-</td>
</tr>
<tr>
<td>freeze-thawed</td>
<td>1.20</td>
<td>1.16</td>
<td>-</td>
</tr>
</tbody>
</table>
xanthine oxidase, nor in two samples of human skimmed-milk.

5.8 LACTOPEROXIDASE

Again no activity of the enzyme was found in BCF. Some activity was found in controls (human skimmed-milk). The aim of making the assay was to compare BCF with milk, which is rich in the enzyme, and also to determine whether the anti-microbial system involving lactoperoxidase (Oram & Reiter, 1966), could be operating in breast cysts.

5.9 AUTOANALYZER

The results for the assay of alkaline phosphatase, SGOT, and SGPT, by the SMA 12/60 AutoAnalyzer, are given in Table 6 and on pages 206 and 208).
The centrifugal-separation studies were made in order to investigate the "milkiness" of BCF. The hypothesis that this may be due to membranes, derived from the 'pink' cells lining the cyst, was tested by the assay of marker enzymes.

6.1 HIGH-SPEED CENTRIFUGATION

The method was used for many BCFs, to attempt to separate out the "milky" material (i.e. "particle-fraction"). It was found to be rather unsuccessful with most specimens, but some had a partial separation yielding a clear layer (i.e. "fluid-fraction") and a cloudy layer (not pure particles, but a particle-enriched fluid-fraction).

These two fractions were assayed for cholesterol, which was found to be highly concentrated in the cloudy layer. Assays were then made for 5'-nucleotidase and cathepsin D, to determine whether the cloudiness could be due to cell membranes, and whether lysosomes were present.

Three BCF samples which gave reasonable separations were used, and the assay results are given below. The cloudy fraction was at the top of 2 samples, and at the bottom in the other.

6.1.1 5'-Nucleotidase

The results of the 5'-nucleotidase assay are in Table 19. They show that in two of the BCFs, the enzyme was concentrated in the fraction containing the particles. In the third, the majority was in the fluid-fraction. The adenosine-deaminase method was used for the assay.

6.1.2 Cathepsin D

The results (see Table 19) showed the activity to be associated with the particle-enriched fraction, with none in the fluid fraction,
### TABLE 19  Analysis of particle-enriched fraction and clear fluid obtained by centrifugal separation

<table>
<thead>
<tr>
<th></th>
<th>5'-Nucleotidase (mIU/ml)</th>
<th>Cathepsin D (ΔE/ml/hr)</th>
<th>Cholesterol (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCF sample 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle-enriched (top)</td>
<td>17.6</td>
<td>0.67</td>
<td>4.62</td>
</tr>
<tr>
<td>Clear fluid (bottom)</td>
<td>2.1</td>
<td>0</td>
<td>1.38</td>
</tr>
<tr>
<td><strong>BCF sample 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle-enriched (top)</td>
<td>29.5</td>
<td>0.11</td>
<td>3.81</td>
</tr>
<tr>
<td>Clear fluid (bottom)</td>
<td>74.4</td>
<td>0</td>
<td>2.17</td>
</tr>
<tr>
<td><strong>BCF sample 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle-enriched (bottom)</td>
<td>221</td>
<td>0</td>
<td>4.20</td>
</tr>
<tr>
<td>Clear fluid (top)</td>
<td>4.7</td>
<td>0.38</td>
<td>1.16</td>
</tr>
</tbody>
</table>
in two samples. The third sample gave exactly the opposite result.

6.1.3 Cholesterol

The majority of the cholesterol was also found to be associated with the particle-enriched fraction (see Table 19).

Since the particle-fraction was not completely separated from the clear fluid, results for this fraction also contain a contribution from the clear fluid.

6.2 Flotation Method

This technique was used to obtain a complete separation of the particle-fraction from the fluid-fraction. It was found that the sodium bromide inhibited 5'-nucleotidase completely, and also interfered with cholesterol assay. The fractions were therefore dialysed in an Amicon ultrafiltration cell, in the diafiltration mode, against 0.1M tris buffer, pH 8.0.

The particles in the BCF were found to float on top of the overlay solution, leaving the clear fluid-fraction beneath. The particles had a white or off-white colour; the fluid fraction was clear and of the same colour as the whole BCF, from which it was prepared. The upper layer, consisting of overlay solution and particles, was removed using a Pasteur pipette, and then made up to 5 ml. This then gave the particles in a volume equal to that of the original BCF sample from which they derived. Analyses for 5'-nucleotidase, cathepsin D, and cholesterol were then made, as before, in order to determine whether they could be found in the particles which were completely (rather than partially, as above) separated from the fluid fraction. The e.m. appearance of the particles is shown in Fig. 27.

6.2.1 5'-Nucleotidase

The levels of the enzyme in the dialysed fractions and the
original whole fluid (unseparated BCF) are given in Table 20. The majority of the activity was lost, and was not regained during dialysis. In two BCFs which had some remaining activity, it was greater in the particle-fraction than the fluid fraction. This may be because the activity in the particles is more stable than that in the fluid-fraction. But it does serve to demonstrate that there is activity in the particles.

6.2.2 Cathepsin D

Cathepsin D was assayed in order to determine whether the activity found in the partially purified particles (see above) was found in the completely separated particle-fraction.

The assay results are also in Table 20, and show the majority of the enzyme to be in the fluid-fraction in all cases, but some activity is seen in the particles.

6.2.3 Cholesterol

The levels of free and esterified cholesterol in both fractions, as well as total cholesterol level, were assayed this time. This was done in order to see whether the cholesterol composition was the same as that in milk (see p. 14), and whether that in the particle-fraction could be membrane-derived.

Both the free and esterified cholesterol levels for the fractions are given in Table 21. The extraction method was used, and was found to give recoveries of 85 to 90%.

The particles were found to contain the majority of the cholesterol in BCF; from 7 to 31% was in the fluid-fraction. Of the cholesterol, in the whole fluid from 90 to 97% was in the free form, in the particle-fraction from 86 to 92%. The estimation of free cholesterol was checked by assaying a control serum, for which a free

*presumably due to inactivation by the sodium bromide
### TABLE 20

Activity of 5'-nucleotidase and cathepsin D in particle- and fluid-fractions of BCF, separated by flotation.

<table>
<thead>
<tr>
<th>5'-Nucleotidase (mIU/ml)</th>
<th>Cathepsin D(ΔE/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole fluid</td>
</tr>
<tr>
<td></td>
<td>Particle-fraction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BCF sample</th>
<th>5'-Nucleotidase (mIU/ml)</th>
<th>5'-Nucleotidase (mIU/ml)</th>
<th>5'-Nucleotidase (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>16.0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0</td>
<td>6.4</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>0.3</td>
<td>16.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BCF sample</th>
<th>Cathepsin D(ΔE/ml/hr)</th>
<th>Cathepsin D(ΔE/ml/hr)</th>
<th>Cathepsin D(ΔE/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.11</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td>0.33</td>
<td>0.88</td>
<td>1.20</td>
</tr>
<tr>
<td>4</td>
<td>0.16</td>
<td>0.36</td>
<td>0.56</td>
</tr>
</tbody>
</table>

---

### TABLE 21

Levels of free and esterified cholesterol in particle- and fluid-fractions of BCF.

<table>
<thead>
<tr>
<th>Whole fluid</th>
<th>Particle-fraction</th>
<th>Fluid-fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Free %Free</td>
<td>Total Free %Free</td>
</tr>
<tr>
<td>BCF sample 1</td>
<td>3.22 3.11 (97)</td>
<td>3.22 2.78 (86)</td>
</tr>
<tr>
<td>2</td>
<td>7.18 -* (-)</td>
<td>4.95 4.48 (91)</td>
</tr>
<tr>
<td>3</td>
<td>3.88 3.62 (93)</td>
<td>3.31 3.03 (92)</td>
</tr>
<tr>
<td>4</td>
<td>2.97 2.68 (90)</td>
<td>2.81 2.50 (89)</td>
</tr>
</tbody>
</table>

All values mg/ml

*Assay spoiled.
value of 30% of total was obtained, the supplier's quoted value for which was 35%

6.3 DENSITY GRADIENT CENTRIFUGATION

Since the use of sodium bromide, in the flotation method (see above), spoiled some assays, an alternative medium was selected. Sucrose was chosen, since it is commonly used for this purpose, and its properties for such use are well-documented. It was also decided that a density gradient should be used, as this would allow the density of the particles to be determined, as well as to show any distribution of the assayed components within the particle-fraction. The particles had been shown to float on most BCF specimens when centrifuged at high speed, and so the specimens had to be made denser than the gradient and layered under it, rather than simply applied to the top of the gradient.

Results of the analyses to estimate protein, cholesterol and 5'-nucleotidase in the fractions from the first two density gradient separations of 6 BCFs are presented graphically in Figures 48 and 49. The light-scattering of the gradient during pump-out (measured as absorbance at 600 nm) for Run 1 is shown in Fig. 47. The assays of the protein and cholesterol of plasma and milk separations are in Figure 51. The density gradient separation of a large concentrated BCF sample was assayed for cholesterol, protein, 5'-nucleotidase, Na^+, K^+- dependent ATPase, acid and alkaline phosphodiesterases, glucose-6-phosphatase, and succinate dehydrogenase. The results for these assays are tabulated in Table 22, and are shown graphically in Figure 50. In all cases, fraction 1 is the light end of the gradient.

6.3.1 Breast cyst fluids

In all samples the fluids showed a separation into two major
Fig. 47  Absorbance at 600 nm of the gradients of BCF separations, Run No. 1. Three different fluids were centrifuged, and are designated A, B and C (also in Fig. 48). Fraction No. 1. is the light end of the gradient in all cases (also in Figs. 48 to 51).
Fig. 48 Assay of 5'-nucleotidase (Δ-Δ), cholesterol (---), and protein (○-○) in BCF separated by density gradient centrifugation. Run No. 1. For explanation of A, B and C, see legend to Fig. 47. Protein (Prot.) and cholesterol (Chol.) are in mg/ml, 5'-nucleotidase (5'-Nuc.) is in mIU/ml.
Fig. 49 Assay of 5'-nucleotidase (ΔΔ), cholesterol (---), and protein (○○) in BCF separated by density gradient centrifugation. Run No. 2. The three BCF samples were different from those used in run No. 1, and are designated D, E and F. Units are as in legend to Fig. 48.
components; a particle-fraction and a fluid-fraction. The latter remained at the bottom of the gradient as a clear fluid of the same colour as the original whole BCF, representing the original sample after removal of the particle-fraction. This was found, after the separation, to be in the lighter portion of the gradient, and appeared as a rather diffuse band. On closer examination this was found to consist of many very narrow, denser bands, of a whitish colour (see also Fig. 27).

The fractions of the gradients also showed this separation quite clearly, particularly in the cholesterol assays. The major peak of cholesterol is in the region corresponding to the particle-fraction (fractions 2 to 5 or 6 in Run 1, and 3 to 10 in Run 2). This fraction is shown by a peak in the light scattering curve for the fluids in Run 1 (Fig. 47), and could also be seen as milkiness in the fractions of the gradient.

The assays reveal the majority of the protein to be in the fluid-fraction, with only trace amounts in the particle fraction, making, nonetheless, a distinct peak. In most separations this peak can be seen to lag behind the cholesterol peak, suggesting the particles at the denser end of the peak had a somewhat higher protein content than those at the lighter end.

The same phenomenon can also be seen with 5'-nucleotidase distribution. There is a peak of activity, in most cases, at the denser end of the particle-fraction. However, the enzyme assays were slightly suspect owing to low levels of activity, and for this reason a run was made using a large sample of BCF, concentrated to boost the enzyme levels.

The results for the large sample (Fig. 50) are rather clearer, but do not conflict with those of the first two runs. The sample was
Fig. 50 Assay of protein, cholesterol, and enzymes in a concentrated large sample of BCF, separated by density gradient centrifugation (see also Table 22). The enzymes are 5'-nucleotidase (5'-Nuc.), ATPase, glucose-6-phosphatase (G-6-Pase), acid phosphodiesterase (Acid P'd'ase), and alkaline phosphodiesterase (Alk.P'd'ase). Protein (Prot.) and cholesterol (Chol.) are in mg/ml, all enzymes in mIU/ml, and fraction density (Dens.) is in g/ml.
**TABLE 22**

Assay of fractions from density gradient separation of large BCF sample

<table>
<thead>
<tr>
<th>Refractive Index</th>
<th>Sucrose Conc. (M)</th>
<th>Density (g/ml)</th>
<th>Protein (mg/ml)</th>
<th>Cholesterol (mg/ml)</th>
<th>5'-Nuc. (mIU/ml)</th>
<th>G-6-Pase (mIU/ml)</th>
<th>ATPase (mIU/ml)</th>
<th>Acid Phospho-Diesterase (mIU/ml)</th>
<th>Alk. Phos-Diesterase (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3437</td>
<td>0.2161</td>
<td>1.0292</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.3452</td>
<td>0.2472</td>
<td>1.0332</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.3480</td>
<td>0.3053</td>
<td>1.0406</td>
<td>0.02</td>
<td>0.38</td>
<td>0</td>
<td>0</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.3525</td>
<td>0.3985</td>
<td>1.0526</td>
<td>0.02</td>
<td>1.82</td>
<td>0.75</td>
<td>0</td>
<td>4.47</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.3577</td>
<td>0.5063</td>
<td>1.0664</td>
<td>0.06</td>
<td>4.68</td>
<td>0</td>
<td>6.00</td>
<td>8.75</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.3632</td>
<td>0.6203</td>
<td>1.0810</td>
<td>0.13</td>
<td>5.21</td>
<td>2.25</td>
<td>3.00</td>
<td>9.47</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.3693</td>
<td>0.7467</td>
<td>1.0972</td>
<td>0.23</td>
<td>6.46</td>
<td>4.50</td>
<td>5.25</td>
<td>2.25</td>
<td>10.8</td>
</tr>
<tr>
<td>8</td>
<td>1.3748</td>
<td>0.8607</td>
<td>1.1118</td>
<td>0.36</td>
<td>4.02</td>
<td>5.25</td>
<td>9.01</td>
<td>10.5</td>
<td>7.93</td>
</tr>
<tr>
<td>9</td>
<td>1.3802</td>
<td>0.9727</td>
<td>1.262</td>
<td>0.33</td>
<td>2.36</td>
<td>8.26</td>
<td>9.01</td>
<td>31.5</td>
<td>5.58</td>
</tr>
<tr>
<td>10</td>
<td>1.3863</td>
<td>1.0991</td>
<td>1.1424</td>
<td>0.22</td>
<td>1.11</td>
<td>11.3</td>
<td>6.75</td>
<td>32.3</td>
<td>5.45</td>
</tr>
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<td>11</td>
<td>1.3930</td>
<td>1.2380</td>
<td>1.1602</td>
<td>0.16</td>
<td>0.90</td>
<td>10.5</td>
<td>1.50</td>
<td>17.3</td>
<td>4.93</td>
</tr>
<tr>
<td>12</td>
<td>1.3997</td>
<td>1.3768</td>
<td>1.1780</td>
<td>0.17</td>
<td>0.91</td>
<td>7.51</td>
<td>2.25</td>
<td>18.0</td>
<td>4.96</td>
</tr>
<tr>
<td>13</td>
<td>1.4058</td>
<td>1.5033</td>
<td>1.1942</td>
<td>0.22</td>
<td>0.96</td>
<td>5.25</td>
<td>5.25</td>
<td>9.76</td>
<td>5.60</td>
</tr>
<tr>
<td>14</td>
<td>1.4127</td>
<td>1.6463</td>
<td>1.2125</td>
<td>0.38</td>
<td>1.11</td>
<td>8.26</td>
<td>2.25</td>
<td>16.5</td>
<td>6.45</td>
</tr>
<tr>
<td>15</td>
<td>1.4193</td>
<td>1.7831</td>
<td>1.2301</td>
<td>1.06</td>
<td>1.50</td>
<td>18.8</td>
<td>4.50</td>
<td>32.3</td>
<td>7.88</td>
</tr>
<tr>
<td>16</td>
<td>1.4272</td>
<td>1.9468</td>
<td>1.2511</td>
<td>10.4</td>
<td>1.87</td>
<td>54.0</td>
<td>-</td>
<td>63.0</td>
<td>12.0</td>
</tr>
<tr>
<td>17</td>
<td>1.4380</td>
<td>2.1292</td>
<td>1.2745</td>
<td>16.0</td>
<td>2.59</td>
<td>92.3</td>
<td>22.5</td>
<td>85.9</td>
<td>14.5</td>
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<td>18</td>
<td>1.4413</td>
<td>2.2391</td>
<td>1.2886</td>
<td>18.5</td>
<td>2.83</td>
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<td>2.3220</td>
<td>1.2992</td>
<td>20.0</td>
<td>1.81</td>
<td>129</td>
<td>15.8</td>
<td>113</td>
<td>15.2</td>
</tr>
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<td>20</td>
<td>1.4463</td>
<td>2.3427</td>
<td>1.3018</td>
<td>19.5</td>
<td>0.52</td>
<td>93.8</td>
<td>20.3</td>
<td>81.8</td>
<td>12.3</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>84.4</strong></td>
<td><strong>41.04</strong></td>
<td><strong>558</strong></td>
<td><strong>140.3</strong></td>
<td><strong>616.5</strong></td>
<td><strong>154</strong></td>
<td><strong>236</strong></td>
<td></td>
</tr>
<tr>
<td><strong>AMOUNT/4ml FLUID</strong></td>
<td><strong>94.4</strong></td>
<td><strong>37.8</strong></td>
<td><strong>662</strong></td>
<td><strong>156</strong></td>
<td><strong>651</strong></td>
<td><strong>146</strong></td>
<td><strong>196</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RECOVERY (%)</strong></td>
<td><strong>89</strong></td>
<td><strong>109</strong></td>
<td><strong>84</strong></td>
<td><strong>90</strong></td>
<td><strong>95</strong></td>
<td><strong>106</strong></td>
<td><strong>120</strong></td>
<td><strong>50</strong></td>
<td></td>
</tr>
</tbody>
</table>

All enzyme activities are corrected for sucrose inhibition using the molar inhibition percentages overleaf.
TABLE 22 (continued)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td>65%</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>50%</td>
</tr>
<tr>
<td>ATPase</td>
<td>41%</td>
</tr>
<tr>
<td>Acid phosphodiesterase</td>
<td>28%</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase</td>
<td>67%</td>
</tr>
</tbody>
</table>

(from Hartman et al., 1974)
run in three tubes, as for the previous individual samples, but the corresponding fractions from all tubes were pooled, after checking that each of the triplicates had nearly identical refractive indices. Thus, three times the usual volume of each gradient fraction was obtained, allowing a greater range of assays to be performed.

The cholesterol peak in the particle-fraction appeared in fractions 3 to 10, corresponding to a density range of 1.04 to 1.14, with a maximum at density 1.10. Again, the corresponding slight peak of protein was at the denser end of the cholesterol peak, with a maximum at density 1.12. The majority of the protein was in the fluid-fraction.

The p.m. marker enzymes, 5'-nucleotidase and ATPase, both show peaks at a higher density than the cholesterol peak, at density 1.14 to 1.16 and 1.13 to 1.14 respectively. The majority of both enzymes, however, is in the fluid-fraction.

Glucose-6-phosphatase (in liver a marker for e.r. membranes - see also p.252) showed a rather random distribution, which casts some doubt on the validity of the results, but has a peak at a lower density (about 1.11) in the particle fraction than the enzymes mentioned above. Again, the majority of the activity is in the fluid-fraction.

Acid and alkaline phosphodiesterase (a lysosomal and a p.m. marker, respectively) had a remarkably similar distribution pattern, with a peak corresponding exactly with that of cholesterol, and a slightly larger peak in the fluid-fraction. The similarity of the distribution of the two enzymes suggests the possibility that the activity is due to one enzyme (possibly the acid phosphodiesterase) operating at both pH values, rather than both enzymes being present. If this were the case, it would explain why alkaline phosphodiesterase
does not have a peak at a density corresponding to that of the other p.m. markers, since the activity would be due to acid phosphodiesterase. The acid phosphodiesterase would suggest that disrupted or altered lysosomes are present in the particle-fraction (not intact lysosomes, since no activation of lysosomal enzymes could be obtained by freeze-thawing BCF).

An alternative explanation is that both acid and alkaline phosphodiesterase are present, and have a very similar distribution in the gradient fractions. Unfortunately, insufficient sample remained to test whether alkaline phosphodiesterase was present by means of assays incorporating inhibitors specific for this enzyme (e.g. EDTA).

Succinate dehydrogenase was not detected in the whole fluid sample and was therefore not assayed in the fractions. The assay technique was verified by using controls of roughly homogenised mammary gland tissues, which gave a positive reaction.

6.3.2 Milk

The milk and blood plasma specimens were run as controls, to compare with the results for BCF. The aim was mainly to see whether or not a particle-fraction was present in either milk or plasma.

Both human skimmed-milk samples showed the entire protein content to be in the fluid-fraction (Fig. 51), with the exception of a small peak at the top of the gradient in sample B. This was due to protein in a small amount of cream, which had been left in the sample due to inefficient skimming. It was floating on top of the gradient, and "tailed" considerably during pumping-out, and therefore appears in fractions 2 and 3.

Cholesterol showed a very varied and erratic distribution, but
Fig. 51 Assay of Protein (o–o), and cholesterol (—) in two samples of human skimmed milk and one sample of plasma, separated by density gradient centrifugation. Samples A and B are skimmed milk, sample C is plasma. Both protein (Prot.) and cholesterol (Chol.) are in mg/ml.
was present in very low concentration. There was no cholesterol peak like that found in BCF runs due to the particle-fraction.

No 5’-nucleotidase assays were made, since the enzyme was not detectable in the starting material used for the separations.

6.3.3 Blood plasma

The plasma sample also showed no cholesterol peak like that of BCF, and nearly all the protein was in the fluid-fraction (Fig. 51). The results for 5’-nucleotidase assay were discarded as unreliable, due to the very low levels of activity. The sample concentration was 3.1 mIU/ml.
DISCUSSION

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DISCUSSION

1. PATIENT DATA

The patient data show that the group from which BCF was obtained for this study was the same age range as that found by other workers (Haagensen, 1971; Bonser, et al., 1961). The majority of patients are in the age range of 40 to 50 years, and are perimenopausal.

Parity and previous history of breast feeding seem to have no influence on production of cysts, but in the younger women, cysts tend to be post-lactational.

The size of cysts and the colour and milkiness of their contents was found to be very variable. It was originally felt that the depth of colour of BCF may increase with age. However, if this is the case, and some evidence has been obtained for it, then age is not related to cyst volume, since there is very little gradation of increasing colour with increasing volume. Therefore, it may perhaps be that all cysts do not continuously enlarge, but some may reach a finite size, where intra-cyst pressure prevents further secretion. This suppression of the secretory process due to alveolar distension is known to occur in lactation (Cowie & Tindal, 1971).

2. PROTEINS

The electrophoretic pattern of pure-type BCF proteins is unlike that of any other body fluid. It bears some resemblance to that of mature human milk, in that the latter has almost no γ-globulin. However, BCF has no discrete bands, unlike milk.

It is possible that the pure-type BCF electrophoretic pattern resembles that of apocrine skin gland secretion but, owing to the difficulty of collecting the latter in pure form, this has not been tested.
The number of proteins present in the pure-type BCF varies with the separation method used. The best resolution was obtained with iso-electric focussing, which revealed at least 14 components in such fluids. SDS-acrylamide-gel electrophoresis showed somewhat fewer bands. Some of these represent plasma proteins present in very low concentrations. It is also possible that some are sub-units of proteins, particularly in the mercaptoethanol-treated samples which are used in SDS-acrylamide-gel electrophoresis. If proteolysis is occurring then it is possible that some of the components are large peptides produced from larger proteins. At least one proteolytic enzyme, cathepsin D, is present in aspirated BCF. The pH of the fluids was found, using narrow-range pH papers, to be from 6 to 10 (with a mean of 7.8, and a standard deviation of 1.4), and this is outside the optimum pH range for activity of cathepsin D, which Barrett (1972) gives as being 3.0 to 3.5. Therefore, proteolysis by this enzyme is almost certainly not occurring in vitro, and unless the pH changes markedly after aspiration, neither does it occur in vivo. However, the presence of one lysosomal protease suggests that others may be present, and some of them have optimum pH values much closer to the pH range of BCF (Barrett, 1972).

The BCF-protein molecular weights estimated by SDS-gel electrophoresis are in the same range as the values found in BCF by Pearlman et al. (1973), employing the same method; however, they found fewer bands.

The proteins of BCF appear to be mainly glycoproteins, as suggested by PAS staining, their low pI values, and difficulty in precipitating them with perchloric acid. At least some of these proteins are precipitated by Cetrimide (cetyltrimethylammonium bromide) which is a known precipitant of some polysaccharides.
(Havez et al., 1971) and glycoproteins.

The only milk protein found in BCF was lactoferrin and this is not specific to milk, being found in other fluids including bronchial secretions, saliva, bile, semen and polymorphonuclear leucocytes (Masson et al., 1969). With immunoelectrophoresis, the BCF lactoferrin was found to have a higher mobility than that in milk. In bovine milk, lactoferrin is reported (Hekman, 1971) to have a lower electrophoretic mobility than the pure protein due to binding to other proteins. Thus, if the same phenomenon occurs with human lactoferrin then BCF lactoferrin may either be bound to a different protein from that in milk, or be in unbound form.

The source of lactoferrin in BCF is not known. Since at least some polymorphonuclear leucocytes are often found in the fluids, it is possible that the protein originates from them.

The occurrence of some serum proteins in BCF, at very low levels, was shown by the presence of corresponding antibodies in anti-(BCF). These could be removed from the antiserum by adding plasma proteins. Not all serum proteins are present, and assuming they are derived from plasma there must be selective passage of certain proteins across the cyst epithelium, or else degradation of some proteins inside the cyst. Degradation would seem more likely, the range of serum-type BCF electrophoretic patterns suggesting different degrees of proteolysis rather than variations of selective passage. However, there is no evidence that the serum-types are eventually transformed to pure-types (for definition of pure- and serum-types, see p.176). It is suggested that they are a different form of cyst, or a cyst which later develops an inflammatory reaction, possibly due to damage to the cyst wall. In support of this view is the correlation of serum-type
electrophoretic patterns with the presence of numerous leucocytes in the fluid. In addition, Bonser et al. (1961) found that inflammatory reactions can occur in cysts following damage to the cyst wall, leading to the entry of polymorphs and lymphocytes. They found this to have occurred in 29 of a series of 100 cysts which they examined histologically. This proportion is very close to the percentage of BCF specimens which are serum-type (24% in the present study, see p.176 and Table 2).

Among the more remarkable features of the pure-type BCF is the very low level of immunoglobulins. The reason for their virtual absence is uncertain. Either they do not appear in the cyst, or else, if present, they are subsequently degraded. The latter explanation seems less likely, as one would expect reasonable levels to be present if there were a continuing passage of immunoglobulins into the cyst, competing with the degradative process. Further, it would be reasonable to expect the ratio of IgG to IgA to approximate to that of plasma, as this is the most likely source of the immunoglobulins; they differ markedly. The absence of other serum proteins in pure-type fluids is an additional argument against such a passage.

The predominant immunoglobulin in exocrine secretions, including milk (see p. 11), is IgA, and this contrasts with the observation that IgG is the major immunoglobulin in 65% of the pure-type fluids investigated. Limited immunohistochemical results using antisera to IgG and IgA suggest that there is an absence of immunocytes producing these immunoglobulins in cystic areas of breast. This lack of immunocytes would explain the lack of secretory IgA, particularly so if Brandtzaeg’s (1977) model is correct. The evidence thus points to the cyst being a somewhat closed system to immunoglobulins from plasma, the levels being further reduced by the lack of local production.
Thus, pure-type BCF bears little resemblance to serum. It is like mature milk in its lack of cathodically migrating material (i.e. IgG) on electrophoresis, but it is unlike milk, because it lacks milk proteins, triglycerides and lactose, and by its overall electrophoretic pattern.

The proteins present are mostly of relatively low molecular weight, SDS-acrylamide-gel electrophoresis showing them to be between 20,000 and 80,000. But in the fluid, some of the proteins may exist in aggregated form, since some are excluded from Sephadex G-200 (molecular weight fractionation range 5,000 to 800,000), although these may be adhering to the particulate material of BCF.

3. PARTICLES

The particles in BCF are responsible for the milk appearance of the fluid. The whole fluid may be separated into a white-coloured particle-fraction and a clear fluid-fraction, that has the same colour as the original whole BCF.

The degree of "milkiness" (estimated visually) of BCF is inversely related to the levels of most chemical constituents, and especially to depth of colour, of the fluids. This would have important implications if milkiness was related to the age of the cyst. If colour increases with cyst age then so would some chemical constituents, whereas the amount of particles (milkiness) would decrease. Obviously there is some difficulty in estimating milkiness in the more coloured fluids since it is obscured by dark colours, but not enough to account for its strong negative association with colour.

The noticeable exception to this general negative association is cholesterol, which is strongly positively related to milkiness.
This is to be expected since the particle-fraction also contains the majority of the cholesterol of BCF. Hence this constituent would decrease with cyst age if colour increased. Whilst this could be further evidence for degeneration of cyst contents, it might also be that the cholesterol is used in anabolic processes for further cell growth or steroid hormone production. Also, if degenerative destruction of cells was occurring, the number of particles would increase with cyst age, since they are membrane-derived.

There is little doubt that some of the particles are of membranous origin. Their appearance in the electron microscope is very suggestive of membranes, and indeed is similar to that of the "fluff fraction" of bovine skimmed milk (Patton & Jensen, 1976) which is largely plasma membrane, including microvilli, of mammary epithelium (see also p. 14). It is of relevance that most of the milk cholesterol, though at much lower levels than in BCF, is in this fraction (Plantz & Patton, 1973) and also is almost entirely unesterified, as in BCF particles. The particle-fraction of BCF contains membranes from a wide range of cellular organelles as judged by their appearance (D. J. Morré, personal communication, commenting on the electron micrographs of negatively stained particles made in this study. See Fig. 27). They presumably derive from cells of the cyst epithelium, although in some fluids leucocytes may be a further contributory source. It is unlikely that connective tissue cells are involved, especially since assays for hydroxyproline and proline hydroxylase (kindly performed by Dr. C. I. Levene) were negative in three BCF samples.

The significant problem is not whether the particles are membranous, but why they arise and how. Their density is of little help in answering these two questions, being over a broad range and

250
somewhat below the levels of cell membranes in general. This must be due to either their gaining less dense, or losing more dense, material. The latter possibility seems more likely, and it is suggested that the particles are membranes from which some of the protein has been removed, leaving an entity with proportionally higher lipid content, which thereby still retains its original gross structure. Thus, for example, if the membrane was in the form of closed vesicles, the outer protein layer of the membrane may be removed, leaving the lipid and inner protein layer which can still maintain the structure. The very high ratio of cholesterol to protein (from 26:1 to 5:1 in the particles of the large-sample density gradient run) would support such an hypothesis. Martel et al. (1973) found the value for human MFGM to be 0.62:1. This hypothesis would also explain the large range of particle densities (i.e. from dense, intact membranes, to the protein-depleted, and thus lipid-rich, membranes having a lower density) and hence also their appearance, after centrifugation of particles in a density gradient, as a series of closely packed but discrete bands.

The other possibility for the altered density, the gaining of low-density material, could be explained either by a presumably non-specific adherence of lipid (probably cholesterol), or by the particles being secreted cholesterol bounded by membrane. The latter suggestion should be investigable by gently rupturing the vesicles and estimating cholesterol in the supernatant and the remaining membranes.

The association of Na\(^+\), K\(^+\)-dependent ATPase and 5'-nucleotidase with the particles, as demonstrated in the assays following density gradient centrifugation, is further evidence for the presence of plasma membrane in that fraction. Both the enzymes are plasma membrane markers. However, although Martel-Pradal and Got (1972) found other

* It should be noted that although most cell membranes contain cholesterol, the levels in e.r. are relatively very low or even zero (Thines-Sempoux, 1974)
p.m. marker enzymes in human skimmed milk, they only obtained
5'-nucleotidase activity in the cream (i.e. associated with milk fat
globule membrane). This suggests that the enzyme is removed from,
or activated in, the membranes of human skimmed milk. This would
not seem to be the case with the particles in BCF.

The fact that the peak of activity of both p.m.-marker enzymes
occurs at the denser end of the BCF particle fraction, as do most of
the other enzymes, further supports the protein-removal hypothesis.
This region also has a higher protein:cholesterol ratio, and densities
more appropriate to cell membranes. Therefore, it probably represents
the more intact membranes, and it is logical that membrane marker
enzymes should locate there rather than in the less-dense region, since
the protein-removal hypothesis would suggest that membranes in that
region have had much of their protein (and therefore most enzymes)
removed.

Acid and alkaline phosphodiesterases have virtually identical
distributions, which differ from those of the other enzymes assayed
in corresponding exactly with the cholesterol peak (i.e. maximal
activity occurs at a lower density). It is not certain whether or not
the identical distribution is due to one enzyme acting at both incubation
pH values. Unfortunately, insufficient material remained to repeat the
assay with an inhibitor of alkaline phosphodiesterase, such as EDTA, in
order to resolve this problem. It is possible that the enzyme(s) may be
non-specifically binding to the particles; this would account for the
activity being separated from the peak of activity of the other enzymes.

Although glucose-6-phosphatase is present, its low activity makes
conclusions uncertain. If the enzyme is a marker for endoplasmic
reticulum, then the presence of that organelle is suggested. There
is some doubt as to the presence, or intracellular localisation, of glucose-6-phosphatase in tissues other than liver (Reid, 1971), where it is a marker for endoplasmic reticulum, though also found in Golgi complex (reviewed by Morré et al., 1973). Keenan et al. (1974) suggest its activity in rat and bovine mammary gland is too variable for it to be of any use as a marker. However, Martel-Pradal and Got (1972) claim it to be a marker for this organelle, in their study of membranes in skimmed human milk.

Some of the particles bear a strong resemblance to lipoprotein secretory vesicles, such as those demonstrated in liver by Glaumann et al. (1975). They are best seen in the thin section of material in a cyst lumen (Fig. 28), and identical structures are also seen in the 'pink' cells (Fig. 24). This is further evidence that secretion is occurring.

4. CHEMICAL COMPONENTS

It had been hoped that assay of some chemical constituents of BCF would provide clues to its source. In fact, the results are rather inconclusive. In part this is due to the wide range of levels found for many constituents and the lack of correlations between them.

The values for total protein are low compared with serum. However, it is somewhat dangerous to compare total protein values in two different fluids on an absolute scale, since although the methods used are accurate enough, the results are standardised using serum protein (or proteins) and, as previously mentioned, the pure-type BCF proteins are not at all like those of serum. The AutoAnalyzer uses the biuret method, and the results for BCF agree well with a manual Lowry procedure, but the absolute values may be open to criticism. The same argument is applicable to albumin...
estimation, but for a different reason. The AutoAnalyzer uses a well-tried dye-binding technique which is adequate for serum; BCF may contain interfering factors which could take up the dye or cause it to bind non-specifically to other proteins.

The calcium levels suggest free diffusion of unbound plasma calcium only into pure-type cysts, but both the bound and free forms into the serum-type. The relatively low levels are another point of dissimilarity with milk.

The high levels of cholesterol are largely due to the particles, although some remains in the fluid fraction after removal of particles. It is occasionally found in the crystalline form, floating on top of the fluids.

The high levels of urate are also interesting. If this compound is arising from catabolism of purines within the cyst then it may be a useful indicator of cyst age, assuming breakdown occurs at a consistent rate. It may be of relevance that the levels show a moderate positive association with depth of colour. Also, in the serum-type fluids the level is much closer to that of serum, suggesting restricted access to plasma in pure-type cysts but freer access to plasma in the serum-type. It seems unlikely that urate enters the pure-type cysts from plasma, since this would require an active process to counteract the concentration gradient into the cyst. Therefore, it must be produced in the cyst. However xanthine oxidase, which would be required for urate production from purines, is reported not to be found in human breast tissue although it is present in the mammary glands of other mammals. No activity of the enzyme could be found in BCF either, but this does not necessarily mean it was not present, at some stage, in the fluid or the cells.
In general, it may be said that the mean levels of chemical constituents in the serum-type cysts tend to be closer to those of serum (especially total protein, albumin, urate and alkaline phosphatase) than mean levels of the same constituents in pure-type fluids. In these latter fluids, the levels are nearer to those of milk. The most noticeable exception is cholesterol, but this is present in a rather specialised form relative to that in plasma. In fact, the mean level in the serum-types is higher than that for pure-types; but this may merely be a reflection of the smaller sample number for the former type, or their high content of leucocytes.

It must also be emphasised that these remarks apply to the mean levels, not individual values, which are highly variable for most parameters.

The correlations between the chemical components assayed are also not very revealing as to the source of BCF. Some of these probably arise because the two parameters are both related to a third. These, therefore, are chance rather than causal relationships, and it is difficult to select them from the rest.

Since the bilirubin in plasma is bound to albumin, their correlation in BCF suggests that both originate from blood. Possibly the same applies to BUN, which also correlates well with albumin. However, the previously mentioned probable impermeability of pure-type cysts suggests that the presence of these constituents must be due to an active process, rather than passive diffusion. Urate is negatively associated with albumin, and this would bear out the hypothesis that it is formed in situ and does not originate from plasma. Colour is positively correlated with BUN, urate and bilirubin and this would demonstrate, if urate was related to cyst age, that the
fluid becomes more deeply coloured as the cyst ages. Milkiness is negatively associated with BCF colour and thus would decrease with age of the cyst.

Ion levels are rather more informative, especially the high levels of potassium, as also reported by Fleisher et al. (1973). Since intracellular potassium concentration is higher than extracellular, the conclusion is that the element arises in BCF due to cellular breakdown. Therefore, potassium levels should indicate the degree of such breakdown and, assuming this occurs at a consistent and constant rate, should correlate with other postulated indicators of cyst age mentioned above. If this were so it would help in several ways: to see if cysts increase in size with age, to help rationalise the high variability of levels of many chemical constituents of BCF - especially enzymes - and to enable determination of which other constituents are related to cyst age and whether they increase or decrease. Unfortunately, no good correlations were obtained between the various parameters and ion levels.

The steroid levels in BCF were unremarkable except for the breast cancer patient who had high levels of 5α-dihydrotestosterone (DHT) in a previous breast cyst. Another patient who later developed breast carcinoma also had high levels of DHT in previous cysts from both breasts. Since the serum DHT level of both these patients was normal, it must be inferred that steroid synthesis was occurring in the breast in these cases. That such a process happens in other cases is not certain, but Miller et al. (1973), using radioactively labelled precursors, found synthesis of testosterone and DHT to occur in normal human breast tissue, and large amounts of DHT in two fibroadenomas. It would be of great interest to know whether such
high levels of DHT in BCF is an early indicator of carcinoma. Cystic disease of the breast may be associated with fibroadenoma, so Miller's explanation would obviously have to be considered. Their results conflict with the work of Jenkins and Ash (1972) who found no DHT in normal breast, but active production in cancerous breast tissue. Further work is necessary to clarify this issue.

The other steroids are possibly plasma-derived, occurring in BCF at roughly the same levels as in plasma. Srivastava et al. (1977) found similar levels, in BCF and serum, of PRL and TSH, and lower levels of LH and FSH in BCF than in serum.

The results for assays of steroid hormones in serum and urine showed no difference between cyst patients and age-matched controls. This would seem to contradict the results of England et al. (1974) who showed slight elevation of 17β-oestradiol in cyst patients, but our sample was only one third of the size of theirs and was not assessable statistically for minor variations. It is certainly true that any hormonal imbalance in these patients is not marked.

The high but variable levels of CEA were of the same range as found by Fleisher et al. (1974). They found the material to elute from a Sephadex G-200 common with the main protein peak and deduced the molecular weight as being at least 200,000. This, they suggest, is either a polymer, or the immunoreactive molecule attached to another protein. Whether the entity is really CEA, or another glycoprotein cross-reacting with the antiserum, is open to debate, but with the high proportions of glycoprotein in BCF proteins the latter seems possible. The levels of CEA-like material do not correlate with other parameters, but if it really is CEA the relationship to subsequent carcinoma should be worth investigating.
5. THE EVIDENCE FOR SECRETION OF BCF

The original hypothesis of this study was that BCF represented, at least partly, a secretion by the cells lining the cyst. Though some of the results obtained suggest an origin for some constituents of BCF from plasma, evidence has also been presented which is in favour of the pure-type cysts being a system isolated from plasma, or tissue fluid, except by active processes. The remaining evidence will therefore be presented as being indicative that secretion into cysts occurs, even if the nature of the secreted material is not fully characterised.

1) The 'pink' epithelium in the breast, and lining cysts, is very similar histologically to that in the secretory coil of apocrine skin glands. This similarity is noticeable using special, as well as general, stains. The ultrastructure of the two cell types is almost identical, and both possess the more unusual features such as the large osmiophilic bodies. This identity then is more than just chance.

2) The functional similarity of the 'pink' cells and apocrine skin gland cells, is shown by their identical reactions with antiserum to BCF. This is further good evidence of a close relationship between the two cell types which current thinking tends to deny. Whether this relationship is functional and could throw some light on the reason for, or even the validity of the existence of, 'pink' cell metaplasia, or whether it merely shows a degradation of the breast cells to a type which has the same embryological origin as apocrine skin gland cells, is not clear. If nothing else, it is
certainly further evidence that the breast is a modified skin gland, as first suggested by Benda (1894). Possibly, the 'pink' cell is a resting breast cell type which secretes all the time but becomes obvious at the menopause when the secretion may be retained in cysts.

The immunological reactions also show a similarity of BCF to apocrine skin gland secretion. The latter is responsible for the 'sweat' odour produced by its bacteriological decomposition. It is of interest that BCF commonly has a strong 'sweaty' odour which is very marked on heating. It is extremely unlikely that the initially sterile BCF is degraded by microorganisms on storage at -20°C, and thus it is suggested that BCF and apocrine gland secretion are chemically similar, and that the method of odour production may not be solely due to bacterial action but perhaps also to change of physical conditions, such as pH. With BCF, the odour is especially marked on heating and after lipid extraction, when it seems to be in the lipid fraction.

3) The ultrastructure of the 'pink' cell shows many characteristics of a secretory cell type, including well developed endoplasmic reticulum, many mitochondria and a prominent Golgi complex. This was first demonstrated by Pier et al. (1970) and is confirmed in the present study. Also present are the dense osmiophilic bodies, corresponding to the PAS-positive granules found in light microscopy, and which could be secretory granules, or may be altered mitochondria. In addition, the presence of lipoprotein secretory vesicles of the type which is seen in liver cells is further evidence that secretion is occurring. Structures resembling them are also seen in the particle-fraction of BCF. This suggests that the particles may be a mixture of
secretion product and membranes; the latter could be the BCF equivalent of the "fluff fraction" (see p.14) of milk.

4) There exists in BCF at least two specific proteins not found in milk or plasma, and for which therefore the only possible source is the epithelial cells of the cyst. It could be argued that they are due to degeneration of cellular elements and not secretion, but in that case it would be expected that they would be found in other tissues or normal intact breast cells. Immunohistological evidence denies this.

5) Anti-(BCF) reacts with sections of breast containing cysts or areas of 'pink' epithelium. The reaction does not occur in cells of 'normal' ducts or alveoli even in the same section. It shows reaction with the material in the lumina both of ducts adjacent to cysts and the cysts themselves (as would be expected since this was the source antigen). The cap of the cell is strongly reactive, but whether this is due to material adherent to the outer surface of the plasma membrane or in small vesicles just below it, as seen in the electron microscope, is uncertain at the light microscope level. There is some suggestion that in apocrine skin glands these vesicles may contain secretory material (Hibbs, 1962; Yasida et al., 1962; Munger, 1965); therefore it would be of great value to determine whether the antiserum is indeed reacting with the contents of such vesicles.

There is some weak overall staining of the cytoplasm but the region of strong staining within the cell is an area apical to the nucleus and of the same size or slightly larger. This area has a
rather tortuous appearance using the light microscope, and from its position would seem to be the Golgi complex. This is well developed and appears in the same position when examined with the electron microscope, and as an unstained, or weakly stained, region by light microscopy. Assuming it is the Golgi complex, the problem is now whether the antiserum is reacting with the membranes or the contents. If the contents are reacting and they are secretory products, the question then arises, why any vesicles which may be moving from the Golgi complex to the apical plasma membrane show no reaction. One possibility is that the vesicles are very small and in the light microscope can only be seen en masse under the plasma membrane, as already mentioned.

The Golgi complex is known (Campbell & Von der Decken, 1974) to be the site of incorporation of at least some carbohydrate groups into glycoproteins, which are the major protein type found in BCF. In some cases, the carbohydrate addition may be initiated in smooth endoplasmic reticulum since this contains some glycosyl transferases. Such a process occurring in 'pink' cells, may account for some of the weak cytoplasmic reaction with anti-(BCF).

The 'packaging' of secretion into vesicles for transport to the plasma membrane also occurs in the Golgi complex (Allison, 1974). The vesicle membrane fuses with plasma membrane to allow passage of secretion out of the cell. Hence the vesicle and "mature" Golgi membranes must have a composition identical, or very similar, to that of plasma membrane (Lucy, 1974; Keenan et al., 1974). If the anti-(BCF) was reacting with Golgi membranes, rather than contents, it would be expected to react also with plasma membrane. This has only occurred in one area of one section, using immunofluorescence, and, except for the cell cap reaction, has not been seen since. On
this evidence it would seem that the reaction with anti-(BCF) is
directed against the content, as opposed to membranes, of the Golgi
complex.

6) There are two types of fluid, and the 'serum-type'
electrophoretic pattern correlates with the presence in the fluid of
large numbers of leucocytes, including lymphocytes. Whether the
latter indicates leakage of blood through cyst walls following damage,
or secretion into the cysts is difficult to determine, but evidence
previously discussed suggests it is not an active process. This is
also borne out by the fact that in many cases, the serum-type fluids
have a gross appearance generally identical to that of pure-types, as
well as an electrophoretic pattern related to that of pure-types;
thus 'normal' cyst fluid production must also be occurring (see also
pp. 176, 192, 247 & 248).

7) Lastly, there is the fact that cysts are formed and increase
in size, and often have a high internal pressure. If secretion does
not occur, then the fluid must accumulate by a passive diffusion
process, often to attain large volumes. That such a mechanism could
operate against the high intracystic pressure is unlikely, and an
active process must be involved.

It would be of great value to attempt to determine the
'permeability' of cysts using labelled substances of various molecular
weights. Thus, for example, a range of radioactively labelled human
peptides and proteins (or even inert, artificial polymers) and ions
could be injected intravenously into cyst patients, and their
concentrations estimated in BCF aspirated after a set time. From these
results, valuable information on partition between BCF and serum of the various sizes and types of compound could be obtained. The largest size of molecule which may enter the cyst by diffusion could thus be determined. Obviously the ethical considerations would have to be seriously discussed before undertaking such an investigation.

6. CONCLUSIONS

This then is the evidence for secretion. It is reasonably strong evidence although the final proof, by demonstration through electron microscopy, of the process actually occurring, has not been obtained. But this does not weigh too heavily against the other evidence. It is possible that the 'pink' cells have a latent period — when secretion ceases due to pressure on the epithelium — as do the cells of skin apocrine glands (Hurley & Shelley, 1960) (see p. 47) and lactating breast epithelium (Cowie & Tindal, 1971). This would reduce the chance of seeing the secretory process occurring, especially without examining larger numbers of specimens, and using high magnification if the secretory vesicles are very small.

There is also some evidence for degenerative change occurring in breast cysts. It could be argued that the proteins in BCF result from at least partial proteolysis of, possibly, plasma proteins, leaving only those most resistant to such degradation (i.e. glycoproteins). However, if this is so, they must be broken down sufficiently to completely alter their antigenicity to prevent them reacting with anti-(whole human serum). Also, this would not explain the presence of the two BCF-specific proteins, unless the degradation is extended to cell organelles and membranes. The particle-fraction may represent degeneration of cells, as could the
high concentrations of urate. But neither DNA nor RNA could be detected in BCF, which seems unaccountable if cellular breakdown is occurring, unless they are very rapidly degraded. So perhaps the two BCF-specific proteins could be cell membrane proteins somehow removed from the membranes, rather than a secretion product. But why, then, does the antiserum not react with cell membranes, as discussed above? The evidence points to the two proteins being secretions.

The presence of cathepsin D is important in this matter. The enzyme seems to be free in the fluid rather than in intact lysosomes, since it was not activated by the classical methods of de Duve et al. (1955), though whether released by the lysosomes, or freed by their osmotic rupture in the cyst fluid, is uncertain. Nonetheless, some proteolysis is probably occurring, and this perhaps accounts for some of the variability of many constituents found during this study, particularly the enzymes (see also p. 206).

In summary, many of the results point towards some of the components of BCF being secreted by the 'pink' epithelial cells. Perhaps the strongest evidence is the location of BCF-specific proteins in the Golgi complex, and the functional relationship (shown by identical reaction) of 'pink' cells to those in a skin gland which is known to be secretory. Few results go against secretion. However it cannot be denied that some degradative processes are occurring.

It is therefore suggested that BCF is, at least partly, a secretion by the 'pink' cells, with some coincident degenerative changes, and that these cells may be an alteration of normal breast epithelial cells to a more basic, or less specialised, cell type. Possibly Higginson & McDonald (1949) are right, and 'pink' cells are
present in the breast all the time (see p. 37).

This study has been undertaken to see how breast cyst fluid is formed. Evidence that the 'pink' cell is the actively secreting cell involved in this process is provided. The cell is a constituent found in the condition of cystic disease of the human female breast, and it is suggested that the function assigned may shed some light on the mechanism whereby such cysts may develop.


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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. E. Reid (University of Surrey) and Dr. M. M. Levene (Regional Cell Pathology and Cytogenetic Service, Wandle Valley Hospital) for their helpful criticism and guidance throughout this work.

Special thanks are due to Dr. D. E. H. Tee (King's College Hospital) for much helpful advice and assistance in raising the antiserum; also to members of staff of the Wolfson Bioanalytical Centre (University of Surrey), and in particular to Dr. Richard Hinton, for their help and encouragement.

I am indebted to the Consultant Surgeons of St. Helier Hospital (Carshalton, Surrey) who have provided the clinical material used in this study, and to Mr. J. Bradbeer F.R.C.S. (Consultant Surgeon, Mayday Hospital, Thornton Heath) for his generous cooperation and assistance.

I would also like to thank Dr. C. I. Levene (University of Cambridge), Professor M. Neville (Director, Ludwig Institute for Cancer Research, Sutton) and Mr. D. Fry (West Park Hospital, Epsom) for performing various assays, Dr. B. Reiter (Nat. Inst. for Research in Dairying) for the kind gift of antiserum to lactoferrin, and Dr. Angela Russell for her advice on the electron microscopy.

I am very grateful to Mrs. Jane Mardling for her patience and the care she has taken with this typescript.

Last of all, but never least, to my wife Barbara, not only for the beautiful drawings but also for her long-sufferance, my eternal gratitude.