A BIOCHEMICAL INVESTIGATION OF A DOMINANT MUTATION
ASSOCIATED WITH CELLULAR INVASIVENESS

Dorien J. Pritchard, B.Sc. (Wales), Dip.Gen. (Edinburgh)

Thesis submitted in requirement for the degree of
Doctor of Philosophy,
University of Edinburgh,
1973
I wish to thank Mrs. Ruth Clayton for her guidance and encouragement throughout the course of this work. I am grateful also to Professors C.H. Waddington and D.S. Falconer for provision of laboratory facilities at the Department of Genetics, University of Edinburgh and to Professor D.J. Manners for making biochemical facilities available to me at Herriot Watt University, Edinburgh.

Special thanks are due to Miss Margaret Ferry for carrying out the electron microscopy; to Dr. W.L. Cunningham for considerable advice on the biochemical analyses; to Mr. E.C.A. Luccy for making the time-lapse film; to Messrs. G.N. Newell, F.M. Holmes, M.J.J. Ireland and D.J. Toghill for building the cell electrophoresis apparatus; and Dr. P.C. Das of the Blood Transfusion Service for collecting samples of my blood.

I also wish to thank Dr. D. Bannister, Dr. J.B.L. Bard, Dr. J.C. Campbell, Dr. W.D. Cooper, Dr. R.B. Kemp, Dr. G. Selman and Dr. D.E.S. Truman for valuable discussions; Dr. J.C. Roberts for animal house facilities; Miss Helen Macrae and her staff for routine maintenance of mouse stocks and for supplies of inbred mice; Mr. R.K. Barrowdale and his staff and Mr. C. Atherton for photographic work; Mr. Forrest of the Edinburgh Corporation Slaughterhouse, George for supplies of bovine eyes; Miss M. Drysdale for unfailing efforts to obtain obscure literature; Mrs. Ann Brown, Mrs. Hilary MacKenzie, Mr. Duncan Easton, Mr. Alan Gillies, Mr. Tom
McKelvie and Mr. Mike Riley for valuable advice on technical matters; and numerous other members of the Department of Genetics for their interest and stimulating conversations. Lastly I wish to thank my wife for the anion exchange chromatography of glycoprotein hydrolysates, for the glucose assays and for advice and moral support in all aspects of the work.

This work was carried out during the tenure of a generous scholarship from the Wellcome Trust for which I am very grateful.
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>i</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 1 REVIEW OF THE LITERATURE ON THE CELL PERIPHERY</td>
<td>3</td>
</tr>
<tr>
<td>PART 1: Cell surface factors in development</td>
<td>3</td>
</tr>
<tr>
<td>(a) Cell adhesion in organisation of the embryo</td>
<td>4</td>
</tr>
<tr>
<td>(b) Specificity in cell migration</td>
<td>5</td>
</tr>
<tr>
<td>(c) Specificity in neural connections</td>
<td>8</td>
</tr>
<tr>
<td>(d) Contact effects</td>
<td>9</td>
</tr>
<tr>
<td>PART 2: The nature of the cell periphery</td>
<td>12</td>
</tr>
<tr>
<td>(a) The plasma membrane</td>
<td>13</td>
</tr>
<tr>
<td>(b) The glycocalyx</td>
<td>17</td>
</tr>
<tr>
<td>(c) Morphological features of the cell surface</td>
<td>28</td>
</tr>
<tr>
<td>PART 3: Theories of cell adhesion</td>
<td>30</td>
</tr>
<tr>
<td>(a) Adhesion of cells to inert surfaces</td>
<td>31</td>
</tr>
<tr>
<td>(b) Tissue dispersion</td>
<td>32</td>
</tr>
<tr>
<td>(c) The reaggregation of dispersed cells</td>
<td>33</td>
</tr>
<tr>
<td>(d) Aggregation-enhancing factors</td>
<td>34</td>
</tr>
<tr>
<td>(e) Specificity in adhesion</td>
<td>36</td>
</tr>
<tr>
<td>(f) The lyophobic colloid theory</td>
<td>41</td>
</tr>
<tr>
<td>PART 4: Surface properties of cancer cells</td>
<td>44</td>
</tr>
<tr>
<td>(a) Contact features</td>
<td>44</td>
</tr>
<tr>
<td>(b) Cancer and the mitotic cycle</td>
<td>45</td>
</tr>
<tr>
<td>(c) Antigenic and molecular features</td>
<td>48</td>
</tr>
<tr>
<td>PART 5: Summary</td>
<td>51</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>THE PRACTICAL PROBLEM</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>A</td>
<td>The sorbitol pathway</td>
</tr>
<tr>
<td>B</td>
<td>Extracellular membranes</td>
</tr>
<tr>
<td>C</td>
<td>Seminal vesicle secretion</td>
</tr>
<tr>
<td>D</td>
<td>General plan of the practical work</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3</th>
<th>MATERIALS</th>
<th>Page</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 4</th>
<th>METHODS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Qualitative analysis of carbohydrates</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Conclusion. Selection of the most suitable methods for qualitative analysis of complex carbohydrates</td>
<td>76</td>
</tr>
<tr>
<td>B</td>
<td>Quantitative methods</td>
<td>78</td>
</tr>
<tr>
<td>C</td>
<td>Enzymic solubilization of lens capsule and seminal vesicle secretion</td>
<td>82</td>
</tr>
<tr>
<td>D</td>
<td>Gel filtration</td>
<td>83</td>
</tr>
<tr>
<td>E</td>
<td>Polyacrylamide gel electrophoresis</td>
<td>84</td>
</tr>
<tr>
<td>F</td>
<td>Analysis of seminal vesicle secretion</td>
<td>86</td>
</tr>
<tr>
<td>G</td>
<td>Examination of the lens capsule</td>
<td>87</td>
</tr>
<tr>
<td>H</td>
<td>Tissue culture of lens epithelium</td>
<td>89</td>
</tr>
<tr>
<td>J</td>
<td>Whole-cell microelectrophoresis</td>
<td>92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 5</th>
<th>RESULTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Determination of sorbitol dehydrogenase in mouse livers</td>
<td>102</td>
</tr>
<tr>
<td>B</td>
<td>Examination of seminal vesicle secretion</td>
<td>102</td>
</tr>
<tr>
<td>C</td>
<td>Examination of the lens capsule</td>
<td>104</td>
</tr>
<tr>
<td>D</td>
<td>Examination of glomerular basement membrane efficiency. Urine analysis</td>
<td>106</td>
</tr>
<tr>
<td>E</td>
<td>Tissue culture of lens epithelium</td>
<td>107</td>
</tr>
<tr>
<td>F</td>
<td>Whole-cell microelectrophoresis</td>
<td>110</td>
</tr>
</tbody>
</table>
## CHAPTER 6  ANALYSIS OF THE BREEDING DATA

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  Comparative effects of different genetic backgrounds</td>
<td>112</td>
</tr>
<tr>
<td>B  Penetrance of the <em>Sey</em> gene</td>
<td>114</td>
</tr>
<tr>
<td>C  The effect of maternal age on expression of the <em>Sey</em> phenotype</td>
<td>117</td>
</tr>
<tr>
<td>D  The significance of differences in regression coefficients</td>
<td>119</td>
</tr>
<tr>
<td>E  Summary</td>
<td>120</td>
</tr>
</tbody>
</table>

## CHAPTER 7  DISCUSSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  Genetic constitution of experimental animals</td>
<td>121</td>
</tr>
<tr>
<td>B  Sorbitol dehydrogenase determinations</td>
<td>122</td>
</tr>
<tr>
<td>C  Analysis of seminal vesicle secretion</td>
<td>123</td>
</tr>
<tr>
<td>D  The lens capsule</td>
<td>125</td>
</tr>
<tr>
<td>E  Prenatal mortality of homozygotes</td>
<td>134</td>
</tr>
<tr>
<td>F  Examination of filtration efficiency of glomerular basement membrane</td>
<td>134</td>
</tr>
<tr>
<td>G  Cell electrophoresis</td>
<td>135</td>
</tr>
<tr>
<td>H  Tissue culture</td>
<td>137</td>
</tr>
<tr>
<td>J  The modification of phenotype by maternal factors</td>
<td>142</td>
</tr>
<tr>
<td>K  Biochemical interpretation</td>
<td>145</td>
</tr>
<tr>
<td>L  The Roseman hypothesis of cell adhesion and its implications</td>
<td>150</td>
</tr>
</tbody>
</table>

## CHAPTER 8  CONCLUSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
</table>

## REFERENCES

161
SUMMARY

This investigation is directed towards an understanding of the nature and significance of cell surface determinants mainly through the analysis of extracellular and secreted material in the Small Eye (Sey) mutant mouse. The results indicate that mice heterozygous at the Sey locus are characterised by abnormalities in the structure and composition of extracellular membranes. This conclusion is confirmed by electron microscopic evidence of the structure of the lens capsule. The results lead to the formulation of the hypothesis that the defects may be due to a deficiency in the action of one or more of the glycosyltransferases involved in the glycosylation of basement membrane collagen.

Since the seminal vesicles are also abnormal in the mutant, a detailed analysis of the composition of normal and Sey mouse seminal vesicle secretion was carried out. The secretions of control and mutant mice contain free glucose and fructose and polysaccharide, consisting of glucose, galactose, mannose, fucose, xylose, hexosamine and sialic acid, associated with protein. The mutant secretion contained lower levels of free sugars and of bound hexosamine and hexose, but sialic acid content was unaffected. The ratio of sialic acid to fucose or hexosamine plus hexose was raised by about 16%.

A collagenase digest of Sey lens capsules was examined by gel
filtration. The pattern of elution of orcinol-positive carbohydrate indicated that the carbohydrate chains of Sey lens capsules are shorter and more polydisperse than normal. The sialic acid content of Sey capsules was increased by about 160% relative to hydroxyproline or hexose. Electron micrographs of Sey capsules revealed occasional banded fibres and other fibrils which varied greatly in diameter. These observations are considered to indicate a reduced, but variable degree of glycosylation of capsular collagen.

The basis of lens cataract is discussed in terms of these results.

Other membranes studied include the kidney glomerular basement membrane. Analyses of urine gave no indication of defects in the glomerular filter, but an abnormality of Retchert’s membrane in the embryo is proposed. It is suggested that the general failure of eye development in Sey mice may originate as a defect in lens induction, due to abnormalities in the properties of embryonic ectoderm basement membrane, or other extracellular material.

Studies of lens epithelial cultures showed that mutant cells are abnormal in their relationships with one another or with the substratum. Outgrowths from mutant explants formed occasional regions of overlapping cells, while normal cultures maintained a strictly monolayer appearance. Time-lapse film analysis revealed tunnelling by a wedge of mutant cells beneath other cells in their path.

The surface charge of disaggregated lens epithelial cells was examined by whole cell electrophoresis. Preparations from mutant mice gave less reproducible values than those from normal animals, but a similar mean surface charge is indicated.
The abnormal behaviour of *Sey* cells *in vitro* and *in vivo* is discussed in terms of current hypotheses of cell recognition.

Penetrance and expressivity of the *Sey* allele are both modified by genetic background. On the basis of ratios of phenotypes in the litters, it is concluded that failure of eye development and/or lens cataract in *Sey* stocks depend on the presence of the *Sey* allele in that individual, its presence in the mother, and the age of the mother. The possibility is discussed that normal foetuses are depleted of an essential factor by growing *Sey* mothers.

Several new methods are described.
INTRODUCTION

"All processes in which cells interact with elements of their environment involve a recognition-step from which a signal is generated. The signal in turns initiates intracellular processes appropriate to the signal. In every case, the first step involves a stereochemical configuration at the cell's surface combining a complementary molecule with sufficient energy to create the signal." (Smith & Good, 1969). There are many known exceptions to this statement, but it is quoted since it serves to express current thought in the field of cellular interaction.

When the cells of multicellular animals undergo differentiation, their degree of adhesion to like and unlike cells becomes altered. These adhesional changes affect the movement and positioning of cells relative to one another, factors which influence the further development of individual cells and their assemblages. Little is known of the nature of the changes responsible for differential cellular adhesion, but they are generally considered to include modifications of the cell surface. This thesis recounts an attempt to discover the nature of the code by which cells become preferentially associated with, or dissociated from other cells, through the analysis of a specific breakdown in this facility.

The materials which characterize the modifications of the cell surface are thought of as enzyme substrates by the biochemist, as structural molecules by the anatomist, as antigens by the immunologist and as electrical
charges by the biophysicist. An attempt is made to collate some of the evidence from diverse disciplines. The field of cellular interaction in the immune response is developing so rapidly that any review is soon outdated (see Metzger, 1970; Roitt, 1971), and a detailed description of its mechanism has not yet been formulated. This topic is therefore not included in the literature survey. Much of the earlier work on cancer is well reviewed elsewhere and is also omitted from the review. Literature relevant to the Discussion which appeared during the preparation of this thesis, is included in the "Discussion" section.

The existence of the blood group and histocompatibility alloantigens implies a genetic distinction between individuals and much of our knowledge of their nature was derived by the use of genetic mutants. A comparable description of cell surface determinants relevant to the differentiation of tissues in higher organisms has suffered from a lack of suitable genetic material. The Small Eye gene is associated with abnormal tissue adhesions, cellular invasiveness and neoplasia, and certain features of this mutant make its investigation uniquely accessible to a traditional biochemical approach. The practical work described below is directed towards obtaining an understanding of the nature and significance of cell surface determinants through the analysis of extracellular and secreted material in the Small Eye mouse. The mutant phenotype and the behaviour of cultured mutant cells are interpreted in terms of deductions based on these analyses. The term 'invasiveness' describes the tendency of tumour cells to infiltrate surrounding normal tissues. Although active invasion has not actually been recorded in Sey mice, the presence of immunologically identifiable cells deep within foreign tissues and the increasing prevalence of these abnormalities with age (Clayton, personal communication) suggest that cellular invasiveness is a characteristic of this mutant.
CHAPTER 1

REVIEW OF THE LITERATURE ON THE CELL PERIPHERY

Part II: Cell surface factors in development

Cellular adhesion is a prerequisite for the existence of the Metazoa, the success of this group may be taken as an indication of the selective advantages conferred by adhesion. The Slime Moulds (Acrasiales) may represent a first step in multicellular organisation and the observation that the surfaces of aggregation-competent cells display antigens undetectable in non-aggregating mutants suggests that specific surface components may be involved in aggregation or the stabilisation of aggregates (Gregg & Trygstad, 1955; Reidel & Gerisch, 1968). Dispersion and reaggregation of cells is unusual in higher animals, but occurs as a normal phase of development in annelid fishes (Wourms, 1965).

The attachment of sperm to egg is frequently highly species specific and appears to involve carbohydrate components of the glyocalyx (Lilie, 1913; Wanner, 1976a). Specific interactions between surface proteins and carbohydrates of opposite mating types of yeasts, bacteria and algae have been reported (Wanner, 1976a).

Changes in intercellular adhesion have also been involved to explain many developmental phenomena.
1 (a) **Cell adhesion in organisation of the embryo**

Tissue development requires adhesion between cells of like function and desmosomal linkages may permit the coordination of metabolic activity (Loewenstein, 1968), the movement of cells as sheets or aggregates, and the moulding of cell associations by physical forces (Gustafson, 1969; Trinkaus, 1969). By definition organs contain more than one kind of tissue. Therefore an overlap of adhesive affinities between unlike tissues within an organ is required for the maintenance of its integrity. Transplantation experiments confirm the general rule that tissues exhibit adhesive properties which "overlap" those of normally adjacent tissues (Chiakulas, 1952), a relationship reminiscent of the heirarchy of tissue adhesiveness described by Steinberg (1964, 1970).

Trinkaus (1963) detected increased adhesiveness of cell surfaces with the onset of gastrulation and Johnson (1969), showed that the mesoderm cells of hybrid amphibians which failed to gastrulate were defective in this respect. Townes & Holtfreter (1965) interpreted morphogenetic movements in development in terms of innate conflicting tendencies of cell associations towards either spreading or invagination, these tendencies being derived from tissue - and stage - specific cell surface properties. However, Curtis's work (1961) seems to disprove this concept as applied to the sorting of cell reaggregates. Working with cultures of brain cells of 'reeler' mutant mice, De Long & Eldman (1970) showed that the ability to aggregate is a separate process from that of histogenetic -self-organisation of cells within an aggregate.

The early processes of morphogenesis in the sea urchin are
summarised by Gustafson (1969). Blastula formation is ensured by the linking of cells (by microvilli) to the surrounding hyaline membrane. Changes in curvature and thickness of the body wall are explained by increases or decreases in the relative mutual adhesiveness of cells, or of cells to the hyaline membrane, the strength of mutual attachment of cells progressively increasing around the animal pole and decreasing at the vegetal pole, where it results in the release of free primary mesenchyme cells into the blastocoel.

The specificity of attachment of filopodial extensions of the secondary mesenchyme cells to the inner contact points of ectoderm cells is the subject of much discussion and may be influenced by the distribution of extracellular material or the low radius of curvature of the projections (see page 4). The activity of the mesoderm cells relative to their contact relations suggests that they may have some affinity for ectoderm which they do not have for their own kind. The distribution of pigment cells also suggests specific affinities which Gustafson (1969) believes may indicate the influence of glycoprolyx material.

The "talpid" mutation in the hen produce characteristic malformations of the limbs and disruption of the feather pattern. In the talpid\textsuperscript{3} mutant these abnormalities have been traced to a basic defect in the adhesive properties of mesenchymal cells (Ede & Agerbak, 1966; Ede, Hincliffe & Mees, 1971). This is associated with decreased motility of mesenchymal cells, a tendency to remain fibrocytic in form when normal cells become fibroblastic, a failure to form organised condensations.

1 (b) Specificity in cell migration

As detailed by P. Weiss (1947) there are only three ways in which
free cells can get from one point to another with specificity in any system: by
guidance involving ordered movement (see p. 9), by selective fixation of
randomly moving cells, or by selective elimination of randomly distributed
cells.

1 (b) (i) Selective localization of extra-vascular cells

Schemes of regionalized differentiation have been proposed in
which locations are specified by three-dimensional arrays of multispeccled
macromolecules in the ground substance (Jackson, 1968). Because of its
self-ordering properties and ubiquitous distribution; collagen has been
considered to play a major part in these schemes (P. Weiss, 1965). Collagen
also has a directional role in coupled morphogenetic effects such as the
positioning of dermal scale primordia in fish (P. Weiss, 1959).

Chick cardiac primordia move as aggregates over the endoderm
to accumulate in the appropriate site (Curtis, 1967), and avian primordial
germ cells also migrate by a partly extra-vascular route to the gonads,
although they also visit other sites (Meyer, 1964).

Cultured melanoblasts at first repel one another under the
influence of diffusible secretions (Twitty & Niu, 1954), but aggregate into
clumps when melanin synthesis is complete (Lehman, 1951). These in vitro
observations parallel the natural dispersion of propigment cells from the
neural crest and their subsequent aggregation into pigment patterns. In the
Axolotl, xanthoblasts migrate from the neural crest after the melanoblasts
and reaggregate before them between the future melanophore bands (Twitty,
1945). Lehman (1957) found that isolated xanthoblasts or melanoblasts lose
their adhesion to the substrate when surrounded by cells of the other type.

Alleles at the \( W \) & \( S1 \) loci in mice are associated with mutually parallel defects in the differentiation of the migratory and proliferative haematopoietic, pigment and germ cells. Transplantation experiments indicate that whereas the \( W \) alleles act on the defective cells per se, the \( S1 \) series appears to act on neighbouring cells (Mayer & Green, 1964). The differentiation of this group of cells therefore seems to involve some form of interaction with other cells.

The neural crest derivatives therefore provide an example of destination specificity in migration and also illustrate the importance of cellular interaction in migratory behaviour and differentiation.

1 (b) (ii) Selective localization of blood-and lymph-borne cells

The major route for the dissemination of migrating cells is the vascular system, but before they can accumulate in any tissue, blood-or lymph-borne cells must pass through the vascular wall. Any mechanism which specifies their destination must therefore act in part at this stage unless the cells migrate a considerable distance after leaving the vessels. It has long been known that the sites of secondary tumours are often in organs where the flow of blood or lymph is slowest. Curtis (1937) therefore suggests that mechanical trapping is a major factor in the accumulation of cells which migrate through the vascular system.

Chick propigment cells injected into the yolk sack circulation of white hosts became specifically sited in the feather primordia (Weiss & Andrea, 1952). Labelled lymphocytes normally accumulate in the spleen and
lymph nodes when injected into the tail veins of rats, but if L-fucose, N-acetyl-D-galactosamine or asialo acid are removed from their surfaces, they accumulate temporarily in the liver instead (Gesner & Ginsburg, 1964; Gesner & Woodruff, 1963; Woodruff & Gesner, 1969). These observations suggest that there is a special relationship between the migrating cells and their sites of accumulation which involves specific carbohydrates on their surfaces.

1 (c) Specificity in neural connections

The precision with which connections are established between nerve cells has been the subject of much speculation (see reviews by Gaze, 1970, and Gaze & Keating, 1972). R.W. Sperry (1951) observed the regeneration of optic nerves and postulated that the growing axons of the retinal cells carry biochemical "labels" possibly on their surfaces, which enable them to contact selectively and exclusively with their opposite numbers in the optic tectum. Spedel (1964) found that nerve regeneration is often faulty, but requires the active participation of the Schwann cells of the distal stump. It was recently concluded that "labels" are applied to nerves, in the early tail bud stage of tadpoles, with reference to two axes of polarity relative to some entity outside the eye cup (see Gaze & Keating, 1972), but the paired retinal and tectal locations so specified progressively alter as the tadpole grows (Gaze, Chung and Keating, 1972). The mechanism of this change has not been deduced. Gaze and Keating (1972) have recently found
that the nerve pattern in Siamese cats confounds their former deductions based on Amphibia and fish. This field is evidently confused at present, but there does seem to be an indication that specific recognition by cells may play some part in nerve connections.

1 (d) Contact effects

Whereas many types of cell reaction probably result from exchange of material, quite a range of effects has been ascribed to mere contact of the cell with another cell or with some special kind of substratum. Cell shape, orientation, movement, some aspects of physiology, and the fine structure of the cell periphery depend on structural inequalities in the substratum (P. Weiss, 1956, 1961). The differentiated state of fibroblasts may require contact with collagen (Attardi, 1954; Elsdale & Bard, 1972) and it has been shown, by comparing tissues in vitro in abnormal combinations, that collagen can determine the precise differentiated state which may be adopted by epithelia (Konigsberg & Hauschka, 1965; McLoughlin, 1961).

P. Weiss (1947) suggested that the substratum may stabilise the location in the cell membrane of molecules which are in some way complementary to the substratum, and Koshland & Kirtley (1968) postulated allosteric changes in membrane proteins following contact with ligands on neighbouring cells.

1 (d) (i) Contact guidance

Guidance of cells along physical structures was explained by P. Weiss (1961) on the theory that fluids exuded by the cell travel preferentially in certain directions due to surface tension effects to form a "ground
mat" of microexudate over which the cell crawls. Trinksus (1966) believed that such a mat could specify orientation but not direction. Directional information could be incorporated if one or more specific components were laid down in varying concentrations by a moving cell. Carter (1965) found that mouse fibroblasts will move up a gradient of non-specific adhesiveness ("haptotaxis"). He (Carter, 1967) described cell movement in terms of competition between cell and medium for contact with pristine surfaces, as opposed to surfaces coated with cell exudate (see "Discussion" section).

1 (d) (ii) Contact inhibition of movement

A normal cell will not use another similar cell as substrate for its locomotion. Contact inhibition of movement was described by Abercrombie (1970) as the cessation of locomotion in the direction which has led to contact. It involves paralysis of the ruffled membrane and the establishment of inter-cellular adhesions, and is succeeded by strong contraction within the cell followed by movement in the opposite direction (Ambrose, 1963). Contact inhibition of movement is often not shown by embryonic cells (Abercrombie, 1971) and appears to be tissue-rather than species-specific (Rubin, 1965). It depends on the relative adhesive stability of cell to cell, and cell to substratum (Weston & Roth, 1969).

Wolpert & Gingell (1968) have suggested a mechanism by which cytoplasmic activity could be controlled by the proximity of other cells through changes in surface potential affecting the configuration of membrane components and the permeability of the plasma membrane to ions. Polyanions and polysaccharides outside the cell produce opposite effects on membrane impedance.
which cause local relaxation or contraction of the membrane respectively
(Wolpert & Gingell, 1988; Gingell & Palmer, 1988).

1 (d) (iii) Contact inhibition of growth

Cell division is inhibited at "S" phase (Abercrombie, 1970) or
"G1" (Trinkaus, 1989) by contact with other cells, although it is usually delayed
for a period equivalent to about one cell generation following simple visible
cell contact (MARTZ & Steinberg 1970). Contact inhibition of growth does not
necessarily coincide with inhibition of cell movement (Stoker, 1967), but is
also effected by macromolecular constituents of the substrate or glycoallx
(Balsara & Jacobson, 1986). Lippman (1968) proposed that glycosaminoglycans
would control cell division by acting at the surface as cation traps. Contact
inhibition of growth is reflected in a drop in the activity of membrane-bound
adenosine triphosphatase and 5'-nucleotidase (LeMevre, Prigent & Paraf, 1971).

Con & Geimer (1965, 1968) found that free L-fucose mimics the
effects of confluence with respect to the morphology and growth behaviour of
mouse fibroblasts. D-mannose and in some cases L-fucose, had a similar
reversible effect on human and monkey cells and also rapidly depressed their
uptake of uridine. It is not known whether these monosaccharides act
intracellularly or at the cell surface.

Many of the phenomena described above appear to involve res-
pponses to the discrimination of structural or chemical features displayed on
substrate or the surfaces of other cells. In the Sey mouse there appears to be
some sort of breakdown in the discrimination of like and unlike cells and
possibly in the relationship of lens epithelial cells to lens capsule.
Investigations into cellular responses have largely centred around a cure for the abnormal proliferative, invasive and migratory tendencies of cancer cells (see p. 44). On the other hand the structure and chemistry of cell membranes and surfaces have been studied from many different points of view. There need be no essential distinction between the blood group and histocompatibility antigens for example, but for historical and practical reasons the two fields are normally considered as separate disciplines each with its own nomenclature. Immunological genetic approaches are much used by workers in these fields, but are rarely used by physiologists and biophysicists in their investigations of membrane functions which may depend on other properties of the very same molecules. The many advantages resultant from this breadth of interest are unfortunately marred by an apparent lack of communication between workers in the different academic areas e.g. theories of cell adhesion do not take into account the biochemical properties which cell surfaces are known to exhibit. For this reason there are few opportunities to integrate the different kinds of information on the cell periphery, with any degree of success, but an attempt is made at such a synthesis in the following sections.

Part 2: The Nature of the Cell Periphery

In agreement with L. Weiss (1967), a distinction is favoured between the "plasma membrane" as the structure on which the main permeability properties of the cell depend, the cell "surface" which is
concerned with interfacial phenomena, and the "peripheral zone", or cell periphery in depth. The term "glycoalyx" is applied to the predominantly polysaccharide coat which Bennett (1963) recognised as a general component of cell surfaces. Certain properties have been referred to an ordered region of the peripheral zone termed the "cortex" (Curtis, 1963), e.g. Sonneborn (1963) suggested that the structure of the cortex on the egg cell plays a part in governing the differentiation of derivative tissues. Evidence for this kind of effect is limited to only a few examples notably the maternal effect on ocelling direction in *Limnaea* (Raven, 1963) and the organising effect of the amphibian grey crescent. The latter example is of great interest since Curtis (1965) showed that mechanical disorganisation of the grey crescent leads to arrest of development at the beginning of gastrulation in succeeding generations, which suggests a continuity of structural organisation of the cortex across the generation gap.

Various enzymic properties are associated with the cell periphery including the hormonally induced production of 3'-5'cyclic adenosine monophosphate (cyclic amp) (Ugelav, 1963; Bennett, 1969a; Cuatrecassas, Desbuguola & King, 1971). Other physiological functions associated with morphological changes of the cell periphery are reviewed by Bennett (1969b).

2 (a) The plasma membrane

2 (a) (i) **Composition**

All animal plasma membranes contain phospholipids and there is significant organ - (Rouser, Nelson, Fleischer & Simon, 1968) and species - specificity (Bretscher, 1972) in their proportions. Phosphatidylcholine and sphingomyelin are chiefly in the outer half of the human erythrocyte bilayer, and phosphatidylserine, phosphatidylethanolamine and protein in the inner half (Bretscher, 1972). The membrane is also asymmetric with respect to carbohydrate which is all on the outer surface (in the glycocalyx) (Bretscher 1972). Some proteins such as those which carry the MN blood group factors span the membrane (Bretscher, 1971) and various transmembrane "carriers" of specific ions and molecules are recognised (Koty and Janacek, 1970; Whittam & Wheeler, 1970).

2 (a) (ii) **Structural model s**

The first generally accepted model of membrane structure was proposed by Danielli & Davson (1935) and later adapted by Robertson (1959) in accordance with electron micrographic evidence. The model conceives a continuous bimolecular leaflet of lipid, the hydrophobic ends of the lipid molecules directed towards the centre of the leaflet. In conventional electron microscope pictures this structure appears as a pair of parallel black lines (see page 42). On both surfaces of the membrane are proteins and on the outside only, glycoprotein extended along the polar surfaces.

Rothfeld & Finkalstein (1968) consider that the real situation probably also includes elements of the opposite extreme described by Benson (1966). In Benson's model protein extends through the entire depth of the
membrane as a hydrophobic framework while lipid molecules are secondarily inserted in association with non-polar portions of the protein.

Ling (1966) found the plasma membrane was no real barrier to the diffusion of small molecules and ions, and considered it to be a system of microscopic channels containing polarized water molecules continuous with similar channels in the cytoplasm.

Various models have been proposed which incorporate globular structures, these elements usually being visualized as micelles of radially oriented phospholipids. As an explanation of the "all-or-none" nature of neuronal excitation, Lehninger (1968) proposed that a transition between bilayer and globular lipid structures may occur during an action potential, conformational transformations of a few subunits being transmitted physically to neighbouring subunits. Transformation of membrane lipids from a stable to an unstable form may also be associated with the increased cation permeability and reduction of electrical resistance which precede cell fusion (Posts & Allison, 1971). On the basis of artificial membranes, Lucy & Glavert (1964) proposed the "hexagonal phase" lipid model which appears to be similar to the globular models usually depicted in section. The hexagonal arrays of subunits seen on the outer surface of plasma membranes are especially characteristic of tight junctions (Korn, 1969; Revel & Karnovsky, 1967) and may represent a particular mode of packing of lipids (Posts & Allison, 1971). Hexagonal structures over entire cell surfaces were observed by Schultz & Asummaa (1970) and Sanders & Zalik (1972).

Willmer (1961) proposed membrane structures based on the patterns
adopted by cardboard replicas of lipids floated on water in different proportions. The implication is that such structures would tend to be self perpetuating by intussusceptive growth.

The most recent membrane model, which incorporates elements of many earlier ideas is the fluid mosaic model of Singer & Nicholson (1972). In this model protein is considered in two locations: at least 70% is "integral protein", an important structural element, while "peripheral protein" is bound to the membrane by rather weak, possibly electrostatic, interactions and is irregularly coiled (Wallach & Gordon, 1969). The integral proteins are coiled in \( \alpha \)-helices and are globular in form, some are glycosylated. They are embedded in a phospholipid bilayer of structure similar to that in the Davson-Danielli model and may react strongly with specific lipids to form lipoproteins. Like the phospholipids, the integral proteins are considered to be amphipathic, the highly polar ends in contact with the aqueous phase and the hydrophobic ends embedded in the membrane interior. Integral proteins may span the membrane if both ends of the globular conformation are hydrophilic. An important feature of the model is the fluidity of the phospholipid bilayer which allows reorganisation of integral protein.

The theory of allosteric proteins was introduced into membrane models by Koshland & Kirtley (1966), as an explanation of ligand-induced permeability changes and transmembrane activation of enzyme activity. In the fluid mosaic model, ligand-binding is considered to induce a conformational change in dispersed integral proteins which favours their clustering and induces similar conformational changes in non-ligated protein.
2 (b) The Glycocalyx

Partly due to its distinctive appearance in electron micrographs, the plasma membrane has proved a popular subject for speculations concerning its gross structure. The glycocalyx, being featureless in most electron microscope pictures, has not been so favoured. It has instead been the object of extremely painstaking chemical and immunological analysis by many geneticists and biochemists with the result that we know a great deal about its molecular composition, but very little about its gross architecture.

Information on the glycocalyx has been reviewed by Bennet (1969a). This layer lies external to the plasma membrane and dependent on tissue type. It may contain glycoprotein, hyaluronate and chondroitin sulphates, lipids, proteins, nucleotides, ribonucleic acid, and crystals of calcium phosphate or carbonate. Some of its components are recognized as blood group and histocompatibility antigens. In contrast to the typical plasma membrane, it is normally easily pervaded by water, ions and small molecules, but may act as a selective filter of substances approaching the plasma membrane. It generally has a low electrical resistance and strong ion exchange properties, behaving like an anionic polymer. Sialic acid in the glycocalyx is involved in the active transport of cations across the plasma membrane (Emmelot & Boz, 1965; Glick & Githens, 1965). The presence of the glycocalyx adjacent to the plasma membrane, which has a different charge distribution, may confer electrical rectifying properties (Bennett, 1969a). Glycocalyces were classified by Bennet (1969a) as "attached" and "unattached". The latter (e.g. intercellular matrices, mucus coats and some basement membranes) are often very thick, while attached glycocalyces are
usually tenous and intimately bound to the plasma membrane. The two
types have characteristic staining properties (Bennett, 1968;.Martinez-Palomo,
1970). Developmental changes in the staining properties of intercellular
material are recorded by McConzachie & Ford (1965).

Much of our knowledge of the chemistry of the cell surface has
been obtained by the combined use of specific glycosidases and antibodies, or
by chemical blocking reagents and whole cell electrophoresis. By these means,
specific sugars and their linkages have been identified (Witzler, 1970) and
carboxyl, amine-, phosphate, sulphate and other groups quantitated
(Mehrsabi, 1970). Sialic acid is especially significant in that it carries a
large fraction of the cell surface charge and so may be relevant to adhesion
(see p.41). It also has a modifying influence on antigenic expression
(Uhlenbruck, Wintzer & Worebörfer, 1967; Currie & Bagshawe, 1968) as is
revealed when cells are treated with neuraminidase. Enzymic removal of
sialic acid from complex carbohydrates affects their antigenicity in two
quite distinct ways. Subterminal structures exposed simply by the removal
of the end group were termed "Friedenreich antigens" by Uhlenbruck &
Wintzer (1970). "Pseudo-Friedenreich antigen" is the term applied to groups
which become accessible to antibody due to reduction of charge in their
vicinity or by removal of steric hindrance, and to structures which become
exposed when a molecule changes shape following the removal of a charged
group (Uhlenbruck & Wintzer, 1970). Conceivably novel antigens might
arise on highly-charged cell surfaces by the converse of these effects due
to high concentrations of sialic acid (see p. 41).
The carbohydrate of cell surfaces is continually synthesised by non-growing cells in vitro, to replace components metabolised or lost into the medium. On the other hand, in growing cultures, membrane glycoprotein synthesis occurs at the same rate with no detectable loss into the medium (Winsler, 1970; Heath, 1971).

Gesner & Ginsberg (1964) point out that the absence of D-glucose from cell surface materials could be explained if carbohydrates of the cell periphery function as recognition sites, since free glucose in body fluids would interfere with recognition of specific glucosidyl residues.

(b) Blood group antigens

The blood group antigens are hereditarily determined glycoprotein and glycosphingolipid components of erythrocyte glycosylayers. Since they are very easily identified by immunological methods and since blood is available in large quantities from living donors, an enormous amount of work has been done in this field. Well over one hundred human red cell antigens are known, about three quarters of which have been traced to fifteen genetically distinct systems (Race and Sanger, 1968; Prokop and Uhlenbruck, 1969; Sanger, 1970). Some blood group substances (e.g. human Le, cattle J, sheep R and pig A) are adsorbed by red cells from the plasma (Hovvitz & Slomlany, 1970).

The A, B, H and Le antigens in red cells are determined by oligosaccharide chains attached to sphingosine. Three genetic systems interact to produce the five specificities based on these groups: Rh, ABO and Le. The genes H, A, B & Le code for glycosyltransferases which catalyse the transfer of sugars from nucleoside diphosphate glycosyl
derivatives to precursor carbohydrate chains. The products of genes L, O and Le are not enzymically active (Watkins, 1970). The antigensities of the carbohydrate units are determined by N-acetyl-D-galactosamine (A).

D-galactose (B) and L-fucose (H & Le) substituents attached by specific linkages to a core of galactose and N-acetylgalactosamine (Spiro, 1969b). Le a activity is associated with L-fucose linked $\alpha$ - 1 $\rightarrow$ 3 to N-acetylgalactosamine and Le b with this linkage combined with L-fucose linked $\alpha$ - 1 $\rightarrow$ 2 to D-galactose (Winsler, 1970a). Krüpe (1958), Kabat (1970) and several others have demonstrated inhibition of haemagglutination by free sugars corresponding to those specific for the blood groups and Kobata, Grollman, Torain and Ginsburg (1970) have demonstrated the appropriate presence or absence of the specific glycosyltransferases.

The M & N blood group antigens are located on extended glycoproteins embedded by one end in the red cell membrane (Winsler, 1970a, b). The M, N-active glycoprotein of molecular weight 31,400 contains 64% carbohydrate linked to the peptide through serine, threonine and possibly asparagine. It contributes 80% of the sialic acid of the red cell surface and therefore a large proportion of the red cell surface charge. (Spiro, 1969b; Winsler, 1969, 1970b). Influenza and other viruses bind to this glycoprotein to cause haemagglutination, this property and M, N antigenicity depend on sialic acid. The amino group of lysine is also relevant to M, N activity (Spiro, 1969b; Winsler, 1970a) and there is some evidence that N substance may be a precursor of M (Hubrikar and Springer, 1970; Winsler, 1970a). The genetics of the system are described by Uhlenbruck (1969).
The antigenicity of M & N and of the genetically unrelated Rh antigen, are depressed in the presence of another allele Ee (\(e^e\)) (Sanger, 1970). The action of this allele may account for some of the anomalous values for the electrophoretic mobility of human red blood cells (Seaman, 1965). The I and RhO (D) antigens (Winzler, 1970) and the P system in humans also seem to involve carbohydrate, P1 specificity being associated with \(\alpha\) -linked galactose (Watkins, 1967).

Some blood group antigens are detectable only on red cells e.g. RH (Coté, 1970), but others are also found on non-vascular cells, their tissue distributions are described by Clayton (1964). Le\(^a\) & A, B or H show a reciprocal relationship in any organ examined (Szulman, 1970) and are mutually exclusive in cells of the gastric and duodenal mucosa (Glynn, Holborow and Johnson, 1957), but the relative quantities vary from organ to organ (Szulman, 1970). A, B, H antigens are present on the membranes of endothelial and many epithelial cells, but Le antigens are not (Szulman, 1970). On epithelia they become progressively restricted during foetal life, to stratified and transitional epithelia only (Szulman, 1966). The capillaries of the placenta have H antigens, but not A or B (Szulman, 1970). M, N, antigens are present on leucocytes, platelets and some tissues, D antigens are present on platelets (Coté, 1970). The Secretor gene (S\(e\)) controls the expression of the H gene at some sites of biosynthesis of secreted glycoproteins and the H substance in these secretions can become elaborated into A or B in appropriate individuals. The Le gene acts on the same precursor molecules in secretions as the H gene, but is expressed whenever it is present in the genotype and is not under the control of S\(e\). Le converts the precursor
substance into $L_e^a$ substance which, in the joint presence of $H$ and $S_e$ alleles can be converted into $H^eL_e^b$ substance which also retains some residual $L_e^a$ activity. The density of group antigens on red cells varies considerably e.g. there are 460,000 - 850,000 $A_1$ and 310,000 - 560,000 $B$ antigen sites per cell in the $A_2B$ adult (Economidou et al., 1967). Genetic dosage and cis/trans factors affect their distribution (Race and Sanger, 1969). Winstler (1969) estimated that the $M,N$-active glycoproteins in red cells would be spaced 125$^a$ apart if evenly distributed. Nicholson, Masurolides & Singer (1971) showed that $R_h$ antigens are randomly distributed on the membrane.

$A, B, H$ antigens are absent from persons with the Bombay gene, with no apparent deleterious effect (Szulman, 1965) which raises considerable doubts concerning their function. On the other hand high $A$ frequencies exist among patients with malignant and benign tumours of several organs, and with other diseases (Gorschowitz & Neel, 1970; J.A.F. Roberts, 1957, 1959; Race & Sanger, 1969). Human adenocarcinoma cells develop $L_e^a$ and $L_e^b$ active glycolipids while $A$ and $B$ antigens are lost (Hakomori and Jeanloz, 1970; Stoffel, 1971), (see p. 50). Low frequencies of $O$ (i.e. $H$ or $h$) are found among rheumatic fever patients and high $O$ frequencies among those with duodenal and gastric ulcers; non-secretors (see $O$) are also more frequent among duodenal ulcer patients (Gorschowitz & Neel, 1970). The geographical distribution of blood groups and the widespread existence of $A$ & $B$ blood group alloantibodies in human serum may be connected with the cross-reactivity between blood group antigens and disease organisms (Gorschowitz & Neel, 1970; Springer, 1970). The effects of mother-child and mother-sperm
Incompatibility with respect to the A, B, H & Rh antigens are reviewed by Prokop & Uhlenbruck (1969), Race & Sanger (1968) and Gerschowitz & Neel (1970).

2 (b) (ii) Histocompatibility antigens

The fate of tissues transplanted from one individual to another depends on the genetic relation of the donor to the host. In each species several alloantigenic factors sited predominantly on the cell surface, but also detectable by immunological techniques on internal membranes, are significant in causing an immune response which results in graft rejection. In mice about twenty loci corresponding to different histocompatibility antigen systems have been described (Davies, 1970). One system is usually of particular significance possibly because it is normally very highly polymorphic. In mice this locus is called H-2, in rats Ag-B, H-1 or R-1 (Palm, 1970), in man HL-A and in humans B. Each allele at a major histocompatibility locus may code for several detectable specificities. About forty H-2 specificities are known and of these, between five and fifteen may be expressed by one individual (Davies, 1970). The expression of one specificity is sometimes dependent on the presence of another (e.g. H-2 and Tl). Groups of specificities are thus known ("inclusion groups"). The different specificities within each inclusion group may represent separate parts of the same molecule (Boyse and Old, 1969). There is evidence that the H-2 and HL-A antigens are structurally similar (Nathenson, 1970). The non H-2 alloantigens in mice were reviewed by Popp (1967). Rejections by mammalian and avian hosts of both sexes to grafts from donors of the opposite sex have been attributed to genes on the
sex chromosomes (Bacon, 1970).

The macromolecules carrying the H-2 specificities are glycoproteins of molecular weight variously estimated as 33,000 to 200,000 (Stimpfling, 1971), possibly built from small subunits (Mathenson, 1970), which bind to lipids and other membrane proteins. The portion of the molecule which protrudes from the surface bears one or several H-2 specificities and carries the carbohydrate portion containing galactose, mannose, fucose, sialic acid and possibly variable amounts of glucosamine (Muramatsu & Nathenson, 1970; Stimpfling, 1971). The carbohydrate of HL-A glycoprotein contains glucose and acetylated galactosamine in addition to these sugars (Sanderson, Cresswell & Welsh, 1971). According to Nathenson (1970) and Stimpfling (1971) the allantigenic sites probably represent differences in the amino acid sequence of the H-2 peptide. Removal of sialic acid has no effect on H-2, H-3, H-4, H-11 or H-26 antigenicity (Sanford & Codington, 1971), but there is evidence that galactosyl-\(\beta-1\rightarrow 4\)-N-acetylgalactosamine is involved in the immunodeterminant portion of H-2 (Davies, 1965; Muramatsu & Nathenson, 1970) and of HL-A (Sanderson, Cresswell & Welsh, 1971). (Shimada and Nathenson (1969) found differences in the contents of neutral carbohydrates, glucosamine and sialic acid, arginine and glutamic acid, between H-2^b and H-2^d antigens. There has been no unequivocal evidence that histocompatibility antigenicity resides exclusively in either protein or carbohydrate moieties, but Shreffler & Klein (1970) and Sanderson et al (1971) achieved a synthesis of the data by suggesting that specificity may depend on the structure of carbohydrate as governed by the sequence of the peptide backbone.
Histocompatibility alloantigens are present in nearly all tissues, but in varying proportions (McDonald, 1968; Snell and Stimpfling, 1966; Nathenson, 1970). The quantitative tissue distribution of HLA in humans approximately parallels that of H-2 in mice (Berah, Hors & Dausset, 1970).

There is a close quantitative correlation between the different H-2 antigens with respect to age of appearance, development and distribution in various organs (Pizzaro, Hoecker, Rubinstein & Ramos, 1961). H-2 antigens appear at the third day (Pizzaro et al., 1961), but murine H-3 and H-6 are detectable at the two-cell stage (Palm, Heyner & Brinster, 1971). Six well characterised systems are represented on mouse thymocytes specified by the loci H-2, Tla, Q, LyA, LyB and MSLA. All except H-2 are regarded as differentiation antigens in that they have their major representation on these cells (Boyse & Old, 1969). Aoki, Hämmerling, de Harven, Boyse & Old (1969) produced electron micrographic evidence that the representation of ferritin-labelled alloantigens on the surfaces of cells from thymus, spleen, lymph nodes & peritoneal cavity was always discontinuous, the fraction of the cell surface covered being characteristic both of the antigen and the type of cell. H-2 is present in far higher amounts on lymphocytes than on thymocytes, whereas the converse is true of Q. Order & Waksman (1969) have demonstrated the change in antigenic character of bone marrow cells which repopulate the thymus after irradiation. Klein (1965) found that tumour lines failed to survive if they lost all H-2 specificity.

The thymus leukemia gene Tla is of particular importance. All mice are phenotypically either TL negative (TL-) or if TL positive they
express specificities TL2 or TL1, 2, 3. Except for TL1, 2, 3 mice, all mice carry repressed structural Tla genes which are invariably derepressed in leukemia. Antigen TL4 occurs exclusively on leukemia cells, but is never expressed in TL1, 2, 3 mice. If a TL+ mouse is immunized against TL+ material and then challenged with a TL+ leukemia, the injected TL+ cells become "modulated" to TL+ so long as antibody is present. The TL antigens modulate in concert and there is a concomitant increase in H-2 on the cell surface as if the TL unit interferes with the normal expression of H-2 (Boyse & Old, 1969).

The genetic fine structure of the major histocompatibility antigens is described by Shreffler (1970) and Stimpfling (1971). The two major loci of the human HL-A region, LA and K apparently correspond to the D & K ends respectively, of the H-2 region in mice (Bodmer, 1972). H-2 and other alloantigens are apparently packed on the cell surface in distinct patches, but at constant density, within each patch (Boyse & Old, 1969). Antibody blocking experiments suggest that TL and LyB are associated with H-2 (D) antigens and LyA with H-2 (K) antigens, but the two groups occupy relatively distinct locations on the surfaces of thymocytes (Boyse & Old, 1969).

This distribution mirrors the genetic map (Nathenson, 1970). Legrand & Bausset (1972) working with the HL-A system showed that such an effect was only shown when the genetic loci were in the cis position.

There is a connection between major histocompatibility loci and the immune system (Burnett, 1970, 1971; Bodmer, 1972). The immune response gene Ir-1 in the mouse controls the level of antibody response to
certain highly unusual, synthetic, branched polypeptides. It lies near the K end of the H-2 region and is not closely linked to the immunoglobulin determining genes. McDevitt, Bechtol, Grumet, Mitchell & Wegmann (1971) have suggested that the Ir-4 gene acts by regulating the synthesis of a new class of receptors located on cell surfaces, which are concerned with cellular recognition in the immune response. Lymphocytes in culture are stimulated to enlarge and divide in the presence of other lymphocytes which differ at a locus closely linked to HL-A. Bodmer (1972) postulates that there may be a large number of genes in this region which control the synthesis of a particular class of differentiation antigens and/or a class of complementary recogniser molecules which he considers may represent the origins of the immunoglobulins.

Statistical analyses reveal that persons with particular histocompatibility antigens are particularly susceptible to certain diseases. These include Hodgkin's disease, systemic lupus erythematosus, leukemia and asthma (see Bodmer, 1972). It has been suggested that multiple polymorphism of the major histocompatibility antigens may be a defence against viral infection or contagious transfer of cancers (see Bodmer, 1972).

The purpose of this survey is to obtain some understanding of the molecular "language" by which cells communicate. Hence the distribution of blood group and histocompatibility antigens between the tissues and upon the surface of individual cells, as indicated by fluorescent and ferritin-labelled antibody and other techniques, is relevant to our enquiry as an indication of the differences in pattern and density of groups displayed on differentiated cell surfaces. If the cell surface factors which mediate in cell adhesion contain complex carbohydrates, it is reasonable to suppose that they are genetically
coded and synthesised in a similar way to the blood group antigens.

The most obvious means of attachment of cells to one another are the desmosomes, but there is evidence that these develop secondarily from less obvious adhesions. Nevertheless large scale structural features of the cell surface must play a major part in intercell relationships. These features are described in the next section.

2 (c) Morphological features of the cell surface

The cell surface displays structures specialised for physiological functions, such as microvilli, ollis, caveolae and folds (see review by Bennett, 1969a, b). Over the majority of cell–cell interfaces the two surfaces appear to abut one another or may be separated by a distance of 100–200 Å, but at intervals their linkage is reinforced by interdigitations or desmosomes.

It is convenient to distinguish between macular desmosomes ("maculae adherentes") which adhere to similar regions on neighbouring cells or unspecialised areas on acellular structures, and zonular desmosomes ("terminal bars") or "zonular adherentes") which encircle a cell like a belt, and bind it to its neighbours which are similarly encircled (Bennett, 1969).

On a functional basis desmosomes may be classified as "adherent", "occluding", or "synaptic" (Bennett, 1969). "Adherent desmosomes" are believed to be specialised for resisting tensile forces and contain dense intercellular material. According to Bennett (1969) the dispersion of tissues by treatment with trypsin or ethylenediamine tetra-acetic acid (EDTA)
depends on the breakdown of this dense intercellular matrix. Adherent desmosomes are linked internally by keratinous tonofilaments. Zonular occluding desmosomes are believed to form a barrier to diffusion and fluid exchange between extracellular fluid compartments; they include "tight junctions" of closely apposed plasma membranes which appear as a trilaminar lipid leaflet in section, and "septate desmosomes" composed of membranous septa passing around each cell like a series of shelves. Septate desmosomes may be confined to invertebrates, which do not seem to have tight junctions (Trinkaus, 1969). Tight junctions are believed to be mechanically weak, (Bennett, 1969) and are usually reinforced nearby by adherent desmosomes, but in Ca\(^{++}\) - free solutions, tight junctions are the most persistent of the two (Mercer, 1965). Groupings of one or more desmosomes are called "junctional complexes". Septate desmosomes provide a path of ionic and molecular communication between cells and are thought to be of the tight-junction and septate type (Bennett, 1969; Trinkaus, 1969).

It has been suggested that the disposition of desmosomes on cells may confer specificity on adhesive relationships (Waddington, 1962) and Mercer (1965) claims that hexagonal patterns of macular desmosomes are revealed in sections of basal layer cells cut parallel to the basement membrane in compound epithelia. However, according to Treistad, Hay & Revel (1967) adherent desmosomes appear to develop from the primitive tight junctions, so the desmosome pattern appears to be secondary to other factors.

Slender projections of cell surfaces connecting cells separated by large amounts of extracellular matrix as in bone, have been grouped as
"plasmodesmata". These junctions have a low electrical resistance, but little is known of their structure (Bennett, 1969a).

Another kind of intercellular junction resembles a knotting together of plasma membranes such as link blastomeres of Limnacea (Waddington, 1962). According to Bennett (1969), such structures, in other situations probably have a role in transport functions.

The main lesson to be learnt from this survey of cell surface morphology, is that cell adhesions do not seem to be initiated at desmosomes so we must look elsewhere for specificity in adhesion. Desmosomes must be a major barrier to tissue disaggregation which therefore probably causes considerable damage to the cell periphery. This fact must be borne in mind when interpreting experiments carried out on freshly disaggregated tissues.

With a detailed knowledge of the structure and chemistry of the cell periphery we are now in a position to assess the value of the various theories of cell adhesion and to consider their success in explaining some of the biological phenomena described above.

Part 3: Theories of Cell Adhesion

Many of the theories of cell adhesion were reviewed in detail by Curtis (1967) and Weiss (1967α).
Adhesion of cells to inert surfaces

Live cells and dead, formol-fixed cells will both rapidly adhere to clear glass except in the presence of protein. These adhesions do not require calcium and are not affected by trypsin. In the absence of extraneous protein, live cells will produce an exudate containing protein and carbohydrate and their adhesion to glass then becomes susceptible to trypsin and EDTA which chelates calcium. If extraneous protein is already present, live cells adhere to glass more slowly, but this adhesion is also disrupted by trypsin or EDTA (Taylor, quoted by Trinkaus, 1967). Carter (1967) analysed cell movement over clear glass in terms of competition between medium and cell for contact with fresh and 'used' surfaces (see p. 10). Berwick and Concinni (1962) found that adhesion of buccal mucosa to siliconized glass was reduced by phosphatases and neuraminidase, but not by EDTA.

Fibroblasts will not adhere to cellulose acetate, but will do so if it is coated with palladium (Carter, 1965); adhesion to polystyrene is inhibited by reagents which block sulphhydril groups (Grinnell & Sere, 1971). Cyclic AMP increases the adhesion of living cells to inert substrata (Johnson & Fastan, 1972). If a uniformly textured substratum is supplied, the spacing of the irregularities must fall within a certain range or live non-transformed cells will not adhere to it (Ambrose, 1967).

It can be concluded that both physical and chemical factors affect the adhesion of cells to inert surfaces, but this evidence need have no relevance whatsoever to the adhesion of cells to one another or to natural surfaces. Nevertheless extrapolations of this kind are still made (e.g. Grinnell & Sere, 1971).
3 (b) Tissue Dispersion

A considerable contribution to our knowledge of cell-bonding agents has been derived by the disaggregation of intact tissues.

In 1890 Ringer observed that addition of calcium bicarbonate to the medium in which tissues of tadpoles were maintained prevented disintegration of the intercellular cement. Herbst (1900) found that sea urchin blastomeres, dispersed by placing in calcium-free sea water, reassociated on replacement of Ca\(^{++}\) ions. Ca\(^{++}\) ions are not equally important to all tissues. In Xenopus embryos, the calcium-dependence of adhesion varies between tissues and with stage of development, being probably most important in the late gastrula and early neurula (Curtis, 1957). Jones and Elsdale (1963) succeeded in dispersing amphibian blastulas by placing them in calcium-free medium, total chelation of calcium by EDTA was required to disperse the neurula, while EDTA together with proteolytic enzymes were necessary for post-neurula stages. This resistance to dispersion parallels a reduction in cell surface charge (Zalk, Sanders & Tilley, 1972). The reaggregative ability of chick lung cells has been found to decrease progressively after twelve days' development, a feature which Grover (1961) considered to reflect the greater difficulty with which older tissues are disaggregated.

Benedetti & Emmelot (1968) discovered that stelic acid groups were localized at junctional complexes of liver cells, their carboxyl groups being revealed only after treatment with EDTA. This finding, which implies that adhesion involves the binding of Ca\(^{++}\) to stelic acid groups, contrasts with that of Berwick & Coman (1962) who showed that acid and alkaline phosphatase and EDTA reduce the cohesiveness of buccal mucosa epithelium.
but neuraminidase does not. The latter authors concluded that cell-cell links in buccal mucosa were by Ca$^{++}$ bridges between phosphate rather than ionic acid groups. There is an isolated report (Rappaport & Howze, 1966) that adult mouse livers and brains are dispersible by means of potassium-complexing agents. Cell adhesions in some tissues at certain stages therefore seem to involve the mediation of cations loosely associated with charged anionic groups on the cell surfaces.

Wilson (1907) dispersed sponge cells mechanically by forcing them through fine cloth and similar techniques were applied to vertebrate embryonic rudiments by Holtfreter (1939) and Moscona (1957) who introduced trypsin as a dissociating agent. Collagenase and hyaluronidase separately and in combination have also been successfully used as tissue dissociating agents (Kemp, 1969).

On this evidence proteinaceous materials must also be involved in cell adhesion.

3 (c) The resaggregation of dispersed cells.

It has been suggested that calcium may assist cell binding by reducing the effective thickness of the electrical double layer surrounding cells (L. Weiss, 1960). Lipson, Dodelson & Hays (1965) found that many divalent cations, including Sr$^{++}$, reduced the net negative charge and hence the double layer thickness around dispersed toad bladder cells, but in Armstrong's (1966) experiments, Mg$^{++}$ and Ca$^{++}$ promoted aggregation of dispersed chick limb bud cells, but Sr$^{++}$ and Ba$^{++}$ did not (cf. L. Weiss - see below). Lipson et al (1965) used the electrical resistance measured between luminal and serosal faces of toad bladder epithelium as an indication of the re-establishment of occluding
deamotosones. The formation of such junctions required ions of calcium, strontium, manganese or aluminium but magnesium and five other polyvalent ions were not effective in this respect. Re-establishment of electrical continuity between reaggregating sponge cells required Ca\(^{++}\) ions and occurs at regions which are apparently not predetermined (Locwenstein, 1968).

Calcium may be involved in the activation of membrane-bound adenosine triphosphatase (probably actomyosin) in connection with locomotion and surface contraction (Gingell & Palmer, 1968). If this were a major factor, cell aggregation should be calcium-sensitive, but L. Weiss (1967a) was unable to demonstrate calcium-sensitivity during reaggregation of cells dissociated by calcium-depletion. He considered that experiments purporting to show that Ca\(^{++}\) ions promote cell adhesion are better interpreted in terms of the hindering of separation of cells by stiffening the cell periphery, but later no correlation between adhesion and surface deformity was found (L. Weiss, 1968). Ca\(^{++}\) ions will bind to steric and ribonucleic acids and other unidentified groups in the cell periphery causing a reduction of surface potential and lowered electrostatic repulsion (L. Weiss, 1967a). In the absence of Ca\(^{++}\), cell surfaces show a "bubbling" behaviour (Trinkaus, 1969) which is coordinated and can be modified by adenosine triphosphate (P. Weiss, 1961). Poste and Allison (1971) suggested Ca\(^{++}\) may maintain the conformation of negatively charged membrane lipids.

3 (d) Aggregation-enhancing factors

Humphreys (1963) found that sponge cells dissociated in calcium- and magnesium-free sea water do not reform aggregates at low temperatures.
(5°C) unlike mechanically dissociated cells. A factor was found to be released into the medium which has species-specific aggregation-enhancing properties (Moscona, 1963). This factor is intercellular in intact tissues, it cannot be synthesized at low temperatures and will only maintain its activity in the presence of divalent cations (Humphreys, 1963). It cross-reacts immunologically with material which appears to be firmly attached to the cell surface (MacLennan, 1969). The factor appears to be composed of glycoprotein containing a large amount of carbohydrate which differs in composition between species, the destruction of its activity by periodate suggests the occurrence of carbohydrate in the binding site (Margolis, Schenk, Hargie, Burokas, Richter, Barlow & Moscona, 1965).

Lillen & Moscona (1967) and Lillen (1968) reported the liberation from embryonic chick retinal cells of a macromolecular substance which is specifically taken up by this cell type, which specifically enhances its reaggregation, and which cross-reacts with materials normally displayed on retinal cell surfaces. Metabolic activity including protein synthesis is required for its utilization. Daday & Creaser (1970) and Creaser & Russell (1971) showed that this material contained protein, but tests for carbohydrate were not carried out. Treatment with trypsin destroyed its activity.

The "conditioning factor" which promotes growth of low density cell cultures, has been identified with aggregation-enhancing material (Rubin, 1966, 1967; Winzler, 1970). Conditioning factor is tissue-, but not species-specific (Rubin, 1965). It is macromolecular with a configuration maintained by weak bonds (Rubin, 1966). There is evidence that conditioning factors and
Intercellular binding materials include collagen (Konigsberg and Hauschka, 1965; Kemp, 1969). Basic proteins such as polylysine can also agglutinate cells (Katchalsky, et al., 1959), but little is known of their properties.

It can be concluded that intercellular collagen and glycoprotein contribute to cell adhesion and do so with some specificity which may depend on the carbohydrate components. Divalent cations such as calcium are probably significant in maintaining the integrity of these materials. It should be noted however that the sponge aggregation enhancing factors described by Margoliash et al. (1965) did not contain hydroxyproline or significant amounts of sialic or uronic acids.

3 (c) Specificity in adhesion

Wilson (1967) and many later workers demonstrated the separation of sponge cells on a species basis during reaggregation in bispecific mixed disaggregates, but Moecon (1957) found that mixtures of two tissue types from mice and chickens would separate on a tissue, rather than a species basis. These conflicting observations may be explained by the differing significance of modification of cellular adhesive properties in the evolution of similar organs in different species (Burdick & Steinberg, 1969). This may be related to the evolutionary age of the species concerned. John, Campo, MacKenzie & Kemp (1971) found that species-specific aggregation in a mixture of two species of dissociated sponges required the interaction of two distinct cell types, one of which was essential for aggregation, the other for specificity. Theories of cell adhesion based on the patterns of cells in reaggregates have frequently
ignored the complexity of the sorting process. Sorting will take place in suspension, but not on a solid substrate (Stoker, 1967) and the primary adhesions between reaggregating cells are between smooth surfaces in sea urchins, by microvilli in chick embryos and possibly by filopodia in sponges (Giovine and Mutolo, 1970).

As pointed out by Abercrombie (1967) any factor which reduces the probability of separation once cells have collided, would theoretically serve instead of adhesion. Jones & Morrison (1969) found that indiscriminate adhesions between unlike cells allowed them to move relative to one another, whereas adhesions between like cells did not, so that aggregates of like cells increased in size. This consolidation of aggregates may involve contact inhibition of movement as well as the formation of intercellular linkages.

The demonstration of distinct patterns in cell reaggregates has been taken as evidence for a code on cell surfaces by which cells can recognize compatible neighbours. In principle three general types of code can be postulated employing differences of order, quantity, or kind (Steinberg, 1964).

3 (e) (i) Codes based on patterns of surface features

Steinberg (1958) suggested that slightly soluble salts of calcium or magnesium may bridge anionic sites on adjacent cells, the pattern of sites being dissoci specific. For the sake of clarity these patterns were illustrated as grids of various dimensions. Cells were considered to form the strongest links with others bearing patterns which are in register. Objections to the theory are based on the non-rigidity of the plasma membrane (Curtis, 1967; Singer & Nicholson, 1972) and overlap of affinities arising from relative
orientations of patterns (Curtis, 1967). Furthermore, cells with sparse patterns would always bind to those with dense patterns, L. Weiss (1967a) allows that such arrays of charged groups could conceivably orientate the cells to permit bonding by other means. Wallach & Gordon (1969) also suggested that charge mosaics may confer stereospecificity, having shown that plasma membrane surfaces have a net electrostatic attraction when near their isoelectric points.

Rappaport & Howze (1966) proposed a theory of cell adhesion based on the structure of minerals. The basis of their argument is that surfaces are held together by the formation of coordination complexes of surface-bound anions, about K⁺ ions. Surfaces adhering only if their anion sites can occupy the available coordination positions about the intervening cation. There has been a notable lack of criticism of this theory, but since K⁺ ions can pass freely through cell membranes (Mag, 1966), the argument does not appear very sound.

The possibility that desmosomes may develop in specific patterns and thereby confer specificity in adhesion was suggested by Waddington (1962) but if such a mechanism exists it would seem to apply to some pre-existing structures rather than to the desmosomes themselves (see p. 29).

3 (c) (ii) Codes based on quantitative factors

Pethico (1961) and Curtis (1967) have listed the plausible physio-chemical forces between cells, and mechanisms have been proposed by which these could be adjusted to confer specific and non-specific adhesive properties. Adenosine triphosphate (ATP) prevented aggregation of chick embryo fibroblasts...
(Knight, Jones & Jones, 1960), but favoured aggregation of amphibian mesoderm (Duchener, 1961). On this evidence P.C.T. Jones (1966) and B.M. Jones (1968) proposed models based on the action of actomyosin which, by contraction, causing ruffling of the cell periphery, was considered either to neutralize positive and negative charges on a cell surface, or to increase the density of negative charges and at the same time obscure the linkage sites. Jones & Morrison (1969) postulated specificity arising from the rate of oscillation of non-specific linkage sites between two positions one favourable and the other unfavourable to adhesion. At this time there was evidence of actomyosin in the membranes of the cells concerned, but Jones, Kemp & Gröschel-Stewart (1970) identified smooth muscle actomyosin at the cell surfaces in chick liver and striped muscle.

Steinberg (1964, 1970) showed that an hierarchy of tissues can be constructed, the most cohesive cells segregating internally to less cohesive cells in bispecific mixtures of disaggregated tissues. As an explanation he suggested that cells display randomly distributed binding sites of density appropriate to tissue-type. (These binding sites may also differ qualitatively between tissues). Cohesion is defined as the work (or energy) of adhesion of one cell surface to another of its own kind, averaged over the area of apposition (Steinberg, 1970). Curtis (1961) found that sorting within aggregates commences at the outside, which suggests that the outer cells are the more cohesive. However, Trinkaus (1969) and Steinberg (1970) believe that such cells may be highly cohesive, but have large areas devoid of adhesive sites.

The dissociated cells of the amphibian gastrula will sort out in culture
so that ectoderm covers the surface of the aggregate, endoderm is at the
centre, and mesoderm separates the two. Roth (1968) deduced that the re-
placement of initially non-selective adhesion between trypsinized cells, by
selective adhesion, indicates the existence of both non-specific and specific
adhesive components, the latter being labile to trypsin. By dispersing
Xenopus midgastrulae, and allowing the endoderm to reaggregate for several
hours before adding freshly dispersed ectoderm and mesoderm from
individuals at the same stage as the original Curtis (1961) showed
that the reaggregative affinities between these tissues depend on the length of time of
recovery of each tissue following dissociation. He (Curtis, 1962) showed
that the viscosity (under shear) of the cell periphery reaches a minimum at
developmental ages characteristic of each tissue type and claimed that
differential cell movement would be promoted by these effects. L. Weiss (1964)
calculated that such factors would be insignificant to actively moving cells
but his calculations were based on experiments with amoebae which use a
locomotory system quite different from that of Metazoan cells.

3 (e) (iii) Codes based on shape of cell-surface molecules

The first theory of specific intercellular bonding was based on
the complementarity of antibody and antigen and was proposed by Tylor (1947)
& P. Weiss (1941, 1947) who envisaged attachments between unlike cells as
"keys" on the surface of one cell and complementary "locks" on the other.
Attachment between like cells can occur if each surface is provided with
both 'locks' and 'keys', or if an intercellular material carries equivalent
determinants. Differences in strength of binding were believed to be due to
differences in the relative abundance of binding groups, or in the degree of steric conformance between complementary pairs of molecules.

The concept of recognition based on enzyme-substrate interaction was put forward by Heinmets (1968) in a theoretical analysis of cellular interaction. In Heinmets' model system the enzyme is supposed to be located on an extension of the cell surface and to react with its substrate on a neighbouring cell. The products of the reaction are supposed to be transported back into the cell, the recognition step and the appropriate response being the result of metabolic "decoding" of the reaction products which act as inducers or repressors within the nucleus.

Recently a new theory of cell adhesion based upon enzyme specificity was put forward by Roseman (1970). This elaborated in the "Discussion" section (p. 570).

3 (f) The lyophobic colloid theory

A considerable stimulus to experimentation in this field was provided by the introduction of physicochemical reasoning by Curtis (1960). Curtis's argument was based on the theory of stability of lyophobic colloids, known as the "DLVO theory" (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948). In this theory a force of attraction, the "London dispersion force", counterbalances electrostatic repulsive forces to confer stability between cells at two positions: at less than 20 Å and at 100-200 Å apart. Curtis found confirmation for the theory in the observation that electron micrographs of contacting cell membranes after permanganate staining, usually appear as
two pairs of dark lines (i.e., two "unit membranes") separated by a gap of about 150 Å, or else tightly fused together as "tight junctions". This superficially highly attractive theory has been the object of much criticism and is not now acceptable as more than a partial explanation of adhesive phenomena.

According to Pethica (1961) the value of a constant used by Curtis (1960) to calculate the attractive force between cells is at least ten times too high (c.f. Brooks, Millar, Seaman & Vassar, 1967), while polyvalent ions in physiological solutions would markedly reduce the electrical potential barrier to adhesion. Bangham & Pethica (1960), arguing within the tenets of the theory, showed that reduction in the radius of curvature of cell processes to less than a tenth of a micron would allow their approach to within 5 or 10 Å and L. Weiss (1964) concluded that this factor combined with active locomotion, would overcome the major physical forces of repulsion supposed to maintain the 100-200 Å separation.

Kemp (1960) dissociated chick muscle cells with different agents so that they carried different surface charges and found that their reaggregative abilities were comparable. For reaggregation to take place, these cells were shown to have an actual requirement for surface-bound sialic acid, the major contributor to the electrostatic repulsive force (Kemp 1969). L. Weiss (1966) also found no correlation between cell surface charge and adhesion of cell to glass.

The apparent 150 Å gap is reduced to 15 Å or less simply by increasing the tonicity of the medium with sucrose (Robertson, 1959). This
observation implies the existence of an intercellular gel which shrinks in hypertonic media, but Brooks et al. (1967) claim that it could arise from a dielectric effect of sucrose. According to Trinkaus (1969) the electron light area may be (i) a real gap filled with fluid or (ii) a "cement" of large molecular weight compounds, or (iii) it may be an artefact in that the unit membrane represents the dense parts of a thicker structure. The electron-light area has in fact been stained with carbohydrate stains (see p. 45) and the real situation would appear to involve a combination of at least two of these explanations.

Drugs which affect transmembrane transport of Na\(^+\) and K\(^+\) ions also affect cell adhesion. Weiss (1972) suggested that this effect is due to a change in transmembrane potential difference which triggers off the unspecified processes leading to cell adhesion. In view of the importance of alkali cations per se in general metabolism (Whittam & Wheeler, 1970) this conclusion would appear premature.

Despite the objections which have been raised against the DLVO theory in its original form, it has served as a focus about which other theories have developed. Brooks et al. (1967) after recalculating the value of a constant, concluded that while inapplicable to cells with special areas of attachment, it does describe the interactions of some reversibly adherent or freely moving cells such as rod blood cells in plasma. However it gives no explanation of specificity in adhesion.
Part 4: Surface Properties of Cancer Cells

Histological examination of the eyes of Small Eye mice reveals adhesions between tissues which do not normally adhere, abnormal proliferation of lens epithelium which tends to invade other tissues, and a high degree of disorganisation of the eye in general. The peripheries of cancer cells typically show antigenic and structural abnormalities which are significant in relation to the local invasion, metastases, disorganisation of tissue form and persistent growth which are also typical of malignant cells (Abercrombie & Ambrose, 1962). Malignant behaviour implies that adhesiveness to normal tissue is greater than to like, i.e. malignant, type (Rubin, 1966). According to Bullough (1971) the basic difference between a tumour and its tissue of origin is not that its cells have changed their nature, but that there has been a shift in the balance between cell gain and loss. Other authors consider tumour cells to be dedifferentiated towards an embryonic condition or redifferentiated towards some novel tissue type. Harris (1971) has shown by cell fusion that malignancy is based on the loss of genetic information. The properties of neoplastic cell surfaces are reviewed in detail by Burger (1971).

4 (a) Contact features

Polyoma transformed cells do not exhibit mutual contact inhibition of movement or growth, but are inhibited in both respects by contact with normal cells (Stoker, 1967; Trinkaus, 1969). Conversely, sarcoma cells may inhibit movement of fibroblasts while they themselves are not inhibited
on contact (Abercrombie, 1971) i.e., the display of, and response to a surface signal relevant to contact inhibition are independent features, either of which may be disrupted in malignant cells.

The morphology and inhibition of growth which characterise confluent fibroblasts is mimicked by isolated fibroblasts in the presence of certain free sugars (see p. // ). The effect of L-fucose on one mouse line was greatly reduced by viral transformation (Cox and Gesner, 1967).

Cancers cells show a more generalised adhesion to surfaces of varying texture than do normal cells. This was shown to be related to the non-uniformity of ruffling of membranes of cancer cells (Ambrose, 1967). This feature may be related to the disruption in spatial distribution of specific chemical groups on the surfaces of transformed cells detected by Nicolson, (1971) by means of ferritin-labelled lectins.

4 (b) Cancer and the mitotic cycle

Burger (1971) advanced an hypothesis which relates the characteristics of cancer cell surfaces to the changes which occur during the passage of a normal cell through the mitotic cycle.

By the use of lectins such as wheat germ agglutinin (WGA), which is divalent and binds specifically to N-acetylglucosamine, it was shown that dividing cells transiently display on their surfaces sites which are otherwise buried within the glyocalyx. These cryptic sites can be exposed by protease treatment, but in all transformed cells they are permanently exposed. Burger suggests that the surface structure characterized by WGA binding sites may be
essential for the initiation of the DNA synthetic (S) phase and that during the phase when it can bind WGA, its growth is not susceptible to contact inhibition. If the normal mitotic (M phase) surface configuration becomes fixed by oncogenic processes, the cell continues to divide without restraint from contact with other cells (see p. 11). The nature of the cover material which obscures the WGA binding sites seems to be unimportant so far as contact inhibition of growth is concerned. Transformed cells can be rendered normal in this respect merely by coating them with a monovalent reagent prepared by splitting the agglutinin. When the artificial cover layer is removed, growth control is lost once more. The cover layer may exert its influence by affecting adhesiveness, membrane flexibility or turnover, or permeability to specific ions, general nutrients or growth factors, or it may be involved in the propagation of messages relating to the control of cell division (Burger & Noonan, 1970).

If normal, contact-inhibited cells are treated with trypsin, they carry out one cycle of growth, but their surface cover layer becomes restored after activity and they again cease division (see p. 11). Hyaluronidase is reported to be secreted in proportion to the malignancy of various tumours (Balaza & Jacobson, 1966). L. Weiss (1967) is not convinced by this evidence, but described collagenolytic activity around tumours. Cells transformed by Rous virus release a substance which counteracts the effect of conditioning factor, which may be a form of collagen (Rubin, 1965). These effects could contribute much to the malignant properties of tumours by destroying intercellular material or by removing the normal cover layer from WGA binding sites.
Cyclic changes in the cell surface have been recorded for some time. The fusion of competent myogenic cells to form multinucleated myotubes probably involves the mediation of cell surface determinants, and this occurs only during the G1 phase (Holtzer et al., 1969). Mayhew (1966) detected an increase in the negative electrophoretic mobility of cells at mitotic peak phase which was ascribed to an increase in the amount of sialic acid on the surface. An increase in the molar ratio of sialic acid to fucose, mannose and galactose was recorded at 'M' phase in synchronized cell cultures by Glick, Gerner & Warren (1971), but this was due to a decrease in the neutral sugars rather than an increase in sialic acid. Sialic acid levels decreased later in the cycle. Biddle, Cronin and Sanders (1970) suggested that the accessibility of the WGA binding sites on normal cells is varied during the cell cycle not by the overlaying of a cover layer, but by the incorporation of different proportions of sialic acid into the surface molecules which carry the WGA binding sites. The implication here is that sialic acid brings about steric or electrostatic changes in the WGA binding site or in neighbouring groups. Support for this interpretation is provided by Warren, Fuhrer & Buck (1972) who demonstrated that the differences between the carbohydrate components of normal and transformed cell surface glycoproteins, which were detected by gel filtration, could be abolished by treating the glycoprotein from transformed cells with neuraminidase. The authors showed that the activity of a sialyltransferase was greatly increased in transformed and dividing cells compared with normal, non-dividing cells.

It was formerly believed that net cell surface charge is increased in all types of tumour due to an increase of sialic acid and that this is
associated with a decrease in general adhesiveness (Ambrose, 1967) in accordance with the lyophobic colloid theory (see page 41). P. J. Ambrose et al. (1959) demonstrated a progressive increase in surface charge as a solid tumour developed into an ascites tumour. On the other hand some virus-transformed lines have consistently depressed contents of sialic acid and there is no positive correlation between sialic acid content and loss of contact inhibition in these lines (Ohta, Pardee, McAuslan & Burger, 1968). Clearly there is more to malignancy than merely the activation of enzymes concerned with the incorporation of sialic acid.

4 (a) Antigenic and molecular features

One characteristic of tumour cells is the apparent loss of certain antigens from the cell surface (Weiler, 1959) and from the intercellular material (Müller & Sutherland, 1971). These observations represent antigen deletion as well as "masking" of antigens by sialic acid (Currie & Bagshawe, 1968; Sanford & Codington, 1971). In human adenocarcinomata blood group A and B-active glycolipids are lost while Le and H antigens simultaneously appear, an indication that the synthesis of complex carbohydrates of the cell surface has been interrupted (see p. 22).

Chemically-induced tumours display antigens which may be unique to that tumour (Globerson and Feldman, 1964; Baldwin, 1967) while the surfaces of cells from tumours induced by viral infection display antigens specified by the virus (Sachs, 1965; Klein, 1970),

Antigens characteristic of foetal stages frequently appear in cancer cells, oncogeny may be "blocked ontogeny" (Alexander, 1972). Carcino-
embryonic antigen (CEA) is glycoprotein material with a characteristic determinant, detectable on surfaces of tumour cells in all adenocarcinomata, (but not benign tumours), of the human gut, in lung and breast tumours and in other disorders (Alexander, 1972). CEA is normally found in the glycoacycyles of cells of the digestive organs, (but not the lungs) of human foetuses of 2–6 months and in only very small quantities in adult colon. According to Alexander (1972) it is accessible to antibody only when in an extended form. The extended structure of glycoproteins is often maintained by charge repulsion between sialic acid groups at the ends of carbohydrate side chains (see p. 18). Sialic acid, or other charged components may therefore be relevant to the antigenic expression of CEA. Prehn (1967) suggested that the different transplantation-type tumour-specific antigens all have a counterpart in embryonic life and are derepressed more or less randomly during carcinogenesis by chemicals. A transplantable murine teratocarcinoma containing undifferentiated derivatives of all three embryonic germ layers was investigated by Braun (1968). He found that the embryonal carcinoma cell was a multipotential cell, highly malignant in the undifferentiated state, but that its well-differentiated derivatives did not have malignant properties. He therefore concluded that malignancy was connected with failure of differentiation.

Burger (1971) summarised the situation with respect to antigenic changes by stating that most reported changes in antigenic properties of cell surfaces associated with neoplastic transformation are due to neocentgens, that antigen-loss may often be partial rather than total and that antigen loss
and gain may be two facets of the same phenomenon. Most surface antigens of virally transformed cells are not identical with antigens on the virion, but may be caused by the activation of host enzymes which degrade surface components. Alternatively the virus genome may specify degradative enzymes, or specific determinants which become incorporated in the membrane. Burger concluded that at present no definite decision can be made whether the surface antigens of tumour cells are a requirement for malignancy.

A great deal of light is thrown on the nature of changes in surface antigens in cancer cells and the enzymic changes which underlie them by a study of membrane glycolipids.

Mora, Brady & Smith (1970) found a consistent and dramatic decrease in the higher gangliosides of various mouse cell lines after viral transformation, while the lower gangliosides were apparently unaffected i.e. the transformed cells had "incomplete" oligosaccharide chains (c.f. the loss of A & B blood group antigens in human adenocarcinomata, p.22). This change paralleled the loss of contact inhibition of growth of the cultures. Later work (see Heath, 1971) has revealed a concomitant increase in the lower gangliosides, precursors of the higher forms. This suggests a specific impairment of key glycosyltransferases, including a styalyltransferase, responsible for particular steps in ganglioside biosynthesis.

Normal cells can extend the carbohydrate chains in the surface glycolipids only after confluent growth has been attained, but this effect is not manifest by transformed cells (Heath, 1971). Contact inhibition of growth may therefore involve the activation of certain glycosyltransferases (see p. 15).
A general picture is now emerging of a glycocalyx which varies cyclically in composition and structure in phase with events which are occurring in the nucleus. The nuclear division phase is characterised by a relatively low content of neutral sugars and a relative excess of stadic acid, certain residues are accessible to lectins and the cell does not respond to contact with other cells. As the G1 phase approaches, the ratio of stadic acid to neutral sugars in the surface glycoprotein decreases, specific chemical groups are obscured by steric changes, and by the end of G1 the surface has attained a differentiated form suitable for interaction with other cells. This interaction may take the form of adhesion, rejection, fusion and/or growth inhibition, but cessation of growth is delayed until the next 'S' or 'G2' phase (see p. 46). If due to genetic damage the surface of the cell becomes fixed in a form corresponding in some respects to the division phase, or the surface glycoprotein characteristic of interphase is removed by lytic activity, the cell continues dividing and fails to interact appropriately with its neighbours. Mutation of the enzymes responsible for the elaboration of the carbohydrate chains of membrane glycolipids and/or glycoproteins (see p. 57) is possibly a primary event in carcinogenesis. Alternatively the failure to complete synthesis of these molecules may be the result, rather than the cause of breakdown in contact inhibition of growth. The outcome of such events is an alteration in the antigenic properties of the cell surface.

Part 3: Summary

It cannot be doubted that animal cells show changes in mutual
adhesiveness, and specificity in their interactions with one another, which are essential for normal embryonic development. Since it is the surface of the cell which first makes contact with other cells and which exhibits antigenic and other abnormalities associated with abnormal intercellular relations, it can be concluded that the cell surface is concerned in some of these interactions. Membrane-bound proteins and carbohydrates which may be of complex and very definite structure, are especially important in this respect.

The use of ferritin-labelled antibodies reveals that differentiated cell types display characteristically different proportions of protein and carbohydrate determinants in discrete patches on their surfaces. On a molecular scale, precise topological interrelations between determinants have been elucidated by antibody blocking experiments. Since the cell periphery seems to be fluid, the proportions and nature, but probably not the large scale pattern of these determinants may be the basis of a code for cell-cell recognition.

The plasma membrane appears to be a fluid organelle possibly capable of changes of state. Physical properties of the cell periphery such as viscosity and charge are probably important factors in contact relations between cells which are not highly motile, but may be overruled by other factors such as cell activity and surface chemistry, in more motile cells.

Adhesion of cells to inert surfaces such as glass or plastic depends on the composition of the fluid medium as well as the inert surface and probably occurs by mechanisms which are quite distinct from those involved in intercellular adhesion. Intercellular materials containing protein and carbohydrate assist the adhesion of cells within tissues and promote the growth and aggregation of cultured cells in a rather specific way. Calcium plays a significant
role in cell adhesion, it may be necessary for the stabilisation of the inter-
cellular material and may also assist adhesion by neutralisation of surface
negative-charges. In mobile cells calcium may affect the activity of
actomyosin and have influence on the stability of cell associations.

There seems to be a quantitative hierarchy of cohesiveness of
cells which may be the basis of some sort of adhesive specificity. In
reaggregation experiments, some sorting phenomena may depend on the
different times of recovery of tissue after disaggregation. Once established,
cell contacts are consolidated by demesomes, which may develop from tight
junctions. There is no evidence that adhesions are initiated at demesomes.

None of the theories of cell adhesion outlined above gives a
satisfactory explanation of specific and non-specific adhesion and its break-
down in cancer cells, in terms of the molecules which are known to
populate cell surfaces and which are known to be abnormal in cancer cells.
In the Key mouse, cellular invasions and abnormal tissue adhesions are
associated with overproduction of seminal vesicle secretion and abnormalities
of the lens capsule. Both of these materials are produced in quantities
sufficient for biochemical analysis by conventional techniques. The key
mutant therefore offers a unique opportunity to advance our understanding of
the biochemistry of specificity in cell adhesions.
The Small Eye (Soy) allele was first detected by Roberts (1967) in a line of mice selected for low body weight, the parents of seven affected mice had normal eyes. The mutation is dominant and lethal in homozygous embryos, but does not appear to affect the viability of heterozygotes. It is incompletely penetrant and its expressivity is variable even in the eyes of one individual. The locus is linked to 'Agouti' (Day, 1970).

The Soy phenotype superficially resembles 'Blind' except that in the latter case the eyelids are open at birth while Soy eyes remain closed for the normal period. Clayton & Campbell (1968) carried out a more detailed investigation. They found that although the major defects are confined to the eye, Soy skulls and brains are sometimes asymmetrical and the optic chiasma is sometimes absent.

One of the most striking features about the Soy phenotype is its great variability and although a catalogue of abnormalities was compiled by Clayton & Campbell (1968) there was little obvious correlation between the separate features. These authors classified the eye defect into five types involving degenerative changes, abnormal adhesiveness of tissues, invasiveness and metaplasia, disruption of fluid balance, and thickening of membranes.
Degenerative Changes

Less than 15% of 

Sey eyes have sensory cells in the retina which usually contains only inner nuclear, plexiform and ganglion layers. The pigment epithelium is often necrotic and retinas and corneas may contain cysts. Most lenses are cataractous and contain disorganised swollen cells, although some lenses have normal fibres.

Adhesiveness

Certain tissues of the eye normally remain unattached although during development they may come into close contact (Coulombre 1965). In Sey eyes the lens may fuse with retina, cornea or iris, or the cornea may fuse with retina or iris. In the embryo the lens may retain its attachment to the external epithelium or may fuse to the optic-cup (Clayton, personal communication).

Invasiveness and Metaplasia

In normal lenses the epithelium is a monocellular layer which covers the anterior surface of the lens beneath the capsule. In Sey lenses the epithelium tends to hypertrophy and form nodules within the capsule or to extrude out of the lens and sometimes to invade the retina. The corneal epithelium and the nuclear layer and pigmented tissues of the retina may also hypertrophy. One eye was invaded by choroid tissue.

Fluid Balance

Lens fibres tend to be swollen and there are fluid spaces between them and in the lens epithelium, but the total water content of lenses tends to
be reduced. The cornea and retina are also often spongy in appearance.

**Thickening of membranes**

The lens capsule may be very thick especially in lenses with hypertrophied epithelium. Lens cell membranes are also recorded as being thickened and the inner and outer limiting retinal membranes and Descemet's membrane in the cornea are also very thick.

**Other features**

The protein content of very defective lenses is low and Clayton & Campbell (1968) claimed that the α-crystallin arc was displaced anodally while the cathodal end of the γ-crystallin arc was lost. However, these features were not confirmed by Day (1970).

The production of excessive quantities of seminal vesicle secretion by old *Sey* male mice was recorded by Clayton (1970) who also reported "evidence of cell surface abnormality in brain as well as eye". PAS-positive material is deposited in the lens, around lens epithelial invasions of cornea and retina and in the extra-ocular muscles, while threads containing carbohydrate form between various tissues of the mutant eye (Clayton, personal communication).

So far as the major theme of this investigation is concerned, the most significant features of the *Sey* syndrome are the invasiveness of lens epithelial cells and the abnormal adhesions between eye tissues. Clayton &
Campbell (1968) drew attention to the apparent abnormalities of carbohydrate secretion in \textit{Sev} mice and also noted the possibility that defective fluid balance might be related to the observed abnormalities in the extracellular ocular membranes.

A. The Sorbitol Pathway

The suggestion by Dr. A. Pirie that the \textit{Sev} gene may exert its effect through the sorbitol pathway is reported by Clayton (1970). The rationale for this suggestion is that the pathway operates in the lens, seminal vesicle, brain and placenta, organs which there is reason to believe may be abnormal in \textit{Sev} mice (Clayton, 1970). The relevant steps in the pathway shown below are taken from Van Heyningen (1969)

\[
\text{Glucose} + \text{NADPH}_2 \xrightarrow{\text{aldo}se \text{ reductase}} \text{Sorbitol} + \text{NADP} \\
\text{Sorbitol} + \text{NAD} \xrightarrow{\text{polyol dehydrogenase}} \text{Fructose} + \text{NADH}_2
\]

Van Heyningen (1962) proposed that lens cataract may result from osmotic uptake of water in compensation for the abnormally high concentrations of sugar alcohols which have been reported in some cataractous lenses. A defect in polyol dehydrogenase (otherwise named sorbitol dehydrogenase, or SDH) in \textit{Sev} mice might lead to accumulation of sorbitol in the lens with consequent osmotic imbalance. Experiments devised to test this possibility are described on p. 80.

B. Extracellular Membranes

Although correlations between the diverse features of the \textit{Sev}
syndrome are not easily made, it is significant that the eye tissues with
abnormal fluid content (the retina, lens and cornea) are bounded by membranes
which tend to be thickened (the retinal limiting membranes, the lens capsule
and Descemet's membrane), or by epithelia which become hypertrophied (the
corneal and lens epithelia). The abnormal tissue fluid levels could therefore
be a secondary effect due to a defect in these membranes or epithelia. The
first major objective in the practical work was therefore to characterise the
abnormality in Sey ocular membranes. It was hoped that such an analysis
would provide data indicative of possible defects in cell surface components.

The eye membrane most easily accessible for examination is the
lens capsule which is secreted by the lens epithelium (see Clayton, 1970). The
capsule is composed largely of highly glycosylated collagen and also contains
acid mucopoly saccharides concentrated around the equator of the lens. (Waley,
1969). Descemet's membrane has a less heterogeneous composition, it
contains no detectable mucopoly saccharide (Dehlman & Balaza, 1955) and no
lipid or phospholipid (Derk, 1955). The permeability properties of Descemet's
membrane appear to be determined solely by the degree of glycosylation and
architecture of its collagen (Fatt, 1969; Spiro, 1969b). On this evidence it
seems plausible that the defects in Sey ocular membranes are concerned with
glycosylated collagen.

None of the extracellular ocular membranes of mice have been
analysed, but the composition of bovine lens capsule is described by Spiro
& Fukushima (1969), Fukushima & Spiro (1969) and Dische & Zelmonis (1969), and
that of canine anterior lens capsule by Kefalides & Denduchis (1969). Bovine
capsule contains approximately 10% carbohydrate of which the majority is in
the form of glucose-galactose disaccharides, or single galactose residues
attached to hydroxylsine. The remainder includes galactose, mannose,
hexosamines, sialic acid and fucose attached to asparagine. Canine lens
capsule has a similar overall composition (Kefalides & Denduchis, 1969).

The main problem in the analysis of mouse lens capsule was
obviously going to be one of scale since the average weight of a normal mouse
capsule was found to be only about 20µg. The problem was solved in three
ways:

(i) by amassing a large number of capsules,
(ii) by developing methods for the measurement of minute quantities
    of carbohydrates,
(iii) by narrowing the scope of the investigation to the minimum
    significant number of molecular species.

C. Seminal Vesicle Secretion

Although lens capsule material is produced in only minute amounts,
another glycoprotein is secreted by male mice in relatively massive quantities.
This is the secretion of the seminal vesicle, which interacts with the secretion
of the coagulating gland to form the vaginal plug after coitus. Seminal vesicle
secretion (SVS) is formed in excessive quantities by old Sey mice (Clayton, 1976).
If the Sey gene is associated with a general defect in glycoprotein synthesis or
secretion, then analysis of the SVS might give an indication of the nature of the
defect. Sey SVS was therefore analysed as a pilot investigation in order to gain
some insight into the most profitable approach to the analysis of the lens
A preliminary objective was therefore the qualitative analysis of the SVS of *Sey* males. Mouse SVS has not previously been analysed. The literature contains a profusion of methods for the analysis of glycoproteins and mucus mucopolysaccharides, but many of these were found to be unsuitable for various reasons. A considerable amount of effort was expended in selecting a suitable set of procedures and most of these methods were used in the actual analysis. Quantitative measurements were also carried out and similar methods were then applied to lens capsule preparations. Confirmation of the general conclusions derived from the latter analysis was provided by an electron microscopic examination of the capsule carried out by Margaret Perry.

The protein and glucose contents of urine were estimated in an attempt to detect filtration or other defects of the cross-reacting glomerular basement membrane. A major objective was to determine whether biochemical abnormalities detected in the lens capsule, are also expressed on the surfaces of lens epithelial cells. The method chosen for this investigation was whole cell electrophoresis. The behaviour in culture of *Sey* lens epithelial cells was examined by time-lapse cinematography.

At the close of the work the causes of variability of expression of the *Sey* phenotype were examined through a detailed analysis of the breeding data.
CHAPTER 3

MATERIALS

Reagents and Standards

All reagents were Analar grade from Hopkin & Williams, Chadwell Heath, Essex, with the exceptions listed below. Chemicals used in the standardization of assay procedures were dried under vacuum over P₂O₅ at 69°C.

Carbazole was resublimed under reduced pressure.

N-acetyleneuraminic acid was from Koch Light, Colnbrook, Bucks.

Sialic acid standards for qualitative analysis were prepared from bovine submaxillary mucin (Sigma type I) by Methods 17 and 20 (see below).

"Clinistix" and "Albustix" reagent strips were from Ames Co., Stoke Poges, Bucks.

Solvents

Ethyl methyl ketone and tert-butanol were from BDH, Poole, Dorset.

Laboratory reagent grade amyl alcohol containing 80% 3-methyl-butanol-1-ol and 20% 2-methyl-butanol-1-ol (May & Baker, Dagenham, Essex) was found to be satisfactory in the Svennerholm (1937) procedure for the estimation of sialic acid. Acetylacetone was redistilled.
Enzymes

Reagents for the enzymic assay of glucose were from Hughes & Hughes, 12a High Street, Brentwood, Essex.

Sorbitol dehydrogenase was assayed with kit no. 16960 TSAB from Boehringer, Mannheim.

Streptomyces griseus protease (type VI) and Clostridium histolyticum collagenase (types I & III) were from Sigma, London.

Chromatography Materials

Cellulose MN 300G and Kieselgel G (Macherey Nagel) were from Camlab, Cambridge.

Aluminium oxide G was from BDH, Poole, Dorset.

Whatman thin-layer Chromedia CC41", filter papers and chromatography papers were from A.R. Horwell, London.

Sephadex G-50 and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden.

"Neatam nov" was from E. Merck, Darmstadt, Germany.

Ion Exchangers

Chromobeads (type 5) for qualitative-analysis of sugar borates were from Technicon, Basingstoke, Hants.

Dowex ion exchange resins (Sigma, London) were cleaned with organic solvents (Montreuil & Scheppler, 1969) and regenerated as recommended by BDH (1970).
Polyacrylamide gel Electrophoresis

Acrylamide was from BDH, Poole, Dorset.

Basic fuchsin and Amido Black 10B were from Edward Gurr, London.

Tissue Culture

Tissue culture dishes were from Nunclon, Roskilde, Denmark and Falcon Platios, Cxnard, CA 93030 U.S.A.

Plastic syringes were from Sterisal, Redditch, Worcs and Becton, Dickson & Co., Ltd., Dublin, Ireland.

Paper face masks were from Robinson & Sons Ltd., Chesterfield, England.

Minimal Essential Medium (MEM)”, Medium 199, Eagle’s Medium Dulbecco Modification, Membrane filtered foetal bovine serum (FBS), penicillin-streptomycin mixture (5000 IU of each per ml) and L-glutamine (2mM) were from Bio-cult Laboratories Ltd, Paisley, Scotland.

Solutions were sterilized by autoclaving at 15lb/ins² for 15 min, or by centrifugation through bacterial filters (“Sterimats”, TB Ford Ltd., Loudwater, Bucks) fitted within twin-bottle assemblies (H.A. Jones, Beaumaris, Anglesey, Wales).

Water

Glass distilled water was used throughout. For tissue culture water was distilled twice and for cell electrophoresis water was distilled first from a Pyrex vessel containing 5% KMnO₄ and 2% KOH and then in a clean Pyrex still.
CHAPTER 4

METHODS

A. Qualitative Analysis of Carbohydrates

Chromatographic separation and staining procedures were tested with purified standards and hydrolytic procedures with fresh, or freeze-dried bovine lens capsules.

Samples in the range 1-10 µl were applied to papers and (thin-layer plates by means of graduated disposable capillary tubes ("Microcaps", Drummond Scientific Co. U.S.A.).

Development of thin layer plates was carried out in sealed glass tanks. Papers were developed by the descending method for 50-100 h in sealed tanks insulated from temperature fluctuations.

Paper Chromatography

1. Choice of paper

Three grades of chromatography paper, Whatman No. 1, No. 4 and No. 3 MM, were compared in solvent system 2 (a).

Comments: Whatman No. 1 paper gave the best separation and the most discrete spots.
2. Solvent systems for neutral and amino sugars

(a) n-butanol-ethanol-water (10:1:2) (Spiro & Spiro, 1965)

Comments: Good separation of all sugars tested (see Fig. 11).

(b) Water-saturated phenol, in an atmosphere of ammonia and cyanide,

(Partridge, 1948).

Comments: The system was considered too dangerous for routine use with the facilities available.

3. Solvent systems for steric acids


Comments: Poor separation, spots were not discrete.

4. Solvent systems for uronic acids

n-butanol-glacial acetic acid-water (30:15:35) (Davidson, 1966).

Comments: No separation of glucuronic and galacturonic acids.

Thin-Layer Chromatography

5. Preparation of thin-layer plates

Glass plates were cleaned with chloroform or toluene, washed in a dilute detergent solution, rinsed finally with distilled water and dried at room temperature. Dry tape ("Elastoplast" or Mallinekrod 250 µ tape) half an inch wide was stuck along parallel edges of one face of each plate and the plates were laid horizontally, taped side uppermost, on a levelled bench.

Slurries were prepared by thoroughly stirring the powdered medium.
(Cellulose, Silica gel, etc) with two thirds of the total required volume of water (or in the case of Method 9 (ii), with 0.1 N boric acid). The remainder of the liquid was then added to the slurry stirred for 15 sec in a fast electric blender. For each 10 g of adsorbent, the volume of liquid required is 60 ml for Cellulose, 17 ml for silica gel, and 16 ml for alumina (SiO₂: 50; aluminium oxide, Kieselgel G).

The slurry was poured immediately onto each plate and a uniform layer obtained by spreading with a glass rod which was laid across the plate and slid over it using the tape as a spacer. The sides of the plate were tapped with a glass rod to remove bubbles and to allow the layer to settle smoothly. After allowing the layer to set and dry, the plate was placed vertically in the oven and activated by heating for 15-30 min at 105-120°C according to the nature of the adsorbent (see below). The tape was then removed and the borders of the layer trimmed by scratching a groove through the layer parallel to the edge of the glass. In some cases it was found advantageous to wash the layers before use by elution with the developing solvent. Washed layers were reactivated before loading.

(i) Cellulose:

Good layers were prepared consistently with MN 300 G cellulose powder, but not with Whatman thin layer Chromedia CC41 either alone or with added binder (10% Ca₃(PO₄)₂·2H₂O). Plates were activated by heating for 10 min at 110°C. (Petrovic & Canto, 1969).

(ii) Silica Gel:

Plates of Kieselgel G were activated by heating for 15 min at 120°C (Granshe, 1962).
(iii) **Silica Gel and Boric Acid:**

Each 4g of Kieselgel G was mixed with 6 or 8 ml of 0.1 N boric acid. Plates were activated for 45 min at 105°C (Patuska, 1961).

(iv) **Alumil:**

Each 25 g of a mixture of equal parts of Kieselgel G and Aluminium Oxide G were stirred with 25 ml water. Plates were used unactivated or activated for 30 min at 110°C (Stahl & Kaltenbach, 1962).

G. **Solvent systems for neutral sugars**

(i) **On activated cellulose:**

(a) Formic acid-methyl ethyl ketone-tert butanol-water (15:30:40:13) (Vomhof & Tucker, 1965).

**Comments:** Very good separation of different classes of sugars, poor separation of glucose and galactose (see Fig. 10).

(b) n-Butanol-pyridine-water (5:3:4) (Petrović & Canić, 1969).

**Comments:** Generally poor separation, but good separation of glucose and galactose.

(c) Water-saturated phenol (Petrović & Canić, 1969).

**Comments:** Good separation of all sugars tested including glucose and galactose.

(ii) **On non-activated alumil:**

(d) n-butanol-glacial acetic acid-water (6:3:1) (Stahl & Kaltenbach, 1962).

**Comments:** Partial separation of the neutral sugars tested.
(e) n-propanol-ethyl acetate-water-glacial acetic acid (4:1:4:1) (Stahl & Kaltenbach, 1962).

**Comments:** No separation of the neutral sugars tested.

(iii) On activated alusil:


**Comments:** Poor separation of the neutral sugars tested.

7. **Solvent systems for amino sugars**

On activated cellulose

Systems 6 (a), 6 (b) and 6 (c) were tested with amino sugar hydrochlorides although they were not formulated for these sugars.

**Comments:** Systems 6 (a) and 6 (b) gave partial separation of amino sugar hydrochlorides, system 6 (c) gave good separation.

(a) n-butanol-pyridine-acetic acid-water (60:5:5:4:30) (Easer, 1965).

**Comments:** Good separation of amino sugar hydrochloride.

8. **Solvent systems for stialic acids**

On activated silica gel


**Comments:** Good separation of stialic acids.

9. **Solvent systems for uronic acids**

(i) On activated cellulose

System 6 (a). This system was tested with uronic acids although not formulated for these sugars.
Comments: Partial separation of uronic acids.

(ii) On activated silica gel with boric acid

(a) Benzene-glacial acetic acid-methanol (20:20:60) (Patuska, 1961)

Comments: Poor separation of uronic acids.

(b) Methyl ethyl ketone-glacial acetic acid-methanol (60:20:20) (Patuska, 1961)

Comments: Poor separation of uronic acids.

(iii) On silica

System $E(t)$ was applied to uronic acid samples on activated and non-activated layers.

Comments: Activated layers gave good separation of uronic acids, non-activated layers gave poor separation.

Stains for Paper and Thin-layer Chromatograms

10. General Carbohydrate stains

(a) Triphenyltetrazolium (Hata & Inouye, 1966).

Papers were sprayed with a solution of triphenyltetrazolium chloride in chloroform (500 mg per 100 ml), dried, sprayed with 0.1M NaOH in ethanol and heated at 100°C. for 5 min.

Comments: Sugars stain red on a pink background, but no distinction is made between different classes of sugars.

(b) Alkaline periodate permanganate (Hata & Inouye, 1966).

Papers were sprayed with a mixture of 4 volumes of 0.1M sodium metsperiodstio with one volume of 0.07M $\text{KMnO}_4$ in 0.1M $\text{Na}_2\text{CO}_3$
adjusted to pH 7.2 chromatograms were examined after 10–30 min.

Comments: Sugars stain red or purple on a yellowish background, but no distinction is made between different classes of sugars. The papers discolour rapidly.

(c) **Silver nitrate** (Trevelyan, Proctor & Harrison, 1950)

The dry chromatogram was dipped in a reagent prepared by diluting 0.1 ml of saturated aqueous AgNO₃ to 20 ml with acetone and adding water dropwise with shaking until the AgNO₃ which separated out was redissolved. After drying, the chromatogram was sprayed with 0.5N NaOH in aqueous ethanol prepared by diluting a saturated aqueous solution of NaOH with ethanol. Excess silver oxide was removed from the paper with a solution of "Artifix"

(3.5.4, Poole, Dorset) in place of the X-ray fixer used by Benson, Bassham, Calvin, Hall, Hirsch, Kawaguchi, Lynch & Tolbert (1952).

Comments: Sugars stain black on a white or grey background. No distinction is made between different classes of sugars.

(d) **Aniline hydrogen phthalate** (Partridge, 1949)

Papers or thin-layer plates were sprayed with a solution of 0.93 g of aniline and 1.66 g phthalic acid in 100 ml of water-saturated butanol, allowed to dry and heated for 15 min at 105°C.

Comments: Excellent results were obtained with careful heating. Hexoses produced yellow-brown spots, uronic acids and lactones stained pink, pentoses purple–pink and ketohexoses green. A very high degree of sensitivity was obtainable by viewing stained chromatograms in ultraviolet light. It was found that heating at 120°C intensified the staining of amino sugar hydrochlorides as brown spots, but all spots failed to fluoresce under ultraviolet illumination.
after prolonged heating at this temperature.

11. Stains for amino sugars

See 10 (d) above.

Ninhydrin (Bayley & Bourne, 1953)

Chromatograms were sprayed with a 0.25% solution of indanotrione hydrate in ethanol and heated at 85°C for 2-3 min.

Comments: Amino sugars stained purple-brown. It was found that the stain could be used on chromatograms already stained with aniline hydrogen phthalate. However, chromatograms of hydrolysates were confused by other materials which stained a similar colour.

12. Stains for sialic acids

(a) Ehrlich's reagent (Svennerholm & Svennerholm, 1958).

Chromatograms were sprayed with a solution of 0.5g p-dimethylaminobenzaldehyde and 8g trichloroacetic acid in 20 ml of 50% aqueous ethanol diluted with 60 ml of n-butanol, and heated at 100°C for 10-15 min.

Comments: Sialic acids gave characteristically coloured violet spots on a yellow background.

(b) Resorcinol (Svennerholm & Svennerholm, 1958).

Chromatograms were sprayed with a solution of 1g resorcinol and 15g trichloroacetic acid in 20 ml of 50% aqueous ethanol containing 0.2 ml of 0.1M CuSO₄, diluted with 60 ml of n-butanol and then heated at 100°C for 10-15 min.

Comments: Sialic acids gave uniformly coloured brown spots on a pink back-
ground. Sensitivity was possibly greater than with stain 12 (a).

13. Recording of Paper and Thin-layer Chromatograms

Chromatograms were preserved untreated or in the case of thin layers, after impregnation with plastic ("Nexitan nov"), but in all cases tested, the patterns faded. Xerox photography and conventional photography by reflected or transmitted white light and a combination of both were tested. The best results were obtained with reflected light, but there were no facilities for photography with ultra-violet illumination. The cheapest and simplest method for recording chromatograms was found to be by tracing onto paper after outlining the spots as seen under ultra violet light.

Preparation of Samples for Chromatography

Hydolysis of glycoprotein-mucopolysaccharide mixtures releases neutral and amino sugars, uronic, stalic and amino acids and peptides. Selective hydrolytic procedures can be employed to release particular classes of carbohydrates and further discrimination can be achieved by chromatographic separation, differential solubilization and selective staining (see above).

For successful paper and thin-layer chromatography, sugar samples must be relatively free from salts, proteins and peptides, one of the principal problems in the use of acid for hydrolysis being the subsequent removal of the acid and its salts. In general a higher degree of purity is required of samples for thin-layer than for paper chromatography.

Sulphate ions can be removed by adding Ba(OH)_2 when they
precipitate as BaSO₄ and excess Ba⁺⁺ ions removed with a cation-exchange resin (Basman & Jackson, 1968; Adams, 1965; see Method 21). This method was found to be successful with Dowex 50W-X8 [H⁺] resin (Sigma), but was too cumbersome for routine use. Chloride ions can be removed from HCl hydrolysates by precipitation as AgCl (Hals & Macek, 1963) or by evaporation of the acid under reduced pressure at elevated temperatures (e.g. 40°C).

The use of ion exchange resins as catalysts during acid hydrolysis permits effective release of free sugars by dilute HCl. The resin can then be filtered out and the acid easily removed by evaporation at 40°C under reduced pressure.

The hydrolytic & purification methods described below were applied to bovine lens capsules and Methods 17 and 20 to bovine submaxillary gland mucin (Sigma type I), in order to select appropriate procedures for the quantitative analysis of mouse seminal vesicle secretion, the composition of the former substances being known.

Hydrolytic Procedues for Release of Monosaccharides

14. Release of neutral sugars

All hydrolyses were carried out at 100°C in an atmosphere of nitrogen within sealed tubes, at a ratio of 3 mg of material per ml. of acid (Spiro, 1966).

(a) In 2N H₂SO₄ for 4h (Fukushi & Spiro, 1969).
(b) In 7N. HCl for 4h (Hais & Macek, 1963).

(c) In 1N. HCl for 6-8h (Bosman & Jackson, 1968)

(d) In 0.5N. HCl for 3h (Pirie, 1951).

Comments: A major factor in the choice of hydrolytic medium was the ease with which it could later be removed. On these grounds all the above methods were rejected in favour of Method 16.

15. Release of amino sugars:

Hydrolysis was carried out in 4N. HCl for 3-6 h at 100°C in an atmosphere of nitrogen within sealed tubes (Fukushi & Epiro, 1968).

Comments: This method was also rejected in favour of Method 16.

16. General method for neutral and amino sugars and uronic acids

Hydrolysis was carried out in 0.1N. HCl with Dowex 50 X 12 - 400 [H+] resin, for 4-6 h at 100°C at a ratio of 1g resin and 7ml acid per 10 mg of glycoprotein (Davidson, 1966). The hydrolysis was carried out in sealed tubes under nitrogen. Neutral sugars were released into solution. Amino sugars bound to the resin and were released by 2N HCl following the method of Boss (1953) (see Method 10).

Comments: This method was originally intended for the quantitative release of uronic acids from mucopolysaccharides, but was found to be the most useful method tested upon glycoproteins.

17. Release of sialic acids

Hydrolysis was carried out in 0.02N H2SO4 for one hour at 80°C in sealed tubes in which air was replaced by nitrogen, at 3mg of glycoprotein
Comments: This method was found suitable for the release of sialic acids from bovine lens capsule and bovine submaxillary mucin (see Method 20).

**The Purification of Acid Hydrolysates**

18. **Neutral sugars**

Neutral sugars may be freed of inorganic ions, charged sugars, amino acids and peptides by passage of the hydrolysate through coupled columns of cation and anion exchange resins (Spiro, 1966). This method was applied to dilutions of a 2N H₂SO₄ hydrolysate of bovine lens capsule, with columns of Dowex 50 X 12 -400 [H⁺] and Dowex 1 X 8 -400 [Cl⁻] resins. The eluent was concentrated by evaporation and examined chromatographically.

Comments: The procedure was very time-consuming and did not purify the sample sufficiently well for chromatography. This method was superseded by Method 22.

19. **Amino sugars**

Amino sugars can be isolated from solution by binding to a column of cation-exchange resin (Bose, 1953). The method was applied to dilutions of a 4N HCl hydrolysate of mouse seminal vesicle secretion, with a column of Dowex 50 X 12 -400 [H⁺] resin. Water and HCl were removed from the eluent by evaporation. The concentrated product was examined by paper chromatography.

Comments: Hexosamine was detectable in paper chromatograms, but the
concentration of impurities was too high for clear thin-layer chromatograms. Although the method was rejected, its basic principle was utilized to isolate amino sugars bound to the Dowex 50[H+] catalyst following hydrolysis of seminal vesicle material by Method 16.

20. Sialic acids

Sialic acids can be purified by isolation on a weakly basic anion exchanger (Spiro, 1966). The method was tested with solutions of N-acetyl-neuraminic acid and a hydrolysate of bovine submaxillary mucin prepared as in Method 17. Dowex 1 X 8 (formate) resin was used.

Comments: The eluent was examined by thin-layer chromatography (Method 8 (a)) and found to be satisfactory.

21. General purification procedure for neutral and amino sugars

Hais & MacK (1963) recommend deproteinizing impure aqueous solutions of sugars by dropping the solution into a ten-fold volume of absolute alcohol. This procedure precipitates proteins and the salts of sugar acids. It was found by experiment that clean samples of neutral and amino sugars, suitable for chromatography, could consistently be produced by extraction of the dried hydrolysate with absolute ethanol.

Conclusion. Selection of the most suitable methods for qualitative analysis of complex carbohydrates

(a) Hydrolytic release. The hydrolytic procedures selected were:

Sialic acids: Method 17, with 0.025N H2SO4 at 80°C.
Neutral sugars, amino sugars, uronic acids: Method 16, with 0.1N HCl and Dowex 50 [H⁺] resin at 100°C.

(b) Purification. No satisfactory procedure was devised for purifying uronic acids, but the following methods were found suitable:

Sialic acids: Method 20, with Dowex 1 (formate) resin.

Amino sugars: Methods 16, 19 with Dowex 50 [H⁺] resin;
Method 21, by extraction of dry samples with absolute ethanol.

Neutral sugars: Method 21.

(c) Identification. The procedures selected for identification of free sugars were as follows:

Neutral sugars: Method 2 (a) (n-butanol–ethanol–water, 10:1:2) on Whatman No.1 paper; Method 6 (a), formic acid–methyl ethyl ketone– tert–butanol–water (15:30:40:15), Method 10 (d), spraying with aniline hydrogen phthalate, heating at 105°C, examination under ultraviolet light.

Amino sugars: Method 2 (a); Method 6 (c), on thin layers of cellulose, with water-saturated phenol; Method 7 (a), n-butanol–pyridine–acetic acid–water (60:45:4:30); Method 10 (d) followed by heating at 120°C; Method 11, staining with ninhydrin.

Uronic acids: Method 6 (f) n-propanol–ethyl acetate–water–glacial acetic acid (4:1:4:1) on non-activated layers of Alusil; Method 10 (d) with ultraviolet illumination.

Sialic acids: Method 8, n-propanol–1N NH₄OH–water (6:2:1); Method 12 (b), resorcinol.

Chromatograms were recorded on tracing paper.
The separation of amino sugar hydrochlorides on cellulose thin layers with water-saturated phenol (Method 6 (c)) has apparently not been attempted previously, although this solvent was used successfully by Partridge (1948) and unsuccessfully by Mukerjee and Sri Ram (1964) for the separation of glucosamine and galactosamine on paper. Partridge (1949) records weak staining of amino sugars on paper chromatograms with aniline hydrogen phthalate (system 10 (d)) after heating at 105°C, but there is apparently no previous record of the intensification of spots by heating at 120°C.

The purification of neutral and amino sugar preparations by extraction with absolute ethanol, and the isolation of amino sugars from hydrolysates by means of cation exchange catalysts (as in Methods 16, 19) are both new methods.

B. Quantitative Methods

Since the release of sugars from glycoprotein by acid hydrolysis normally causes some destruction of the released free sugars, quantitative assays for glycoprotein carbohydrates were selected which did not require their prior release.

Total Hexose

The total hexose contents of digests of lens capsules and ethanol-insoluble seminal vesicle secretion (SVS) were assayed by the method of Winzler (1955) against standards of D-glucose (0.20μg). Standards of D-fructose were used in the analysis of ethanol-soluble secretion. Optical
densities were determined at 540 nm in 20 mm micro-cells.

**Amino Sugars**

Amino sugars in lens capsules and ethanol-insoluble secretion were quantitated by the method of Gatt & Berman (1966). The method was standardized with D-glucosamine and the values obtained in the analysis of SVS were converted to galactosamine equivalents by reference to the authors' published values for the two sugars.

**Fucose**

The fucose content of the ethanol-insoluble fraction of SVS was determined by the Dische & Shettles (1948) assay for methyl pentose. The assay was also applied to the lens capsule digests.

**Sialic Acid**

The sialic acid contents of the SVS and lens capsule digests were determined by the method of Svenssengholm (1957) scaled down so that quantities in the range 0-10 μg could be assayed.

Samples and standards containing 0-19 μg of sialic acid in 0.5 ml of water were placed in Pyrex test-tubes (internal diameter 11mm) with 0.5 ml of the resorcinol-HCl reagent. Water blanks and standards containing 40-80 μg of glucose were also examined. Blank reagent lacking resorcinol was added to duplicate samples to correct for non-specific colour development. 1.25 ml of isoamyl alcohol was added and optical densities were determined in 20 mm micro-cells.

**Fructose**

The fructose content of the ethanol extract of SVS was determined by the assay for keto-sugar described by Dische & Borenfreund (1951).
Glucose

Free D-glucose in the ethanol extract of SVS was assayed by the glucose oxidase method of Morley, Dawson & Marks (1968). This determination was kindly performed by Dr. P.J. Pritchard of the Poultry Research Centre, Edinburgh.

Protein

The protein content of SVS hydrolysates was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with the reagents and volumes modified as described by Miller (1959). Optical densities were determined at 750 nm. The method was standardized with a series of dilutions of bovine serum albumin.

Hydroxyproline

The hydroxyproline content of the lens capsule preparations was determined by the Jackson & Cleary (1967) modification of the Neuman & Logan (1954) method, with standards of 4-hydroxy-L-proline. The method was applied to enzyme digests treated with 6N HCl for 24 h at 100°C.

Dry Matter

Dry matter determinations were carried out by weighing unfractionated SVS before and after heating overnight at 105°C.

Sorbitol Dehydrogenase

The sorbitol dehydrogenase (SDH) activity of extracts of mouse livers was assayed according to Boehringer Corporation (London) Ltd. Test Handbook "SDH.Determination of Sorbitol Dehydrogenase Activity in Serum".
with Boehringer Mannheim test kit No. 15960TSAB. Variations in enzyme levels due to sex or stage of oestrus were eliminated by use of male mice only.

Adult mice were killed by cervical dislocation and the livers removed, washed in ice-cold 0.9% NaCl and chopped rapidly with a razor blade on a glass surface. Aliquots of liver were weighed, rapidly homogenised in a glass/glass conical homogeniser with 100 vols of ice-cold saline (0.145 M) buffered to pH 7.1 with 0.067 M phosphate. Homogenates were centrifuged at 38,000 G for 30 min at 4°C and the supernatants filtered through glass fibre tissue (Whatman GF/A). The filtrate was diluted with an equal volume of 0.145M NaCl and assayed immediately. Incubations were carried out at room temperature and readings of optical density taken at 366 nm.

A small scale investigation of the activity of SDH in mouse livers was carried out to test the hypothesis that the Sex gene codes for this enzyme (see p. 57). The method was applied in two separate experiments, to two control and two mutant young adult male mice and three genetically identical mice of widely different ages, in order to test the effects of age. The two sets of data are not directly comparable however since no standard samples of SDH were available for standardization. The mutant mice were the product of five backcrosses to the Fv and C57Bl strains (see p. 27) and were selected since they expressed the mutant phenotype to a marked degree.

Glucose & Albumin in Urine

Urine was obtained from live mice by light finger pressure on the bladder, and its glucose and protein content estimated by use of "Clinistix" and "Albustix" reagent strips. The Clinistix test detects glucose in urine at
concentrations down to 0.1%. Albumin may be detected in the range 5 to over 1000 mg per 100 ml of urine and assessed as "5-20" mg, approximately 30, 100 or 300 mg, or "over 1000 mg" per 100 ml.

C. Enzymic Solubilization of Lens Capsule and Seminal Vesicle Secretion

Quantitative analyses of ethanol-insoluble SVS and of lens capsules were performed after enzymic solubilization.

The quantitative assay of carbohydrates is affected by traces of salts (W. L. Cunningham, personal communication: Gatt & Berman, 1966). A volatile buffer was therefore selected so that all buffer components could be removed from the preparation after the digestion was complete. A colidine-HCl system gave buffering over the range of pH appropriate for the enzymes used.

Seminal Vesicle Secretion

The ethanol-extracted SVS was suspended at 100 mg per ml, in Gomori's volatile buffer containing 0.05M, 2:4:6 colidine adjusted to pH 7.5 with 0.1 M HCl (see Long, 1961) and treated with 2 mg Streptomyces griseus protease (Sigma type VI) and one drop of toluene. A further 0.5 mg of protease in 0.5 ml of buffer was added after incubation at 37°C for 66h and the incubation continued for 24h. Approximately 0.5% (W/w) of the material remained unsolubilized and was centrifuged out. The buffer components were removed by evaporation under vacuum at 40°C and the residue was dissolved in water at 2.7 ml per 100 mg dry material.
**Lens Capsule**

The dry capsules and lens preparation were weighed, submerged in water by degassing under suction, then centrifuged out and ground in a glass/glass homogeniser. The ground material was suspended in Gomori's buffer at 25 mg per ml. To the suspension were added 0.20 mg of *Clostridium histolyticum* collagenase type I and 0.3 mg of type III (Sigma) per ml of buffer. One drop of toluene was added to each suspension. Incubation was carried out at 37°C for 72 h. The material was then further digested for 48 h with 0.5 mg of *Streptomyces griseus* protease (Sigma type VI) per ml of buffer. The very slight insoluble residue was centrifuged out and the water and buffer components removed by evaporation.

**D. Gel Filtration**

Enzyme digests of SVS and lens capsules were examined by filtration through Sephadex gel. Carbohydrate in the effluent was monitored by a colorimetric assay based on that of Winzler (1955).

**Preparation of column**

Sephadex G-50 gel (fractionation range 1000-50,000 daltons) was allowed to swell in an excess of water. "Fines" were decanted and the suspension degassed under suction. The gel was slurried in pyridine-acetic acid buffer pH 6.0 containing 5 mM acetic acid and packed under gravity into a column 50 cm x 1 cm. The natural flow rate of the column was 54 ml per h.
Fig. 1. Apparatus for automatic monitoring of column effluent for total carbohydrate.
Examination of column effluent

Samples of 0.1 ml of 0.2 ml of the digests were applied to the column which was eluted with pyridine-acetic-acid buffer pH 6.0. The effluent was monitored continuously by an automatic system comprised of a standard Technicon proportioning pump, colorimeter, etc. (Technicon Corp., Ardsley, N.York) and chart recorder (Honeywell, Greenford, Middx.) The column effluent was mixed with 1.6% aqueous orcinol and 72% H₂SO₄ and heated at 80°C for 20 min. The optical density of the sample was measured at 420 nm in a 15 mm flow cell (see fig. 1).

Calibration of column

The column was calibrated with solutions of Blue Dextran 2000 and D-glucose each containing 0.2 mg of carbohydrate. Blue Dextran is totally excluded from the column while glucose is completely retarded, these two molecules therefore define the capacity of the column. The elution volume of Blue Dextran 2000 was 13.9 ml and that of glucose was 35.5 ml.

E. Polyacrylamide Gel Electrophoresis

The seminal vesicle secretions of normal and Sox mutant mice were examined by polyacrylamide gel electrophoresis following the method of Notides & Williams-Ashman (1967) which was originally designed for guinea pig SVS.

Fresh secretions obtained by squeezing the seminal vesicles of freshly killed mice, were extracted with 1.5 ml per donor, of a buffer solution
of pH 8.7 containing disodium EDTA (0.0014 M), boric acid (0.012 M) and Tris base (0.03 M). Insoluble material was centrifuged out and sucrose (0.3 g) was added to each supernatant to increase the specific gravity. Samples of 100 or 200 μl were layered on top of polyacrylamide gels and electrophoresed at 50v for 15 min, then at 150v for 120 min, at about 1.3 m.amps per gel.

Gels (80 x 5 mm) were composed of 9% acrylamide and 0.45% methylenebisacrylamide in Tris–borate–EDTA buffer. Electrode compartments were filled with the same buffer and current was supplied by a constant voltage, direct current "Voltem" power source (Shandon SAE 2761).

Gels were stained for protein and carbohydrate. In order to exclude extraneous carbohydrate (sucrose) from the sample, the latter was also applied to conventional gels, within a "spacer gel" containing 2.5% acrylamide and 1.25% methylenebisacrylamide.

**Staining for protein**

Gels were immersed in a 1% solution of Amido Black 10B in 7% acetic acid at room temperature for 20–30 min. Destaining was by immersion in 7% acetic acid and by lateral electrophoresis between pads of paper tissue-soaked with 7% acetic acid.

**Staining for carbohydrate**

The ORTEC (1970) procedure designed for staining polyacrylamide gel slabs was modified to avoid non-specific staining of gel cores. In the modified procedure, gels were fixed in 7% (V/V) acetic acid for 75 min at room temperature, oxidised in 0.2% (W/V) periodic acid for 60 min at 4°C and
stained with Schiff's reagent (Pears, 1954) for 60 min at 4°C. Gels were destained at room temperature in 10% (v/v) acetic acid.

F. Analysis of Seminal Vesicle Secretion

Small eye male mice and Ju control males 16-20 wks old were the source of seminal vesicle secretion (SVS). The mutants were the product of 5 backcrosses to the Ju strain (see p./2/). Mice were killed by cervical dislocation, the seminal vesicles immediately excised and the secretions squeezed out. Dry matter determinations were carried out as described above. Pooled secretions were exhaustively extracted with 80% (v/v) aqueous ethanol and the extract assayed for component sugars.

For qualitative analysis of the ethanol-insoluble material the neutral sugars and hexosamines were released by hydrolysis with 0.1 M HCl and Dowex 50[H+] resin (Method 6). Hexosamine was liberated from the resin by 2 M HCl (Method 9). The released sugars were taken up in absolute ethanol (Method 2), 50% (v/v) aqueous ethanol, or water.

Qualitative analysis

Free neutral sugars in the ethanolic extracts and neutral and amino sugars released by acid hydrolysis, were examined by paper and thin-layer chromatography by Methods 2 (a), 6 (a), 6 (c), 7 (a), 10 (a) and 11. Uronic acid was not detected in paper chromatograms and malic acid was present in quantities too small for chromatographic identification.

Identification of neutral sugars was confirmed by anion exchange
chromatography on a column (75 x 0.6 cm) of chromobeads (Type S) with the Technicon AutoAnalyzer and the boric acid-sodium chloride buffer system of Catravas (1987) modified according to Technicon Development Bulletin 124. The absorbance of sugars detected by the orcinol $\text{H}_2\text{SO}_4$ reagent (Catravas, 1967) was recorded at 420 nm. This analysis was kindly carried out by Dr. P. J. Pritchard of the Poultry Research Centre, Edinburgh.

Buffer extracts of SVS were examined by electrophoresis in polyacrylamide gel (Method E) and enzyme digests (Method C) were examined by filtration through a column of Sephadex G-50 gel (Method D).

Quantitative Analysis

Quantitative assays for glucose and fructose were performed on the ethanolic extract and assays for protein, total hexose, hexosamine, fucose and sialic acid were carried out on ethan-insoluble material solubilized enzymically as described in Method C. Blank solutions containing the enzymes used in the digestion were also examined.

G. Examination of the Lens Capsule

Lens capsules were collected from mice of various stocks carrying the Small Eye gene. All mice had small eyes and/or lens cataract. Capsules were also collected from control mice which had no known deleterious genes and from bovine eyes. Sey lenses are often small and irregularly shaped and the capsules cannot easily be removed, in these cases whole lenses were collected. Normal mouse whole lenses (CBA strain) were
exhaustively extracted with 0.145 M NaCl and water, then solubilized and assayed as a control on the contribution to the Sgy material of non-capsular lenticular carbohydrate.

The capsules of 575 control mice killed by cervical dislocation, were immediately stripped off the lenses without removing the zonules. The whole lenses or capsules of 485 Sgy heterozygotes were similarly collected and all samples were intermittently stirred for 48h in several changes of 0.145 M NaCl at 4°C. Lens fibres and other contaminants were decanted off or removed by pipette. The remaining material was washed with several changes of distilled water at 4°C, with intermittent stirring for a further 48 h. Large amounts of zonule, which at this stage turned opaque, were removed by pipette. Bovine eyes were removed immediately after death and stored on ice for no more than one hour, before the lens capsules were removed and cleaned as above, but with continuous mechanical stirring. The freeze-dried capsules and lenses were stored dry at -20°C.

Quantitative analysis

The capsules were solubilized enzymically (Method C) and their carbohydrate components were assayed for hexose, hexosamine, fucose and sialic acid. Values were related to hydroxyproline as an index of collagenous protein.

Electron Microscopy

An examination of the ultrastructure of the capsules of whole mouse lenses, by electron microscopy, was kindly carried out by Miss Margaret Perry at the Institute of Animal Genetics, Edinburgh. One normal lens was
examined and four mutant lenses which were grossly abnormal in either shape, or size, or both.

Lenses were dissected from the eyes of normal and mutant mice, and double-fixed in Karnovsky's glutaraldehyde-formaldehyde fixative and buffered osmium tetroxide. Some lenses were then stained in uranyl acetate. After alcoholic dehydration, the material was transferred through epoxypropylene and embedded in Araldite. Sections were double-stained with uranyl acetate and lead citrate.

H. Tissue Culture of Lens Epithelium

Primary explants of normal lens epithelium and lens epithelium from Small Eye mutant mice were cultured in plastic dishes in order to gain some insight into the dynamic characteristics of moving and growing ey lens epithelial cells. Single cultures were examined visually and by time lapse cinematography. The interaction between normal and mutant cells was investigated in a culture containing explants from both normal and mutant mice.

Working surfaces were sterilized by wiping with absolute ethanol, in an atmosphere previously exposed for at least 12h to ultraviolet irradiation. Dissecting instruments and glassware were sterilized by maintaining at 180°C overnight.

The microscopes used were Gillet & Sibert (London) inverted, phase-contrast Conference microscopes. For time-lapse filming the microscope was fitted within an insulated box, the internal temperature of which
Fig. 2. Gas perfusion chamber used during filming of tissue cultures.
was maintained at 37°C. A Kodak Ciné special camera mounted coaxial with the photographic aperture of the microscope light beam splitter, was operated by a Wild Intervalometer (McCulloch Bros. & Wilson, Glasgow). The camera was loaded with Ilford Pan F film. During filming, culture dishes were contained within a gas perfusion chamber designed by Mr. Eric Lucey for this purpose (see fig. ). This chamber was made from two plastic petri-dish bases approximately 90 mm diameter, which could be pushed tightly together to allow sufficient space to accommodate a Cooper-style dish (60 x 15 mm) between them. Circular holes were cut in the top and base of the chamber to allow access of the microscope objective or condenser, while maintaining the gas-restricting seals at all joints. A mixture of 5% CO₂ in air was introduced into the perfusion chamber through a hypodermic needle cemented into its wall. The gas was moistened and its flow rate checked by bubbling through water contained in Wolff's bottle.

Mice were killed by cervical dislocation and the eyes removed. Whole eyes were dipped briefly in 70% ethanol, washed in sterile 0.145M saline and transferred with sterile forceps to sterile "Nunclon" petri dishes containing Minimal Essential Medium. The lenses were removed and the capsules together with the lens epithelium were stripped off with sterile instruments, while submerged in the medium. Capsules and attached epithelia were placed in plastic, Cooper-style dishes containing growth medium (see below). Each culture was held in a suitable position for viewing, by the weight of light glass "roof" made from a strip of glass 25 mm x 4 mm cut from a microscope slide coverslip. The end of each strip was softened in a Bunsen flame and bent at
right angles. The culture dishes were placed in an air-tight plastic box filled with a 5% mixture of CO₂ in air and incubated at 37°C. Within three days, cultures had normally adhered to the floor of the dish and the glass roofs could be removed. The box containing the cultures was then placed on an oscillating table (Gallenkamp & Co., Ltd., London) and incubation at 37°C was continued. Growth medium was replaced at 3-day intervals and the box re-gassed every day.

(a) Selection of Culture Medium

Lens epithelium has been cultured in a variety of media. In recent years Dulbecco’s modification of Eagle’s medium (Green, Goldberg & Todaro, 1966) and Medium 199 (Von Callman, Grimes & Albert, 1969), both supplemented with foetal bovine serum, have been favoured. These two media were compared. Explants of lens epithelium from Ju & Sey (5 x Ju) mice (see p.21) were cultured for 1-2 weeks in plastic dishes in the two media supplemented with 10% foetal bovine serum, penicillin (100 IU per ml) and streptomycin (100 IU per ml).

(b) Comparison of Normal and Mutant Lens Epithelia

Explants of lens epithelium taken from Sey (8 x Ju) mice (see p.21) and their normal sibs were separately cultured in plastic dishes in Medium 199 supplemented with 10% foetal bovine serum plus penicillin and streptomycin (100 IU per ml of each). About 20 normal and an equal number of mutant cultures were examined at intervals. Two normal cultures taken from mice 39 days old were filmed over the periods 3-10 and 10-14 days in vitro. One
So culture from a mouse 42 days old was filmed from 6 to 17 days in vitro. Exposures were made at intervals of 300 seconds.

(c) Interaction between Normal and Mutant Epithelia

Explants of lens epithelium of *Soy* (8 x Jd) mice and their phenotypically normal sibs were together cultured in Medium 199 supplemented with 10% foetal bovine serum and 100 IU of penicillin and of streptomycin per ml. The explants were held in close proximity by glass "roofs" until they had become established.

When both explants were placed in culture at the same time the *Soy* tissue did not readily adhere to the plastic substratum, but it was found that adherent cultures of both tissues could be obtained by introducing a fresh normal explant into an established *Soy* culture. A double culture was set up in this way from a *Soy* (8 x Jd) mouse 44 days old and its normal sib, the material from the normal mouse being introduced three days later. A region including both tissues was filmed by time-lapse photography over the period 7-11 days from the commencement of the *Soy* culture.

J. Whole Cell Microelectrophoresis

The surface charge of normal and Small Eye lens epithelial cells was examined by whole cell microelectrophoresis.

An apparatus based on that of Bangham, Flemans, Heard & Seaman (1958) was constructed in the workshop at the Institute of Animal Genetics, Edinburgh. Basically the apparatus consists of a transparent chamber fitted with non-gassing electrodes in which a suspension of particles under controlled
Fig. 3. Apparatus for cell electrophoresis, after Baugham, Flemans, Heard & Seaman (1955).
conditions can be observed by means of a microscope.

The Microelectrophoresis Chamber and Electrodes

The chamber and electrodes were obtained from Rank Bros., Bottisham, Cambridge. The chamber consisted of a cylindrical precision capillary of "Veridia" glass, of refractive index 1.47. The bore of the chamber was measured as 2.04 mm (see p. 76). Quickfit and Quartz B10 "Pyrex" sockets were fused to each end of the capillary tube and the viewing region was formed as a polished flat on the capillary wall. The capacity of the chamber was 3-4 ml.

The electrodes were of platinum foil of dimensions 12.5 x 20.0 mm formed into a cylinder and welded onto a platinum wire. Each wire was joined to a copper lead and embedded in the base of a B10 soda glass stopper. The electrodes were blacked by electrolysis in a solution of platinum in concentrated HCl, containing a small quantity of lead acetate which acts as a catalyst (W.D. Cooper, personal communication). The electrodes were stored always in doubly-distilled water. Occasional cleaning of the electrodes was carried out by immersion for about 10 min in concentrated HCl.

The chamber was cleaned with permanganic acid. This cleaning was carried out routinely before use and was followed by fifteen rinses with doubly-distilled water.

The inter-electrode distance was measured by an electrical method (see below).

The chamber holder

The electrophoresis chamber was mounted horizontally in a holder
attached to a vertical traverse mounted on a crossbar fixed across the top of a water bath. The position of the chamber relative to the axis of the microscope, could be ascertained to within ±0.002 mm by means of an adjustable zero dial indicator. The chamber and holder were immersed in the water bath with the chamber parallel to the surface of the water. Levelling of the chamber was achieved by means of adjustable feet on the water bath.

The temperature of the bath was maintained at 25°C (± 0.2°C) by means of a 'Tecan TEI Tempette' thermostatted heater and pump and a cooler made from a length of thin-walled copper tube (36 x 0.25 ins) attached to the cold-water tap.

**Optics**

The microscope tube (Ernst Leitz Wetzlar, No. 374435) was firmly attached to the water bath frame in a horizontal position, perpendicular to the axis of the chamber. It was fitted with a x43, 4 mm objective (Bausch & Lomb) with a numerical aperture of 0.65 and a 25 mm, x10 eyepiece incorporating a chessboard graticule with 10 x 10 squares of 0.25 mm side. The microscope tube length was calibrated for magnification by means of the graticule and a stage micrometer marked with a 1 mm scale calibrated to 0.01 mm. The scale was viewed immersed in water. An overall magnification of 400 diameters was obtained by extending the microscope tube to 201.5 mm.

The objective was inserted into the bath through a water-tight rubber grommet. An adjustable-zero dial test indicator calibrated to 0.002 mm was mechanically linked to the fine adjustment of the microscope to ensure reproducible focussing.
Fig. 4. Circuit diagram.

$A$ = galvanometer
$D$ = reversing switch
$P_1, P_2$ = linear potentiometers
$PP$ = power pack
$R$ = 1 megohm resistor
$V_1, V_2$ = voltmeters
Illumination was by a Kohler-type microscope lamp (G. Baker, London) mounted coaxial with the microscope.

The Circuit

The circuit is shown in Fig. 4. The potential was supplied from a rectified and smoothed mains supply (Ediswan Stabilised Power Unit, type R1095) which was tested for A.C. contamination by means of a Telequipment oscilloscope. The power pack incorporates a voltmeter (V1 which covers the range 100-200 V. Voltmeters V2 (100 μA, 10kΩ/V) covered the range 0-100 V. Potentiometers P1 and P2 could be adjusted to stop down the potential drop across the electrodes to 0-100 V. The potential gradient across the chamber was obtained directly from the applied potential and the measured inter-electrode distance. The galvanometer A (Fye 'Scalamp', 22) was used only to check for polarization of the electrodes.

Determination of inter-electrode distance

The precise distance between the electrodes was measured in terms of the electrical resistance of a solution of known specific conductivity.

An aqueous solution of KCl (approximately 0.01 M) was placed in a standard conductivity cell and its specific conductivity at room temperature was determined by means of a conductivity meter (Model P310, Portland Electronics Ltd., Oldham, England). The conductivity of the same solution was measured in the electrophoresis chamber and the distance between the electrodes calculated from the equation:

\[ K = \frac{1}{A} \cdot L \]

where \( K \) = the specific conductivity of the solution.
\[ L = \frac{1}{R} \] = the experimentally determined conductivity (mhos)

I = the inter-electrode distance (cm)

A = the cross-sectional area of the cell.

For the chamber used in these determinations the inter-electrode distance was calculated as 14.77 cm.

**Setting up the apparatus**

The electrophoresis chamber was positioned with respect to the microscope by the following sequence of operations:

1. The chamber was levelled by adjustment of the tank feet so that the chamber bore was parallel to the water surface.
2. The optical flat on the front of the chamber was set perpendicular to the objective by focussing on dust on the glass surface and moving the chamber past the objective by means of the vertical traverse.
3. The objective was directed at the centre of the chamber bore by focussing on the upper and lower surfaces of the capillary and recording the values shown on the vertical traverse dial indicator. The chamber was then moved to a point midway between the two extremes. In order to carry out this adjustment the X43 objective was exchanged for one of magnification X10. The adjustment was carried out with the water bath stabilized at 25°C. At the same time the radius of the chamber was measured.
4. The position of the stationary level was determined experimentally by recording the mobility of cells at a range of distances from the wall of the chamber at two different values of pH (see below). The horizontal traverse dial indicator reading corresponding to this value was recorded and all...
subsequent readings of mobility were taken at this setting.

**Preparation of suspending medium**

Except during the determination of the stationary level, all cell mobility values were obtained with the cells suspended in 0.145 M NaCl. The purity of the water used for the preparation of this medium was found to be the most critical factor determining the reproducibility of results. Water was prepared as described on p. 63 and used within 24 h.

**Preparation of red blood cell standard**

My own venous blood (0; M; Rh⁺) was collected by Blood Transfusion Service standard procedure and mixed with 3.8% (W/v) sodium citrate at a ratio of 9 volumes of blood to one volume of citrate. The red cells were centrifuged out and washed five times with five times the original blood volume of 0.145 M NaCl adjusted to pH 7.0 - 7.5 by addition of 1.0 M NaHCO₃. The cells were centrifuged out after each washing, at 1400 g and 4°C for 10 min (Bangham, Pethica & Seaman, 1958; Heard & Seaman, 1960).

The cells were then 'stabilised' by treatment with 2% redistilled acetaldehyde in 0.145 M NaCl at pH 7.0 - 7.5, for a total period of 20 days at 4°C (Heard & Seaman, 1961). They were then washed four times with five times the original blood volume of 0.145 M NaCl and stored at 4°C in 0.145 M NaCl adjusted to pH 7.5 with 1.0 M NaHCO₃, containing 0.002% sodium azide.

**Determination of electrophoretic mobilities**

The rate of migration of cells was found by timing their transit
over a given number of squares of the chessboard graticule, selected so that
the time taken was not less than 5 sec and not more than 15-20 sec, at an
applied potential of 2-5 V/cm.

Determination of the Stationary Level

The position of the stationary level was determined according to
Seaman (1965).

Washed, stabilized red cells were suspended in a dilute solution of
haemoglobin in 0.145 M NaCl adjusted to pH 6.2 with 0.145 M HCl, or pH 8.7
with 0.145 M NaOH. Mobilities were determined at intervals of 0.05 mm
between the wall and the axis of the chamber. The average values obtained
for 10 - 20 cells at each position were plotted against the square of their
displacement from the chamber axis. Lines of best fit were drawn by
calculation of the regression of mobility on (displacement)$^2$ (see fig. 5).

The two regression lines crossed at a position corresponding to a
stationary level 0.292 mm from the near wall of the chamber. The radius of
the chamber, determined by use of the vertical traverse dial indicator was
found to be 1.02 mm. The theoretical location for the stationary level is
therefore $0.293 \times 1.02 = 0.299$ mm from the chamber wall (Bangham, Flemans,
Hoard & Seaman, 1958). The difference of 0.007 mm between experimentally
determined and theoretical values is probably close to the optical limits of the
apparatus (Seaman 1965).
Fig. 5. Mobility vs. (displacement from axis)^2 relationship for human red blood cells: ○ pH 6.3; ○ pH 8.7; ——— stationary level.
Fig. 6. Times taken by human erythrocytes suspended in 0.145 M NaCl to travel 37.5 μ at 3.0 v/cm and 25°C.
Check on correct functioning of apparatus

The mobility of washed, stabilised red cells was determined as a check on the correct functioning of the apparatus. The suspending medium was unbuffered 0.145M NaCl. The cells were timed over a distance of 37.5 μ under a potential of 3.0V/cm. The results for a mobility determination on a sample of 35 cells, expressed as a frequency distribution are shown in Fig. 6. In the table below, these values are compared with comparable values quoted by Seaman (1965).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mobility (μ/sec/V/cm)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present results</td>
<td>1.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Bangham et al (1958)</td>
<td>1.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Seaman (1965)</td>
<td>1.07</td>
<td>0.03</td>
</tr>
<tr>
<td>Brody (1954)</td>
<td>1.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Furchgott &amp; Ponder (1941)</td>
<td>1.10</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Electrophoretic mobility of human red cells in 0.145M NaCl at neutral pH and 25°C.

Although the standard deviation of the present results is considerably larger than the published values, the average mobilities are in good agreement.

Slight inconsistencies in the mobility of red cells from the same preparation, were recorded in separate determinations. These were found to be due largely if not entirely to variations in the purity of the distilled water.
Determination of the Mobility of Normal and Mutant Lens Epithelial Cells

(a) Disaggregation of lens epithelium

The lens capsules together with the attached lens epithelia, were stripped off the lenses of normal and mutant young adult mice which were the product of 6 or 9 backcrosses to the inbred Ju line. The material from about a dozen mice of each group was combined and incubated for 30 min at 37°C with 0.15 mg/ml collagenase (Sigma, Type I) in 0.145 M NaCl, buffered at pH 7.0 with 0.06 M phosphate. This procedure loosened the cells and also digested away much of the capsule. The treated epithelium was then disaggregated by flushing 15 times through a hypodermic syringe needle. The separated cells were collected in a siliconized glass centrifuge tube, washed three times in 0.145 NaCl and resuspended in 0.1 ml of saline.

(b) Determination of electrophoretic mobilities

To compensate for inconsistencies due to variations in the purity of the water used in the preparation of the suspending medium, red cells were examined routinely at the start of each experiment with lens epithelium. The mobilities of the epithelial cells were then corrected by reference to the published figure of 1.07 μ/sec/V/cm for the true mobility of red cells (Seaman, 1963).

Since the volume of epithelial cell suspension was so small compared with that of the chamber (3-4 ml), sufficient cells could be examined only if the suspension was introduced directly into the field of view. This was accomplished by means of a hypodermic syringe fitted with a narrow polyethylene cannula.
Cells were timed over 37.5 μ in unbuffered 0.145 M NaCl under a potential of 4.46 V/cm at 25°C (± 0.2°C).
A. Determination of Sorbitol Dehydrogenase in Mouse Livers

The results are shown in Table II and Fig. 7. The two sets of data are not directly comparable since no standard samples of SDH were available for standardization.

The values indicate a considerable variation in sorbitol dehydrogenase activity in the liver, which is not related to genotype. A general increase of activity with age is suggested, but this increase is probably negligible over the range of ages of the mice used in the second experiment.

There is no evidence of any abnormality in the activity of SDH in the livers of Sey heterozygotes in either mutant line.

B. Examination of Seminal Vesicle Secretion

(a) Dry Matter

The dry matter content of Sey & control samples was similar and averaged 29.3% (σ = 1.36) of wet weight.

(b) Protein

The protein content of the samples was similar and represented 29.4% (σ = 3.3) of wet weight, measured against a bovine serum albumin standard.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age, days</th>
<th>Activity, mU/mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Determined Values</td>
</tr>
<tr>
<td><strong>Expt I:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ju</td>
<td>28</td>
<td>3.0, 2.9, 3.1, 3.0</td>
</tr>
<tr>
<td>Ju</td>
<td>77</td>
<td>3.9, 3.9</td>
</tr>
<tr>
<td>Ju</td>
<td>230</td>
<td>4.5, 4.8</td>
</tr>
<tr>
<td><strong>Expt II:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ju</td>
<td>74</td>
<td>5.7, 4.7</td>
</tr>
<tr>
<td>Scy (Jut)</td>
<td>112</td>
<td>6.3, 6.0</td>
</tr>
<tr>
<td>C57Bl</td>
<td>80</td>
<td>6.4, 6.3</td>
</tr>
<tr>
<td>Scy (C57Bl)</td>
<td>107</td>
<td>5.8, 5.9</td>
</tr>
</tbody>
</table>

**Table II** Sorbitol dehydrogenase activity in mouse livers
Fig. 7. Activity of sorbitol dehydrogenase in mouse livers. Variation with age.
(c) Free carbohydrate

Fructose and glucose were identified on both paper and thin-layer chromatograms of the 80% ethanol extract (Figs. 8, 9). A very faint spot with a mobility similar to that of lactose was detected on freshly stained papers, but the sugar was not positively identified.

The concentrations of fructose and glucose are shown in Table IIIa. Each value represents the mean of three determinations. Both sugars are present at lowered concentrations in SSV secretion; fructose levels are down by 7% and glucose by 22%.

(d) Bound carbohydrate

Chromatograms of acid hydrolysates of the material not soluble in 80% ethanol were apparently identical and revealed hexosamine tentatively identified as galactosamine, and five major neutral sugars: glucose, galactose, mannose, fucose and xylose (see Figs. 10, 11). No hexuronic acid was detected. Several minor, low mobility components were detected in both samples when papers were viewed under ultraviolet illumination. These may correspond to small, unidentified peaks which appeared in the oligosaccharide region after separation of hydrolysates of whole SSV on anion exchange resin (see Fig. 12). They probably represent incompletely hydrolysed material. Sialic acid is present in SSV, but at a very low concentration; it was not identified by chromatography.

Quantitative results are shown in Table IIIb. Each value represents the mean of three determinations. The concentration relative to protein of all sugars except sialic acid, is lower in the secretion from mutant mice.
Fig. 8. Diag. of paper chromatogram of ethanolic extract of SVS developed with butanol–ethanol–water (10:1:2). Stained with aniline hydrogen phthalate.
Fig. 9.

Diag. of chromatogram of 80% ethanolic extract of SVS on activated cellulose thin-layer developed twice with formic acid - methyl ethyl ketone - tert-butanol-water (15:30:40:15). Stained with aniline hydrogen phthalate.

Fig. 10.

Diag. of chromatogram of acid hydrolysate of ethanol-insoluble SVS on activated cellulose thin-layer developed twice with formic acid - methyl ethyl ketone - tert-butanol-water (15:30:40:15). Stained with aniline hydrogen phthalate.
Fig. 11. Diagram of paper chromatogram of acid hydrolysate of ethanol-insoluble SVS developed with butanol-ethanol-water (10:1:2). Stained with aniline hydrogen phthalate.
Fig. 12. Profile of elution from anion exchange column, of carbohydrates in acid hydrolysate of whole SVS.
Fig. 13. Elution profile of Sephadex G-50 column.
Protease digest of ethanol-insoluble fraction of seminal vesicle secretion of control or Sey mice.
G: Elution volume of D-glucose.
Fig. 14. Polyacrylamide gel patterns of buffer-soluble fraction of seminal vesicle secretion, control or Sey.

a. Anodal material stained for protein.
b. Anodal material stained for carbohydrate.
c. Cathodal material stained for protein.
### Table IIIa. Free carbohydrates in mouse seminal vesicle secretion

Values are expressed to two significant figures per g protein in whole secretion. Errors were calculated from the variation between replicate determinations.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Concentrations</th>
<th>Difference in Sev</th>
<th>Max. error in any reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>Control: 470 μM, Sev: 440 μM, ±10 μM</td>
<td>-7%</td>
<td>± 2%</td>
</tr>
<tr>
<td>Glucose</td>
<td>Control: 27 μM, Sev: 21 μM, ±1 μM</td>
<td>-22%</td>
<td>± 4%</td>
</tr>
</tbody>
</table>

### Table IIIb. Protein-bound carbohydrates in mouse seminal vesicle secretion

Values are expressed to two significant figures per g protein.

Errors were calculated from the variation between replicate determinations.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Concentrations</th>
<th>Difference in Sev</th>
<th>Max. error in any reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose</td>
<td>Control: 330 μM, Sev: 300 μM, ±9 μM</td>
<td>-9%</td>
<td>± 3%</td>
</tr>
<tr>
<td>Fucose</td>
<td>Control: 31 μM, Sev: 27 μM, ±2 μM</td>
<td>-13%</td>
<td>± 6.5%</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>Control: 124 μM, Sev: 107 μM, ±2 μM</td>
<td>-14%</td>
<td>± 2%</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>Control: 0.76 μM, Sev: 0.79 μM, ±0.02 μM</td>
<td>+4%</td>
<td>± 2.5%</td>
</tr>
</tbody>
</table>
(c) Gel filtration

The elution patterns of samples from control and mutant mice were identical (see Fig. 13). The peak values corresponded to an elution-volume of 27.4 ml.

(c) Polyacrylamide gel electrophoresis

Only one major band of the three major and three minor anodal components stained for carbohydrate. Carbohydrate was not detected in the material which remained at the origin, or in the diffuse cathodal components (see Fig. 14). There was no recognizable difference between normal and mutant patterns.

C. Examination of the Lens Capsule

(a) Quantitative determinations

Molar quantities of carbohydrates relative to hydroxyproline in bovine and normal mouse capsules, and the corrected values for Sey capsules are shown in Table IV. Each value represents the mean of three readings. A correction for non-capsular lens material in the Sey preparation was derived on a dry-weight basis from the values for normal mouse whole lens and normal mouse capsule.

The hexose content of Sey capsules is normal and equivalent to that of bovine capsules. While the normal value for hexosamine in the capsules of mice is higher than that for bovine capsules, there is no evidence that Sey capsules have an abnormal hexosamine content. On the other hand
<table>
<thead>
<tr>
<th>Carbohydrate/g hydroxyproline</th>
<th>Bovine Control</th>
<th>Mouse Control</th>
<th>Sey Capsule</th>
<th>Difference in Sey e.f. control mouse</th>
<th>Maximum error in any reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexose</td>
<td>10.4mM</td>
<td>10.2mM</td>
<td>10.8mM</td>
<td>+6%</td>
<td>± 5%</td>
</tr>
<tr>
<td></td>
<td>(±0.2mM)</td>
<td>(±0.5mM)</td>
<td>(±1.0mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexosamine</td>
<td>2.83mM</td>
<td>3.30mM</td>
<td>3.76mM</td>
<td>+14%</td>
<td>± 20%</td>
</tr>
<tr>
<td></td>
<td>(±0.14mM)</td>
<td>(±0.20mM)</td>
<td>(±0.75mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sialic acid</td>
<td>0.096mM</td>
<td>0.149mM</td>
<td>0.390mM</td>
<td>+182%</td>
<td>± 9%</td>
</tr>
<tr>
<td></td>
<td>(±0.004mM)</td>
<td>(±0.001mM)</td>
<td>(±0.035mM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table IV. Carbohydrates of normal bovine capsules, normal mouse capsules and Sey capsules corrected for non-collagenous carbohydrate.
the salicylic acid content of normal mouse capsules is about 30\% higher than that of bovine capsules and the mutant shows a further increase of 162\%. In the methylpentose assay, replicate determinations were not sufficiently consistent for accurate assessment of fucose levels in samples of the size available.

(b) Gel filtration

The profiles of elution of orcinol-positive-carbohydrate from the Sephadex column are shown in Fig. 15. The elution volumes of the peaks of bovine and control mouse samples were 32.5 ml while the second peak was flattened and retarded to an average elution volume of 34.1 ml. All peaks of excluded material were recorded in all three samples at elution volumes of 14.6 ml.

(c) Electron microscopy

In the control lenses the thickness of the capsule varied from 17\( \mu \) at the anterior face to 3\( \mu \) at the posterior face (Fig. 16). In the four mutant lenses which were examined, the true, discrete part of the capsule was on average only half this width and some capsules showed the normal gradation in thickness from anterior to posterior.

The control capsule consisted of a homogeneous, dense material which appeared fibrous in the outermost region where it was less densely packed. Near the zonule and extending in a posterior direction was a thin layer of loose fibrils scattered around the capsule. The fibrils were 10-15 nm wide and in some areas, in samples stained with uranyl acetate, they showed a cross-banded pattern with a major periodicity of 50 nm (Fig. 7).
Fig. 15. Elution profiles of Sephadex G-50 column.
(I) Bovine capsule digest; (II) Control mouse capsule digest;
(III) Sey mouse capsule digest.

BD = elution volume of Blue Dextran 2000.
G = elution volume of D-glucose.
Fig. 16. Normal mouse lens capsule.
(a) Anterior; (b) posterior.
Fig. 17. Thin striated fibrils near posterior surface of normal capsule.
Fig. 18. Sey capsule showing lamination, normal cross-striated fibrils (SF) and a bundle of thin fibrils lacking striation (TF).
Fig. 19. Mass of fibrils probably of thin striated type associated with the Sey capsule.
Fig. 20. Large, striated fibrils in sex capsule.
Fig. 21. Sery capsule showing fibrils and granules in the capsule, and capsular material penetrating the epithelium.
In general the appearance of the mutant capsules was similar, but in some areas they were less densely packed and occasionally showed laminations (Fig. 18). The lens epithelium and capsule interpenetrated one another in some areas (Fig. 21) and there was a marked increase in the quantity of fibrils outside the true capsule, particularly near the zonele, (Fig. 19). In one instance a group of abnormally large fibrils of diameter 80-140 nm and a major period of 60 nm, was embedded in a matrix of light, particulate material (Fig. 20). In other regions outside the true capsule were bundles of thin, non-striated filaments (Fig. 21), membrane-bounded bodies (Fig. 21) and masses of material of different densities.

D. Examination of glomerular basement membrane efficiency

**Urino Analysis**

Urine was obtained from 65 mice of 100 which were handled. Of these 65, 12 were normal females, 20 normal males, 15 very females and 21 very males. Urine was expelled more readily from males than females and from adults more easily than from young mice.

With the Clinitest test, no urine sample gave a positive reaction for glucose.

Protein content fell within the range zero to approximately 100 mg/100 ml and values were roughly in parallel with a visual estimate of intensity of urine colour. A frequency distribution of protein concentration within each group of mice is shown in Fig. 22. The normal female group included a relatively high proportion of the class which had only trace amounts (5-20 mg/100 ml) of protein. The mice in this class were all sibs of the C3H1 line.
Fig. 22. Protein content of mouse urine. Shaded areas indicate the contribution of the C57Bl genome. (I) Control QQ (12); (II) Control CD(20); (III) Sey QQ (15); (IV) Sey CD(21).
The contribution of the C57Bl genome to each group is therefore illustrated in Fig. 22, but apart from this example, the protein content of urine of C57Bl mice is unexceptional.

E. Tissue Culture of Lens Epithelium

(a) Selection of culture media

The cultures grew well in both media, but growth appeared more vigorous in supplemented Medium 199.

(b) Comparison of normal and mutant cultures

(i) Normal tissue: Normal cultures became established within three days and practically all explants grew well in vitro. At the commencement of outgrowth from the explant, fibroblastic cells moved out onto the plastic. They were usually very flattened and they showed ruffling membrane activity and contact inhibition of movement (Fig. 23). The cells divided and remained well separated until growth resulted in confluence. It was only at this stage that cells began to show any orientation relative to one another. The confluent cells moved away from the explant and when moving in concert they were aligned approximately, parallel to one another and to the direction of movement (Fig. 23). This movement of the cell sheet seemed to be related to ruffling activity which was predominant on the clean plastic. Activity was greater in some regions and led to a distortion of the front of the sheet, so that the outline of a culture was often star-shaped about the explant. A sparsely populated area separated the outward-moving front from the explant. In this area the cells resembled splintered glass, being very
FIG. 23. Cultures of normal lens epithelium. (x200)

(a) Cells on clean plastic showing ruffled membranes.
(b) Cells on "used" surface without ruffled membranes.
(c) Parallel alignment of moving confluent cells.
(d) Epithelioid appearance of stationary confluent cells.
Fig. 24. Still taken from film of eye lens epithelial culture showing spindle-shaped cells. $(\times 80)$

Fig. 25. Still taken from film of eye culture showing wedge of cells being forced beneath cells in its path. $(\times 80)$
Fig. 2.6. Cultures of ey lens epithelium showing overlapping cells.

(×200)
Fig. 27. Still taken from film of double culture showing relative degree of outgrowth from normal (lower left) and eye explants. (×80)
angular in shape, often with concave sides as if under internal tension, while remaining anchored to the substratum at only a few points (Fig. 23). Confluence in this region was eventually achieved by an increase in the mutual area of adhesion between cells, and stretching of the cells. Cells became more epithelioid in character as confluence in this region was approached. The confluent culture had a generally smooth appearance (Fig. 23).

Cultures were maintained for more than three weeks in vivo, but during this period there was no evidence of differentiation into lens fibres or of secretion of fibrous material. Evidence of normal cells moving over or beneath one another in culture was never seen.

(ii) Mutant tissue: In general Sey explants took a longer time to become established and about a quarter failed to produce vigorously growing cultures.

The pattern of outgrowth from Sey cultures showed several peculiarities, but these were not seen in every Sey culture nor in all parts of any one culture.

The Sey cells which first grew out onto the plastic surface were generally more closely bunched and in the early stages of outgrowth some cells were seen which were long and spindle-shaped. The latter cells did not adhere well to the dish, or show marked ruffling membrane activity, but tended to form criss-crossed, disorderly masses (Fig. 24). Elongated heaps of overlapping cells were seen in several Sey cultures apparently at regions where streams of cells had converged (Fig. 24). These appeared usually in a generally radial pattern relative to the explant. Overlapping cells could also
be seen in other areas.

The tendency of cell sheets to migrate away from the explant was not pronounced in Sey cultures. A sparsely populated zone around the explant was therefore seen only occasionally, this region being occupied in some Sey cultures, by a thick heap of cells (Fig. 25). The movement of Sey cells was possibly less active than normal and the film showed a spectacular growth of cells which originated on the explant and pushed forwards among the cells packed together on the dish surface. This wedge of cells appeared to creep beneath elongated stationary cells lying perpendicular to its path. The stationary cells became forced away from the dish (Fig. 25) and their attachment to the dish was eventually broken at one end so that one by one they snapped back out of the path of the moving mass.

(iii) Interaction between normal and mutant tissue.

There was definite evidence of physiological interaction between normal and mutant tissues when cultured together in the same dish. When normal and Sey explants were together placed in the same dish, although the normal cultures became established within the usual time, the mutant explants did not adhere to the plastic or show much evidence of growth. When normal explants were introduced into dishes containing established Sey cultures, the growth of the normal tissues was at least as vigorous as normal, but that of the Sey cultures appeared to be depressed (Fig. 27).

The film showed that inhibition of movement was a feature of contact between all combinations of pairs of cells. However, because of their superior rate of growth, the normal cells succeeded in forcing back the Sey cells and establishing a confluent sheet composed largely of normal cells, on
areas previously occupied only by \textit{Sey} cells.

\textbf{F. Whole Cell Microelectrophoresis}

When freshly disaggregated and washed lens epithelial cells were examined, it was found that the majority of cells were stationary, while some moved towards the anode and some towards the cathode. In older preparations positively charged cells were not detected and many more cells showed negative mobility. In preparations examined 2–3 h after washing practically all cells showed negative mobility. Figure 29 shows the mobility of the fastest cells in a \textit{Sey} lens epithelial preparation, plotted against the time elapsed since the commencement of the electrophoresis run. It can be seen that the mobility of the fastest cells reached a plateau value about 40 min after the commencement of the electrophoresis run. The cause of this change in mobility was not investigated but in all later experiments only the (negative) mobility of the cells from the plateau region were included in the frequency distributions or used as a basis for calculation.

The results of three separate experiments with pooled normal cells and three with pooled \textit{Sey} cells are represented as frequency distributions in Fig. 29 and Fig. 30. The combined data are shown in Fig. 31. Statistical values calculated from the combined data are shown in Table V.

The values derived from the combined data for normal and for mutant samples are superficially rather similar. The most obvious difference between the two populations is in the reproducibility of values, the frequency
Fig. 28. Change of mobility of lens epithelial cells with age of preparation. Cells were suspended in 0.145 M NaCl and timed over 37.5 μ at 4.46 V/cm and 25 °C.
Fig. 29. Frequency distribution of times taken by control lens epithelial cells to travel 37.5 μ at 4.46 V/cm. Three separate determinations.
Fig. 30. Frequency distribution of times taken by Sey lens epithelial cells to travel 37.5 μ at 4.46 v/cm. Three separate determinations.
Fig. 31. Frequency distribution of times taken by lens epithelial cells to travel 37.5 μ at 4.46 v/cm. Combined values from I. Three experiments with control cells, II three experiments with Sey cells.
Table V. Analysis of data from electrophoresis of lens epithelial cells

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>Scy/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of experiments</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total no. of cells timed</td>
<td>240</td>
<td>260</td>
</tr>
<tr>
<td>Modal class (sec)</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>Mean (sec)</td>
<td>10.49 (σ=3.17)</td>
<td>12.02 (σ = 3.74)</td>
</tr>
<tr>
<td>Median (sec)</td>
<td>9.72</td>
<td>11.39</td>
</tr>
<tr>
<td>First quartile (sec)</td>
<td>8.29</td>
<td>9.04</td>
</tr>
<tr>
<td>Quartile deviation (sec)</td>
<td>1.95</td>
<td>2.60</td>
</tr>
<tr>
<td>Pearson coefficient of skewness</td>
<td>0.73</td>
<td>0.50</td>
</tr>
<tr>
<td>Mean mobility (μm sec⁻¹ v⁻¹ cm⁻¹</td>
<td>0.80 (σ=0.27)</td>
<td>0.70 (σ = 0.24)</td>
</tr>
</tbody>
</table>

Distance travelled: 37.5 microns; potential: 4.46 v cm⁻¹; medium: 0.145M NaCl; temperature: 25.0 ± 0.2°C.
distributions of the individual Sey preparations being less consistent than normal. This lack of consistency is expressed in the larger quartile deviation of the combined Sey results. The quartile deviation is a more relevant measure of these data than the standard deviation, since the latter statistic is very much affected by extreme values. The mean, median and first quartile times are all higher for Sey cells, but the modal class is 8-9 sec in each case. The normal population is obviously more skewed than the Sey and this difference is expressed in the Pearson coefficient of skewness, Sk.

\[
Sk = 3 \frac{\text{mean} - \text{median}}{\text{standard derivation}}
\]
CHAPTER 6

ANALYSIS OF THE BREEDING DATA

Detailed data concerning the characteristics of genetic mutants can be considered reliable only if the effects of other genes are excluded. It was therefore decided to isolate the *s ey* allele on a homozygous background so that it could be compared directly with its normal allele against the same background. The simplest way to achieve this situation is to backcross the gene to an established inbred line. Three easily distinguishable inbred lines were selected for this purpose: *Ju* (white), *C57 Black* and *Jb* (agouti with a white belt). The first matings with each line were carried out by the same three male sibs of the *XSF* inbred strain (agouti and small eyes).

Heterozygous *s ey* offspring were identified by eye size and/or lens cataract at the age of three weeks. Identifications were checked at six weeks when male or female mice which expressed the phenotype well were selected for further breeding. Breeding cages normally contained two females and one male. If three or four females were placed in the same breeding cage there were frequent losses of young. Young mice were also eaten or neglected in cages which were disturbed during the first week after the birth of a litter.

A: Comparative effects of different genetic backgrounds

Differences in the breeding capacity of the three strains were soon apparent. *Ju* and *Jb* bred readily and mating did not appear to be affected by overcrowding. On the other hand *C57 Bl* stocks did not reproduce so readily
although breeding performance was increased if the mice were isolated in pairs. On several occasions the breeding performance of the whole C57Bl colony went down for unaccountable reasons.

The *Sey* phenotype was expressed to different degrees on the three backgrounds, there were differences in both the expressivity and penetrance of the *Sey* gene. In mice which were basically of *Ju* genotype the *Sey* gene often produced very small eyes, but lens cataract was not so easily detected due to the lack of pigmentation of the eye. On the CB7Bl background there were fewer very small eyes, but cataractous lenses were obvious in the darkly pigmented eyes. The C57Bl strain occasionally produced one or two very small offspring in a litter of normal-sized mice. At six weeks of age these small mice resembled their sibs at half that age. This effect was not associated with expression or non-expression of the *Sey* phenotype.

The *Sey* phenotype was not well expressed on the JFt background, and as the percentage of *JFt* genes in the background increased, it became progressively more difficult to distinguish *Sey* mice from their normal sibs. Not only were the eyes of *Sey* (*JFt*) mice fairly normal in size, but their lenses were also clear. Many litters were eaten by *JFt* or *Sey* (*JFt*) parents and casualties among adults were also relatively high.

The existence of occasional individuals among the *JFt* inbred stocks (which had not been mated with *Sey* mice) with some characteristics similar to those of *Sey* mice, was brought to my notice by Miss Helen Tait to whom I am indebted for the following information: The affected mice are very small and have eyes which are small in proportion to their bodies, their coats are
often pale and their heads asymmetrical. They are subject to "fits" which resemble epileptic fits and they die usually within four weeks of birth. The mother of these mice develop sores on their tails after giving birth to several litters. All affected mice are descended from one individual.

Since the phenotypic effects of the \textit{Sey} gene on the \textit{JBe} background were likely to be masked by other features of the \textit{JBe} strain, backcrossing of the \textit{Sey} gene to this line was discontinued after five generations.

Since mice of the \textit{Jk} line reproduce most abundantly and the \textit{Jk} genome favoured the expression of the \textit{Sey} phenotype better than the other two, this line was selected as the most suitable for provision of defined mutant and control material. The breeding programme with the \textit{C57Bl} line was also continued in case the \textit{Jk} background proved unsuitable for some other reason.

3. Penetration of the \textit{Sey} gene

Roberts (1967) records that the \textit{Sey} gene has variable expressivity even within one individual and this observation was confirmed in the present work. This fact indicates that there is some influence on the expressivity of the gene which is not a straightforward expression. Furthermore, since apparently normal parents may give rise to \textit{Sey} progeny, the gene can be regarded as incompletely penetrant (Roberts, 1967). The effects of different genetic backgrounds on the expression of the \textit{Sey} phenotype are described above, but in this section a quite different phenomenon is examined, \textit{viz.} the modification of expression of the \textit{Sey} phenotype by the parental genome.

If a mouse heterozygous for a dominant mutation is mated with a normal homozygote, half the progeny should be normal and half should express
the mutant phenotype. The ratio of phenotypically Sey to phenotypically normal mice in the progeny of the C57Bl breeding programme are:

\[ \frac{158}{178} \] Sey: 178 normal

This ratio is obviously close to 1:1, and the probability (P) of such a ratio occurring as merely a sampling error in a population which is in fact an equal mixture of both types can be calculated by applying the \( X^2 \) test. In this example \( P = 0.26 \), i.e., in a hundred samples taken from a population containing equal proportions of two types, a deviation from the expected 1:1 ratio as great as, or greater than the deviation of this sample, could occur in 26 samples due to chance alone.

In the C57Bl breeding programme, the Sey gene was introduced by either parent in approximately equal numbers of litters. The figures can be divided according to maternal genotype and when this is done we find a new pattern of ratios emerging. The following figures were obtained:

Mother C57Bl: 66 Sey; 95 Normal; \( P < 0.02 \)

Mother Sey (C57Bl): 92 Sey; 63 Normal; \( P = 0.48 \)

It can be seen that the proportion of mutant mice born to Sey (C57Bl) mothers is in agreement with the expected ratio of 1:1, but the ratio born to normal C57Bl mothers is improbable on Mendelian theory, there being more offspring than expected.

Unless the Sey gene affects the viability of young mice in the heterozygous condition as well as in homozygotes, this pattern would normally be considered to indicate that the gene is incompletely penetrant on the C57Bl background when the mother carries only the normal allele at the Sey locus,
but completely penetrant when she carries one copy of the mutant allele.

The equivalent total figures for the \textit{Ju} breeding programme are shown below. In this programme the \textit{Sey} gene was always carried by the mother except in the first few matings, the breeding figures for which are not included in these values.

Mother \textit{Sey (Ju)}: 414 \textit{Sey} ; 347 normal; \( P < 0.02 \). Here there is an excess of mutant offspring which is so large that it should not occur by chance in even 2\% of samples. The figures clearly indicate that on a background which is basically \textit{Ju}, the presence of the \textit{Sey} gene in the mother is associated with a higher proportion of phenotypically \textit{Sey} offspring than was expected.

In this case the concept of penetrance is not applicable since the \textit{Sey} phenotype was expressed in mice which, on the basis of Mendelian theory were not expected to carry the \textit{Sey} gene. An explanation of this situation could be based on the theory that normal foetuses or young mice are less viable than their mutant sibs, but this is unlikely since the \textit{Sey} gene is lethal in the homozygous embryo.

Unfortunately insufficient matings have been carried out between \textit{Sey} males and normal \textit{Ju} females to examine the effect of a normal \textit{Ju} maternal genome on the proportions of normal and mutant mice in the offspring.

In both the \textit{C57Bl} and the \textit{Ju} programme, matings were always carried out between effectively completely homozygous members of the inbred strains, and phenotypically \textit{Sey} mice containing 50\% or more of the
Inbred strain genome, which expressed the Ey phenotype to a very marked degree. No genetic analysis was carried out on the mice which expressed the Ey phenotype in a mild form, or on the normal sibs of Ey mice. We therefore have no reliable genetic evidence for, or against the hypothesis that the Small Eye phenotype is not necessarily associated with the Ey gene in that individual. However the evidence suggests that expression of the Ey phenotype is a function not only of the genome of that individual, but also of the genome of the mother.

**C. The effect of maternal age on expression of the Ey phenotype**

Since maternal physiology seems to bias the phenotype of the offspring, age of the mother might also be expected to have some influence.

Of the offspring born to C57Bl mothers, more than half were apparently normal and the deviation from the expected 1:1 ratio was statistically significant. When the numbers of Ey and normal offspring per litter* born to normal C57Bl mothers, are independently plotted on the 'y' axis against maternal age on the 'x' axis, the two lines of regression of y on x are approximately parallel and decline steeply with maternal age, (see Figs. 32, 33, and Table V). In the straight-line equation,

\[ y = a + bx \]

for normal young, \( a = 4.536; b = -0.059; S_b = 0.024 \),

for Ey young, \( a = 3.793; b = -0.066; S_b = 0.020 \)

\((S_b = \text{standard error of } b)\)

* In some cases, litters born to female sibs in the same breeding cage became mixed. These young were recorded as if they belonged to the same litter. For the purposes of statistical analysis, litters recorded as exceeding ten mice were regarded as two equally proportioned litters.
There is no evidence whatsoever that the slopes (b) of the two lines are different ($P < 0.30$, see below). This pattern is what might be expected on the theory that the *Sey* gene is incompletely penetrant.

The offspring of *Sey (C57Bl)* mothers mated to *C57Bl* males were equally distributed between the *Sey* and normal phenotypic categories. However, when the numbers of each phenotype were plotted against maternal age, an unexpected pattern emerged (see figs.33, 34). The number of phenotypically *Sey* mice born to young mothers was apparently higher than the number of normal young, while in the litters of old mice the situation was reversed. The regression coefficients (b) were -0.044 for normal and -0.048 for *Sey* young. Only at a mean maternal age of 36-37 weeks was the 1:1 ratio upheld in accordance with Mendelian theory. However, the sample size was relatively small (31 litters) and the standard errors of 'b' were correspondingly large (0.020 and 0.014 respectively). Therefore although the graphs are suggestive of some difference in the distribution of the two phenotypic categories with respect to the age of the mother, the distinction cannot be regarded as statistically highly significant ($P = 0.17$, see below). However considerable supportive evidence that this pattern indicates the real situation is provided by the equivalent figure for the litters of *Sey (Ju)* mothers.

The litters born to *Sey (Ju)* mothers contained in toto a very significant excess of phenotypically *Sey* young and this phenomenon cannot easily be explained in terms of the conventional concept of penetrance. When plotted against maternal age we see that *Sey* young are predominant only in the litters of young mothers, whereas in the litters of older mothers the ratio is inverted (see Figs.34, 35). The pattern is in fact very similar to that
Fig. 32. Phenotypic composition of litters, variation with maternal age. (I)
Fig. 33. Phenotypic composition of litters, variation with maternal age.
Fig. 34. Phenotypic composition of litters, variation with maternal age.
Fig. 35. Phenotypic composition of litters, variation with maternal age.

- Mother C57 BI
- Mother Sey(C57 BI)
- Mother Sey(Ju)

Summary of results.
Phenotypically normal young; phenotypically Sey young.
described for the litters of *Sey (C57Bl)* mothers. In the *Sey (Ju)* example the regression lines cross at a maternal age of 33.94 weeks. The number of litters examined was 109, the values of $b$ are -0.008 and -0.060, and the standard errors are 0.010 in each case. The difference in the slopes of these lines is significant at the 0.1% level of confidence (see below) which indicates a real change in the composition of the litters as maternal age advances.

The evidence of the *Sey (Ju)* mothers therefore provides very strong support for the possibility that the pattern described with *Sey(C57Bl)* mothers does resemble the true situation, although taken alone the *Sey (C57Bl)* data is inconclusive.

D. The significance of differences in regression coefficients

A test for the significance of a difference between two regression coefficients is described by Mather (1943). In this test the difference 'd' is compared with its standard error by a 't' test. The standard error ($S_d$) is found by taking the square root of the sum of the variances of the two coefficients.

\[ S_d = \sqrt{(Sb_1)^2 + (Sb_2)^2} \]

\[ d = b_1 - b_2 \]

and \[ t = \frac{d}{S_d} \]

The number of degrees of freedom (N) is equal to the sum of the numbers available for the estimation of $Sb_1$ and $Sb_2$. In this case $N$ is double the number of litters born to mothers of each class. $P$ is determined from tables of $t$. 
<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>No. of litters</th>
<th>Total no. offspring (phenotype)</th>
<th>p (ratio)</th>
<th>Intercept a</th>
<th>Regression coefficient b</th>
<th>Standard error of b</th>
<th>p (regression coefficients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57B1</td>
<td>35</td>
<td>normal: 95 Sey: 66</td>
<td>&lt; 0.02</td>
<td>4.538</td>
<td>-0.069</td>
<td>0.024</td>
<td>&gt; 0.90</td>
</tr>
<tr>
<td>Sey (C57B1)</td>
<td>31</td>
<td>normal: 83 Sey: 92</td>
<td>0.48</td>
<td>3.033</td>
<td>-0.014</td>
<td>0.020</td>
<td>0.17</td>
</tr>
<tr>
<td>Sey(Ju)</td>
<td>109</td>
<td>normal: 347 Sey: 414</td>
<td>&lt; 0.02</td>
<td>3.063</td>
<td>-0.005</td>
<td>0.010</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table VI. Analysis of breeding data from Sey/+ X +/+ cross
By this method the significance of \( d \) was calculated for the three pairs of regression coefficients. The difference in the slopes of the pairs of lines was highly significant in the case of \( \text{Sey}(\text{Ju}) \) mothers (\( P = 0.001 \)), but not for \( \text{Sey}(\text{C}3\text{H}/\text{He}) \) (\( P = 0.17 \)) or \( \text{C}3\text{H}/\text{He} \) mothers (\( P > 0.90 \)).

Summary

This analysis of the phenotype composition of litters born to mice carrying the \( \text{Sey} \) gene provides a new insight into the basis of thresholds of expression with respect to both penetrance and expressivity of the \( \text{Sey} \) gene.

Penetrance and expressivity were both dependent on genetic background. Among mice which were basically \( \text{Ju} \), cataracts lenses and small eyes were detectable in fewer individuals than in stocks which were basically \( \text{Ju} \) and the abnormalities of the former mice were less severe than those of the latter.

Phenotype was also related to maternal genotype. Fewer small-eyed mice were born to normal mothers than to \( \text{Sey}/+ \) mothers and in both mating systems deviations from the expected even ratio were significant. In litters born to \( \text{Sey} \) mothers, there was an association between the phenotypic composition of the litter and the age of the mother, young mothers produced an excess, and old mothers a deficit, of phenotypically \text{Small-Eye} young.

A possible explanation of these results is presented in the "Discussion" section.
Comparisons between the effects of a mutant gene and its normal allele can be considered valid only if all other parameters are eliminated. The simplest way of isolating a dominant gene and its normal allele on the same genetic background is by repeated backcrossing of heterozygotes to a completely homozygous stock. If a dominant gene is transferred by successive backcrosses to an inbred line, after 't' crosses, the mean length of chromosome introduced with the gene is approximately $\frac{100}{t}$ centimorgans (cM) on each side of the gene, or $\frac{200}{t}$ cM altogether. The total map length of the twenty chromosomes of mice is approximately 2000 cM, so $\frac{200}{t}$ cM represents $\frac{10}{t}$ percent of the total chromatin. The proportion of chromatin not associated with this gene, which is still expected to be heterogeneous i.e. which was present in the first mutant, is given by $1 - F$,

$$ F = \frac{1}{2} + \frac{1}{2} \frac{2}{2} + \frac{1}{2} \frac{3}{2} \ldots \ldots + \frac{1}{2} \frac{t}{2} $$

(see Falconer, 1964).

The Sey gene was crossed onto three different backgrounds (see p. 112). Seminal vesicle material and S.D.H. extracts were from mice which were the product of five backcrosses to the Ju strain. The chromatin of these mice
would be expected to contain 95% of the Ju genome, while the other 5% included the Sey gene and its normal allele. Cell electrophoresis and tissue culture experiments were carried out with material from mice which were the issue of eight or nine backcrosses and which should therefore contain the Sey locus with 98-99% of the Ju genome. In view of the deductions made concerning the maternal modification of Sey phenotype (see p. 142) it should be stressed that in the latter experiments the mutant material was from individuals which expressed the phenotype to a very marked degree, and were almost certainly true heterozygotes. The control animals were their phenotypically normal sibs.

Lens capsules were required in large quantities for the biochemical analysis and were collected from any mice which expressed the Sey phenotype regardless of genetic background. Control capsules were collected from mice of various strains, but never from lines which were known to carry genes for eye disorders, or any other major abnormality.

**B. Sorbitol Dehydrogenase Determinations**

The assay of sorbitol dehydrogenase activity in mouse livers revealed a considerable variation between mice of the same genotype, but no features were revealed which were peculiar to Sey mutants.

The liver is the best source of SDH and it would be expected that a defect in the activity of SDH in other organs would be expressed in the liver. In the absence of such a defect we must look elsewhere for an explanation of the causes of lens cataract and the other abnormalities associated with the Sey gene.
The composition of mouse seminal vesicle secretion (SVS) has not previously been determined, but Mann (1954, 1964) and Mann & Lutwack-Mann (1958) described the composition of SVS's of related species.

Electrophoretic variants of mouse SVS proteins in a different electrophoretic system are reported by Flatz & Wolfe (1969). In contrast to the present results, these authors failed to detect a PAS-positive fraction.

Free fructose is present in mouse SVS at a concentration of 280 mg per 100 g fresh whole organ (Hestirin & Salmen, 1951) and 458 mg per 100 g wet weight of secretion (Fouquet, 1971). The present results are equivalent to 250 mg fructose and 14.2 mg glucose per 100 g wet weight of normal secretion. The unidentified free sugar was also reported, but not identified, by Fouquet (1971).

The results suggest an association of the Sex gene with a slight, but general reduction in the ratio of both free and bound carbohydrate to SVS protein. The difference is not uniform for all carbohydrate species. There is a significant decrease in hexose and hexosamine, the average values for fucose differ by the summed range of experimental variation while the sialic acid content is essentially unchanged.

Although only one band, separable by polyacrylamide gel electrophoresis, stained for carbohydrate, the variety of bound sugars present suggests that mouse SVS contains more than one type of carbohydrate chain bound to protein. It is impossible on the evidence available to determine whether one or more of these chains is deficient in the mutant secretion.
There is no evidence that the average size of the carbohydrate chain is greatly affected, since the orcinol-positive units released by protease from the normal and mutant material were eluted from the Sephadex column in a similar pattern. The possibility remains that orcinol-negative carbohydrate (e.g. hexosamine and sialic acid) is eluted separately from the bulk of the carbohydrate, but we have no evidence on this point.

The seminal vesicle basement membrane cross-reacts immunologically with the lens capsule (Midgley & Pierce, 1963), which is grossly abnormal in Sey mice. Possibly the slight abnormalities in the production of free sugars and glycoprotein by Sey mice are a result of slight differences in the structure of this membrane.

The function of the SVS is to form a vaginal plug after coitus, which prevents sperm loss and may stimulate the anterior pituitary of the female (Marshall, 1956). The clottable component of guinea pig SVS is a glycoprotein (Notides & Williams-Ashman, 1967). Despite the deficiencies in the carbohydrate of Sey SVS, the mutant is capable of forming an effective vaginal plug.

The properties of Sey SVS are not detectably very different from normal and its analysis gives little indication concerning the basic biochemical lesion of Sey mice. However, of the carbohydrate species which were assayed, it is noteworthy that the levels of sialic acid alone were normal. The ratio of sialic acid to other sugars is therefore considerably increased in the mutant secretion. This increase is of the order of 17% relative to fucose, or 15% relative to hexose plus hexosamine.
Those results therefore lead us to consider that the \textit{Sey} gene may possibly have some effect on the synthesis of sialic acid or its incorporation into glycoprotein, or on the production of a minor component which contains sialic acid. This very tentative conclusion is given considerable support by the figures derived by analysis of the lens capsule.

D. The Lens Capsule

(i) Biochemical Analysis

In this experiment the bovine lens capsule was included as a control since its composition is known (Dische & Zelments, 1966; Fukushi & Spiro, 1969; Spiro and Fukushi, 1969), but the published results are not directly comparable since one group examined only the polar parts of the capsule while the other group subjected their material to extraction procedures. By analogy with the work of Spiro & Fukushi (1969) the major carbohydrate peaks recorded in the column effluent should contain sialofucosylglycan material linked to asparagine and glucose-galactose disaccharides linked to hydroxyllysine. The peak produced by \textit{Sey} material is retarded and flattened suggesting either an excess of disaccharide, or a large number of short heteropolysaccharide chains. The relative concentrations of sialic acid and hexose in the \textit{Sey} material support the latter explanation i.e., the \textit{Sey} capsule contains heteropolysaccharide units which are smaller than normal and have a much higher sialic acid content. However, it should be pointed out that we have no direct evidence concerning the position of sialic acid or hexosamine in the column effluent since these carbohydrates do not form products which absorb at 420 nm with orcinol under these conditions.
We cannot exclude the possibility that grossly defective *Sey* lenses included in the sample may contain contaminants of extralenticular material, but on the basis of histological examination of *Sey* eyes, it may be concluded that such material would make a negligible contribution to the determined values (Clayton, personal communication). Since it is impracticable to separate very defective *Sey* capsules from their lenses, it was necessary to assume that the carbohydrate composition of *Sey* non-collagenous lens material is the same as that of normal mouse lens non-collagenous material. Histochemical examination shows that most of the carbohydrate of normal lenses is in the capsule or, in *Sey* lenses, in deposits around lens epithelial cells. The percentage composition of sialic acid in the *Sey* preparation was fifteen times the calculated value for normal capsulot lens, so any errors introduced by these assumptions are unlikely to affect the major finding that sialic acid levels are appreciably increased in the *Sey* lens as a whole, and that the major part of this increase is probably represented by capsular components.

Sialic acid and fucose in glycoproteins tend to be reciprocally interrelated since both occupy terminal positions in side chains. No previous analyses of normal basement membranes of mice could be found in the literature, but the normal ratios of sialic acid to fucose in the lens capsule and Descemet's membrane of other mammals are in general lower than those in alveolar, glomerular and choroid plexus basement membranes, or in tendon collagen (Dische & Zelmenis, 1966; Fukushi & Spiro, 1969; Kefalides, 1969, 1970; Spiro, 1970). In *Sey* mice the former membranes are defective, but
the latter group are not known to be structurally abnormal although thorough investigations have not been carried out. An increase in the sialic acid content of a membrane in which this ratio is normally low might be expected to exert a more profound effect than in a membrane where the ratio is already high.

(ii) Electron Microscopy

The typical banded appearance of collagen is not revealed in electron microscope pictures of normal lens capsule or other basement membranes. This is because typical collagen fibres fail to form since the orderly aggregation of tropocollagen monomers is inhibited by the large amount of carbohydrate attached to the protein backbone (Spiro, 1969b). In collagen of low carbohydrate content, cross links are formed between unsubstituted lysine and hydroxyllysine residues or their derivatives (Robins, Shimomokami & Bailey, 1973) on adjacent chains, but in basement membranes such as the lens capsule, many of these sites are occupied by neutral hexose. In addition, asparagine-linked heteropolysaccharide chains are believed to obstruct the layering of sheets of collagen fibres (Spiro, 1969b).

Around the outside of the normal mouse capsule are scattered thin, banded fibrils with a major periodicity of 50 nm. Cohen (1965) has observed fibrils, with a 67 nm periodicity, around the human lens capsule. If these fibrils are some form of collagen, since they have a banded pattern they are probably of low carbohydrate content. Mutant lenses were characterised by a marked excess of these fibrils.

The banding pattern of the exceptionally wide fibres in the mutant capsules (fig. 20) resembles that of native collagen of low carbohydrate content.
The presence of these fibres within the capsule therefore provides further evidence of a defect in the glycosylation of collagen associated with the *Sey* gene.

Cross-striated collagen fibres have been observed in human lenses with anterior polar cataract (Pau & Caesar, 1967). Henkind and Prose (1967) described a case in which the opacity was caused by a plaque of collagen fibres between the lens epithelium and the capsule. The fibres were 28 nm wide and as in the *Sey* capsule, had a banding period of 60 nm. The plaque contained spindle-shaped cells considered to be lens epithelial cells which had undergone metaplasia. The subjacent epithelium showed proliferation and there was generalised cortical degeneration. The details of this example are very similar to those of *Sey* lenses except that the opacity of *Sey* lenses does not appear to be due solely to collagen *per se* (see p. 127).

In some areas *Sey* mutant capsules had a laminated appearance unlike that of normal capsules. Cohen (1961, 1963) describes a laminated structure of the capsule in normal mouse and human lenses. The discrepancy between Cohen's observations and the present ones probably arises from the use of different fixation procedures (M. Perry, personal communication).

In some areas, the mutant capsules appear less densely packed than normal and typically there are fibrils of various diameters within the capsule and scattered about it. Schofield, Freeman and Jackson (1971) have shown that the diameter of collagen fibrils depends on their carbohydrate content.

We therefore have two lines of evidence arising from this work...
which support the previous conclusion that the *Sey* gene affects the glycosylation of collagen. These are the sporadic occurrence of banded fibres and the variation in the diameter of fibres in mutant capsules.

In the cartilage of the chondrodystrophic (cho) mouse mutant are abnormally wide collagen fibres, with a cross-banded pattern not usually seen in cartilage collagen. In this tissue, collagen is normally associated with a Seagmillar, large amount of acid mucopolysaccharide. Fraser & Sheldon (1971) suggest that this acid mucopolysaccharide is depleted in cho mice. On the other hand, in the *Sey* mouse lens capsule the excess of sialic acid suggests that it is the glycosylated collagen or other sialofucoglyco protein component (Dische & Murty, 1973), rather than the mucopolysaccharide, which is abnormal. The abnormality of Descemet's membrane in *Sey* mice supports this conclusion since Descemet's membrane contains no mucopolysaccharide (see p. 58).

(iii) The Basis of Lens Cataract

Several hypotheses may be advanced in an attempt to account for the lack of transparency of *Sey* lenses. Physical collapse of the lens and the existence of optical inconformities due to thickened fibre membranes and fluid spaces within the lens must have a major effect (Philipson, 1973). Structural abnormalities in the capsule may also have a large effect (see p. 29), but the most frequently cited cause of lens opacity is the precipitation of proteins which are normally retained in solution (Barber, 1973).

For normal vision, the cation concentration of the lens must be kept sufficiently high to ensure solubilization of the lens proteins (Fruton &
Simmonds, 1963), but not so high relative to that of the surrounding fluid, that the lens swells due to osmotic uptake of water. Hydration & swelling of the lens are frequent major factors in cataract (see Barber, 1973) and may arise due to disruption in the balance of inorganic diffusible ions (Duncan & Croghan, 1969), or accumulation of non-diffusible sugar alcohols (Van Heyningen, 1971). In the Sey lens, there are swollen fibres with fluid spaces between them (Clayton & Campbell, 1968), but the lens as a whole is generally soft or collapsed, as if the osmotic pressure approached that of the surrounding fluids, or the semi-permeable containing membranes have broken down.

The bovine capsule carries a net positive charge (Spiro & Fukushi, 1969). By comparison it can be shown that the sialic acid in Sey capsules probably reduces the normal positive charge by about 5%. Sialic acids tend to be prominently exposed, so the effective charge reduction may be considerably greater than this. Furthermore the protein content of Sey lenses is typically reduced (Clayton & Campbell, 1968). The combined effect should be to reduce the potential difference across the lens surface and so depress the Gibbs-Donnan cation-attractive forces, and the passive intake of diffusible ions would be correspondingly lowered. This situation probably contributes to the lack of turgor in Sey lenses.

The active transport of cations in other systems is influenced by sialic acid on the cell membrane (Emmelot & Bos, 1965; Glick & Githens, 1965) and in the lens, by wrinkling of the lens capsule (Harris & Gruber, 1963). There is therefore good reason to suppose that active transport of cations in Sey
lenses may be disrupted although this has not been established.

The temporal and topological distribution of sodium in the normal lens parallels that of "albuminoid" (Paterson, 1970) which contains insoluble \( \alpha \)-crystallin (Weley, 1969) and glycoprotein associated with cell membranes (Dische, 1965). Paterson (1970) suggested that the fraction of the total sodium which is non-exchangeable is bound intracellularly to albuminoid within the lens nucleus. Paterson did not consider the role of membrane glycoproteins, but another possibility is that glycoprotein in the lens fibre membranes may affect the distribution or exchangeability of \( \text{Na}^+ \) ions within the lens. Clayton & Campbell (1968) reported that the lens fibre surface membranes are sometimes thickened in Sey lenses. Such abnormalities may contribute to water imbalance through disruption in the distribution of non-exchangeable \( \text{Na}^+ \) ions.

Glycoprotein abnormalities in the Sey eye may therefore affect the distribution of non-exchangeable cations within the lens and also modify both the passive and active transport of cations into and out of the lens. All of these factors could contribute to the breakdown of water balance in the lens with consequent precipitation of lens proteins.

(iv) Developmental Aspects

The eye begins development as the product of reciprocal inductive interaction between outpushings of the diencephalon (the optic vesicles) and the overlying ectoderm (see Coulombre 1965). Interaction between the two ectodermal tissues is necessary for the differentiation and development of both retina and lens, while other eye structures develop as a result of interaction with mesoderm. The ectoderm is influenced to differentiate towards lens by
successive interactions with the underlying endoderm of the foregut and with portions of the presumptive heart mesoderm, but the tip of the optic vesicle plays the final role in inducing lens from ectoderm and in aligning the lens precisely with the rest of the eye. During the inductive period, a material containing carbohydrate develops between the two tissues and radioactive label, introduced as amino acids into the optic vesicle, can be transferred to lens at this stage.

The lens originates as an invagination of the ectoderm and the lens cells differentiate into fibres under the influence of the neural retina except for a layer on the ectodermal side which forms the lens epithelium. The lens becomes completely invested within the ectoderm basement membrane, the precursor of the lens capsule, which continues to be built up by the synthetic activity of the lens epithelium.

The size and shape of the lens are controlled by regulatory factors which control the number, size and shape of the lens cells. These factors are of two types: retinal factors which promote growth and differentiation, and lens inhibitory factors arising from the lens itself (Coulombre, 1965). There is a strong correlation between size, and rate of increase of size of the lens, and retinal surface area. It has been shown that eye size is controlled to some extent by the size of the lens (Harrison, 1929; Coulombre & Coulombre, 1964).

It is difficult to explain the failure of eye development in Small Eye heterozygotes beyond stating that there are defects in both retina and lens which may mutually inhibit the normal development of the other. Irregularities in eye cup formation may be due to disruption of water regulation in the brain (Clayton, personal communication). At the site of lens induction two basement
membranes separate the interacting tissues and these membranes may be continuous at the neuropore (Cohen, 1961). Any materials passing between optic vesicle and ectoderm must pass through these two basement membranes. Examination of these membranes and the carbohydrate produced between optic vesicle and ectoderm during induction might reveal defects in permeability or charge relevant to transfer of materials between the two tissues. Both retina and lens are of ectodermal origin, but Descemet's membrane of the cornea is also defective in Sey mice and this develops apparently due to the activity of the corneal posterior epithelium which is of mesenchymal origin (Coulombre, 1965). Even within the early embryo the enzyme defect must be expressed in several tissue types (see p. 55).

Hyperproliferation of Sey lens epithelium is probably encouraged by wrinkling of the capsule (Bito & Harding, 1965; Duke-Elder, 1969; Srinivasan & Harding, 1965). In Cataract Fraser (Cat FR), overproduction of capsule is associated with hypertrophy of lens epithelium following degeneration of the lens nucleus (Zwaan & Williams, 1969). Hypersecretion of collagenous material in both mutants may reflect the increased rate of proliferation of the secreting cells (Manner, 1971).

The biochemical analysis and electron microscopic examination of the lens capsule indicate fairly conclusively that the Sey allele is associated with a fault in the glycosylation of capsular collagen. Lens cataract would seem to be a result of this and related faults. The general failure of eye development is possibly related to similar biochemical lesions in the basement
membrane of the presumptive lens epithelium, or in carbohydrate secreted during induction of lens by optic vesicle.

In terms of the abnormal invasive behaviour of lens epithelial cells the most significant defect in the capsule is the gross increase in sialic acid (see p. 48). This discovery afforded an opportunity to extend the investigation to the level of the cell surface since the sialic acid content of cell surface material can be readily assessed by whole-cell electrophoresis (see p. 135).

(E) Prenatal Mortality of Homozygotes

The Sex gene is lethal in homozygous embryos at 5-6 days' development (Campbell & Clayton, in preparation). It is at the stage that Reichert's membrane, one of the extra-embryonic membranes, is produced as a secretion of the distal endoderm (Snell & Stevens, 1966). Reichert's membrane cross-reacts immunologically with the lens capsule (Midgley & Pierce, 1963) and is considered to be the best source of collagenous basement membrane antigen. There is therefore good reason to propose that this membrane is implicated in the prenatal death of the homozygote. In addition cellular organisation is disrupted in the 5-day embryo (Campbell & Clayton, in preparation).

(E) Examination of Filtration Efficiency of Glomerular Basement Membrane

The lens capsule cross-reacts immunologically with the glomerular basement membrane (Roberts, 1957) and biochemical analysis also shows that the two membranes have a similar composition (Spiro 1969b). In human patients with diabetes mellitus, there are structural and biochemical deficiencies
of these membranes, associated with lens cataract and excretion of glucose (Spiro, 1969b; Van Heyningen, 1962). The absence of detectable glucose in the urine of Sey mice suggests there is no connection between the Sey syndrome and the human diabetic conditions characterised by raised levels of urinary glucose.

Since the glomerular basement membrane seems to be the principal barrier to excretion of protein (Cohen, 1962) it was considered that structural defects in this membrane might be indicated by abnormally high levels of albumin in the urine. A rapid survey revealed no abnormalities in the albumin content of the urine of Sey mice. We can therefore infer that there is probably no major filtration defect in the glomerular basement membranes of Sey mice.

G. Cell Electrophoresis

No investigation was made into the basis of the change in mobility of epithelial cells with ageing of the preparation, but this change is consistent with the theory that negatively charged components are synthesized by the disaggregated cells and incorporated into their surfaces. If this is the case, the greater variability of values obtained for mutant cells may indicate a deficiency in their ability to replace charged surface components. Alternatively this may reflect a lowered accessibility of the intercellular material to the collagenase so that subsequent disaggregation of the epithelium by physical methods causes more damage to the surfaces of mutant cells than is the case with normal tissue.
Cell electrophoresis was introduced to test the hypothesis that the negative surface potential of lens epithelial cells from heterozygous *Sey* mice may be elevated compared with controls. This hypothesis is clearly not supported by the present data. Indeed estimates of average mobility (the mean and the median) indicate that the converse may be the case. The first quartile values also indicate that the fastest *Sey* cells are not as fast as the fastest control cells.

We can conclude therefore that the mutant cells are not more highly charged than normal cells under these conditions and there is some evidence that they may be less highly charged. The mean mobilities were 0.80 μ/sec/V/cm (σ = 0.27) for control cells and 0.70 μ/sec/V/cm (σ = 0.24) for *Sey/+* cells.

It should be stressed that whole cell electrophoresis provides information only on the extreme surface layers of cells. An increase in charged components which are buried within the surface material has no direct effect on electrophoretic mobility. If two samples show no significant difference in mobility this cannot therefore be considered as proof that cell peripheries in both samples are the same. Although it is probably safe to assume that the surface molecules relevant to cell recognition and adhesion are on average not different in charge.

We may conclude that the surfaces of *Sey* lens epithelial cells do not carry a higher negative charge than normal. The mean charge is in fact reduced, but not significantly so. The wider variability of values for *Sey/+* cells may indicate a difference in the accessibility of the intercellular material to collagenase, or a deficiency in the ability of mutant epithelia to replace
charged components of their surfaces. (Autoradiographic evidence indicates considerable metabolic differences between individual epithelial cells in Stx lenses (Clayton & Campbell, in preparation).) Such a deficiency could be relevant to the contact relations of the epithelial cells, but any abnormality in affinities cannot be ascribed to an increase in the negative charge of the surface (cf. prA, 474).

Tissue Cultures

The culture of lens epithelium was reviewed by Lucas (1965). Mann (1948) recorded what he believed was the beginning of differentiation of mouse lens epithelium into lens fibres at about the tenth day in culture.

Platigorsky & Rothschild (1972) showed that polar lens epithelium taken from chick embryos up to fifteen days old would differentiate into fibres in culture, but this ability was lost before nineteen days. Mamo & Leinfelder (1958) found no evidence for lens fibre formation, but observed that human lens epithelium from cataractous lenses grew earlier and quicker in culture than explants from normal lenses. This observation contrasts with the present results in which the Sex epithelial explants became established in culture less readily than normal tissue. Green, Goldberg & Todaro (1966) recorded that their rabbit lens epithelial culture preserved a strictly epithelioid appearance in culture, but Von Sallman, Grimes & Albert (1969) noted that in areas of low cell density particularly at the edge of the outgrowth zone, rainbow trout lens epithelial cells were often fibroblastic in appearance. No account could be found of lens epithelium maintaining a fibroblastic appearance until confluence was attained as described above.
The form adopted by a cell is a function of the properties of both the cell and the substratum (see p. 9). On clean plastic, normal and mutant lens epithelial cells were usually seen to maintain large areas of contact with the substratum and they showed much ruffled membrane activity (fig. 23). This suggests that their adhesion to plastic was strong. On the other hand their adhesion to 'used' i.e. cell-conditioned, plastic surfaces, which were presumably coated with exudate, was apparently relatively poor. On cell-conditioned surfaces, cells had only a few small areas of contact and displayed little ruffled membrane activity (fig. 23). However in normal and ZM cultures on both 'used' and clean surfaces, adjacent cells could be found which had very different appearances in these respects.

The means by which cells move from place to place is still not understood. Ambrose & Forrester (1968) believed that locomotory power was provided by the ruffling wave, but not by large-scale expansion of the leading edge of the cell. Cells tend to move up a gradient of adhesiveness (Carter, 1965, see p. 10). More precisely, it is claimed that cells 'compete' with the medium for contact with the substratum, the competition being based on the relative capacities of cells and medium to 'wet' the substratum (Carter, 1967). That is, if the angle of contact (in terms of surface tension) between cell and substratum is less than the angle of contact of the medium with that same region of the substratum, then that area of substratum will become occupied by the cell. Otherwise the cell will retract and the substratum will become occupied by medium.

On page 107, attention was drawn to the progressive centrifugal movement of outgrowths of normal lens epithelium. This movement was associated with ruffled membrane activity which was predominant on the clean
plastic. The ring-like sheet of cells spread outwards and broke its connection with the explant, which was left isolated at the centre of the sheet. The movement was filmed and its results were seen on many occasions in cultures which were not filmed. In *Sey* cultures this behaviour was seen only occasionally, the region immediately surrounding the explant being occupied in some cases by heaps of cells.

The radial movement of sheets of normal cells may be interpreted in two ways. We could say that it indicates that adhesion of cells to clean plastic is stronger than adhesion to plastic coated with exudate. Or we could say that the "angle of contact" between cell and plastic may be less than that between medium and plastic (since cells replace medium on plastic) and also that angle of contact between medium and exudate is less than that between cell and exudate (since medium replaces cell on plastic coated with exudate). The relative lack of movement of *Sey* outgrowths may indicate that the adhesion of mutant cells to plastic is not appreciably greater than their adhesion to their exudate; or that the relative angles of contact of cell to plastic, cell to exudate, or medium to exudate are perturbed so as to lessen the differentials which favour movement.

Overlapping heaps were seen at what appeared to be junctions between confluent streams of *Sey* cells, but unfortunately the development of these heaps was not observed in the film. A possibly analogous phenomenon which was filmed, was the outpushing of a large wedge of *Sey* cells beneath a group of cells which had blocked its path. An impression is gained from the film that the movement of this wedge is caused by growth from the rear rather than traction from the tip. There is no evidence of breakdown of contact
Inhibition of movement when isolated *Sey* cells contact one another or normal cells. It may be that abnormal overlapping occurs only when the tip of a stream of *Sey* cells contacts other mutant cells. If some of the motile power for the advance of cell streams is supplied from the rear, this movement would hardly be blocked completely by contact inhibition of movement of cells at the tip.

It should be stressed that *Sey* cultures were not consistently unusual and although the unusual features described above were selected because they were never seen in normal cultures, they were not always obvious in *Sey* cultures either. For example, spindle-shaped *Sey* cells which adhered poorly to plastic and which seemed insensitive to the proximity of other cells (p. 77) were seen only during early stages of growth *in vitro* and in only a few cultures.

Extracellular strands of material, presumably capsular collagen, were detected by Von Sallmann, Grimes & Albert (1969) in two-week old cultures of rainbow trout lens epithelium. The behaviour of lens epithelial cells in culture may be related to the properties of collagen which they secrete (see p. 77). Elsdale & Bard (1972) investigated the role of collagen in a dynamic system. They found that the ruffled membrane form of human foetal lung fibroblasts was accentuated by collagen deprivation (i.e. presence of collagenase). The bipolar spine form which was not subject to contact inhibition, appeared as confluence was approached and it was suggested that collagen contributed to the stabilization of this form. Grobstein & Cohen (1965) also concluded that contact with collagen was essential for the differentiation of epithelia (see also p. 77).
Elsdale & Bard (1972) found that collagen was also required for the growth of orthogonal multilayers of fibroblasts, which were formed above, (not below), the pre-existing layers. The spindle-shaped cells in these layers were aligned in parallel, in masses which merged if the angle between the axes of the cells was less than $20^\circ$. If this angle was greater than $20^\circ$, a trench was formed between the two cell masses. Overlap between such masses was not recorded.

In the present investigations, no fibrous secretions were observed in the culture dish and due to lack of time, no attempt was made to determine any possible effects of secreted collagen. Elsdale's & Bard's observations show that collagen is relevant to the morphology and behaviour of the cells which secrete it, and also to the interrelationships between cell masses. The abnormal form and behaviour of Sey lens epithelial cells in culture may therefore reflect abnormalities in collagenous capsule components which they secrete.

Sey epithelial explants did not establish themselves in culture as rapidly as normal explants and the presence of normal lens epithelium in the same culture dish further inhibited their development. These observations confirm that Sey lens epithelium is metabolically defective and further suggest that its well-being is dependent on the supply of some factor in the medium, which is depleted by growing normal lens epithelium. This situation presents an interesting comparison with the deductions made on p. 143 regarding a maternal effect on the expression of the Sey phenotype.
The Modification of Phenotype by Maternal Factors

It was shown that penetrance and expressivity of the *Sey* allele are both dependent on genetic background. Phenotype was also related to maternal genotype and maternal age.

If it is assumed that equal numbers of *Sey/+* foetuses are conceived by all mothers regardless of their genotype, and also that foetuses homozygous for the normal allele of *Sey* are no less viable than *Sey/+* foetuses, then the high proportion of phenotypically *Sey* mice in the litters of young *Sey (Ju)* mothers must be due to some effect of the maternal genome. That is, young *Sey (Ju)* mothers seem to favour the development of their own defects in genotypically normal progeny, while conversely old *Sey (Ju)* mothers seem to favour lowered penetrance of the gene in their heterozygous young. The overall recorded excess of phenotypically *Sey* young born to *Sey (Ju)* mothers seems to be a function of the effect of the maternal genome, the preponderance of matings set up using young mothers and the decline in litter size with maternal age.

Possibly a similar pattern occurs with *Sey (C57BL)* mothers although the data leaves this in doubt, but the situation is clearly quite different with normal *C57BL* mothers. In this case there is a significant excess of phenotypically normal mice in the litters and there is no reason to assume that this sample is unrepresentative. Differential viability does not explain these figures since mutant foetuses would hardly be less viable than normal in normal mothers, but more viable than normal in mutant mothers.

A possible explanation for this unusual situation is that the young *Sey/+* mother competes with her foetus for some factor which is required by
both parties. The level of this factor in some \(+\slash+\) foetuses could become reduced below the threshold required for the expression of the normal phenotype, with the result that the genotypically \(+\slash+\) foetus expresses the \(\text{Sey}\) phenotype. With older \(\text{Sey}/+\) mothers and in \(+\slash+\) mothers of all ages, the supply of this factor may be sufficiently high to maintain normal levels in \(+\slash+\) foetuses and also to raise the level in some \(\text{Sey}/+\) foetuses above the critical threshold, so that the \(\text{Sey}\) phenotype is not expressed in young mice which are genotypically \(\text{Sey}/+\).

When grown in culture \(\text{Sey}\) lens epithelial explants took longer to become established than normal. When normal and \(\text{Sey}/+\) lens epithelia were cultured together in the same dish the normal tissue grew very well, but the mutant tissues were even more retarded, (p. 100). The nature of this interaction has not been investigated, but the mutant epithelium was possibly in competition with the normal tissue for components of the medium, in a way which may be comparable to the hypothetical competition between mother and foetus. However there is no real evidence for competition in either case.

The concept of competition between mother and foetus is an unusual one. It is generally believed that the mammalian mother will invariably supply all available nutrients to her unborn young even if this is detrimental to herself. Possibly this rule does not apply to mothers who are themselves handicapped metabolically. A gene for atavistic-polydactyly in the guinea pig is, like the \(\text{Sey}\) gene, incompletely penetrant and is also expressed asymmetrically within individuals. The percentage of abnormal young decreases rapidly with maternal age (from about 80\% to about 10\%) and Wright (1934) concluded that in this case also, physiological competition between growing mothers and
foetuses lowers the concentration of an essential factor below a threshold which dictates the limits of expression of the phenotype.

Current work on the nature of collagen cross-links may throw some light on the way in which maternal age could influence the composition and structure of basement membranes in foetuses. Robins, Shimokomski and Bailey (1973) have demonstrated marked changes in the proportions of different types of cross-links in bovine collagen as the animal grows older. All the cross-links are based on lysine, hydroxylysine, or their derivatives, but some of these residues form condensation products with glucose or mannose instead. The concentration of these glycosylated residues increases demonstrably with age. The *Sey* allele seems to affect the assembly of collagen in basement membranes through an effect on its glycosylation (see p. 144). The age-related maternal effect on expression of the *Sey* phenotype could be connected in some way with the normal age-related increase in glycosylation of collagen.

One feature of the data which is not explained is the relatively much greater decrease in total litter size with age of the *C57Bl* mothers, compared with *Sey* mothers. The probable explanation is that the *C57Bl* mother, being effectively completely homozygous, is less fit than her outbred cousins, and is not so capable of bearing large litters except when young. That is, this effect is probably related to the genetic background rather than the pair of alleles under examination.

On the basis of the ratios of phenotypes in the litters, we can
therefore conclude that the expression of the *Sey* phenotype in an individual seems to depend on the presence of the *Sey* allele in that individual, its presence in the mother and the age of the mother. The modifying effect exerted apparently by the maternal system can be explained in terms of the sharing of an essential factor between growing mothers and their young and may be related to normal changes in the glycosylation of collagen which occur with ageing. Genetic background also controls both the penetrance and expressivity of the syndrome and in addition some as yet unidentified element seems to act locally in the region of the eye since the ocular abnormalities of *Sey* mice are frequently asymmetric.

**K. Biochemical Interpretation**

The analysis of the lens capsule indicates that the *Sey* allele is associated with a defect in its composition and structure.

The synthesis of collagenous basement membrane is described by Spiro (1969 b). After the assembly of the polypeptide on the ribosome, lysine and proline residues become hydroxylated and subsequently hydroxyllysine becomes the site of attachment of galactose residues through the action of a specific galactosyl-transferase. A specific glycosyltransferase then transfers glucose to all the hydroxyllysine-linked galactose residues. At the same time, a heteropolysaccharide chain composed of galactose, mannose, hexosamines, sialic acid and fucose, is assembled on another part of the chain through the stepwise action of a series of glycosyltransferases. This chain is attached to the polypeptide through asparagine. The peptide chains, with the carbohydrate attached then pass out of the cell where cross-links form between lysine and
unsubstituted hydroxylysine residues or their derivatives (Robins et al., 1973) and the insoluble membrane becomes established. Pathological or other factors may influence the structure of the membrane at several postribosomal steps such as hydroxylation, glycosylation, or cross-link formation, or by breakdown of the completed membrane. Typically in any glycoprotein sample, many carbohydrate chains are incomplete and it can be assumed that this is also the case in the basement membranes.

Specific cofactors are required at certain of the postribosomal stages. Ascorbate and Fe$^{++}$ ions are necessary for hydroxylation and there is a general requirement for Mn$^{++}$ ions during glycosylation (Roseman, 1970). Spiro (1967b) states specifically that Mn$^{++}$ ions are essential for the attachment of both galactose and glucose.

A plausible explanation of the results derived from the work on the lens capsule is that the Sey gene affects some stage in the glycosylation of basement membrane collagen. It was deduced that the average size of the carbohydrate chains is smaller in Sey mice, that the range of sizes of these chains is wider than normal and that the chains contain an excess of sialic acid (see p./25). The electron micrographs reveal details which are compatible with this deduction (p./29).

At certain stages in the extension of an oligosaccharide chain, two or possibly more glycosyltransferases, with the same acceptor specificity, may compete for the same attachment site. Success in this competition must depend on the relative activities of the enzymes, the availability of the relevant sugar nucleotides and cofactors, and possibly the topographical distribution
of the enzymes within the Golgi apparatus (Heath, 1971). The $\textit{Sey}$ gene possibly affects the activity of a sialyltransferase, or a glycosyltransferase which competes with a sialyltransferase for an attachment site on the partially completed heteropolysaccharide chain. If the functional activity of the sialyltransferase is raised or that of the competing glycosyltransferase is depressed in $\textit{Sey}$ mice, this could result in preferential substitution of sialic acid at this site, with a consequent premature termination of the chain. Such a situation occurs in the submaxillary glands of sheep, where submaxillary mucin with short carbohydrate chains is synthesized although all the enzymes required for the synthesis of the more elaborate porcine mucin are present (McGuire, 1970). In ovine glands the relevant sialyltransferase is sixteen times as active as that in the glands of pigs.

In the submaxillary mucin biosynthetic system, all the glycosyltransferases except the sialyltransferase require manganese as an essential cofactor (McGuire, 1970). Abbreviated carbohydrate chains as in ovine submaxillary mucin might therefore be expected to be produced if there is a deficiency of $\text{Mn}^{++}$ even if all the porcine submaxillary mucin enzymes are present at their normal levels. On p. 143 it was suggested that the apparent maternal modification of phenotype might involve the exchange between mother and foetus of a factor deficient in young $\textit{Sey}$ mothers. It is also possible that the establishment and growth in culture of primary explants of $\textit{Sey}$ lens epithelium is dependent on the supply of one or more factors in the medium which is seriously depleted by growing normal lens epithelium (see p. 14/). These factors could be cations or other cofactors required in the glycosylation of collagen and/or other extracellular material. Ascorbate and iron are
components of Medium 199, but Mn\textsuperscript{2+} would be present only as an impurity, or component of the foetal calf serum supplement.

The nature of collagen cross-links changes with maturity and the change is accompanied by a systematic increase in the binding of monosaccharides to the lysine and hydroxyllysine residues which in a younger animal might be involved in cross-linking (Robins, et al, 1973). The maternal age-related modification of the \textit{Sey} phenotype is possibly a consequence of mobilization of extra glycosylation activity in maturing animals.

Basically two hypotheses have been advanced as an explanation of the apparent breakdown in the glycosylation of basement membrane collagen. These are concerned with the activity of glycosyltransferases and the availability of cofactors required for their action. Other explanations could be advanced in terms for example, of the availability of their substrates, the nucleotide sugars.

An alternative hypothesis is that the activity of lytic enzymes may be defective in \textit{Sey} tissues. The eyes of rats contain neuraminidases which may control the composition and charge of ocular glycoproteins and glycolipids (Tulsiani, Nordquist and Carubelli, 1973). A deficiency in the activity of neuraminidase in the eye might cause an increase in the sialic acid content of the capsule, but this would not account for other features such as the abnormally short carbohydrate chains.

Having characterised a biochemical lesion in secreted glycoprotein, one of the major aims of this work was to detect similar abnormalities in materials on the surfaces of the cells which secrete that abnormal glycoprotein. The behaviour of \textit{Sey} lens epithelium in culture indicates an abnormality in the relationship of cells to one another or to their substrata, and the wide variation
in the cell electrophoresis results with Sey cells as opposed to control preparations, suggests a defect in the capacity of cells to restore their surfaces damaged during disaggregation (p.35). We have no evidence that the surface carbohydrates of Sey lens epithelial cells have an abnormality comparable with that detected in the capsule, but it can be deduced that their surfaces probably are abnormal and an explanation of the biochemical lesion in terms of deficient carbohydrates and defective glycosyltransferases is especially attractive in view of a recent hypothesis of cell adhesion (Roseman, 1970).

One of the primary events in carcinogenesis is the failure of certain key glycosyltransferases (see p.51). Transformed cells begin to display "incomplete" oligosaccharide chains at the same time as contact inhibition of growth breaks down (see p.50). The Roseman hypothesis puts the mechanismism of cell adhesion, its breakdown in cancer cells, and the biochemical lesion suggested as an explanation of the major abnormalities associated with the Sey allele all within a single conceptual framework.

Another hypothesis which may contribute to an explanation of the invasiveness of Sey cells concerns the relationship of cells to collagen. This relationship is important with respect to cell behaviour and the state of differentiation (see pp. 7, 140), and we know that the collagen of the lens capsule is abnormal in Sey mice. Abnormalities in the capsule or capsular components secreted by lens epithelium in vitro may upset their normal differentiation and behaviour. This is possibly related to electrical charge effects due to the high content of sialic acid in the capsule. Electrical charge
could affect contact between cells and collagen (p. 4) or the distribution and movement of ions through the plasma membrane of cells in its proximity (p. 10).

II. The Roseman Hypothesis of Cell Adhesion and its Implications

None of the theories outlined in the Literature Survey is completely satisfactory in explaining the known features of cell adhesion in terms of the molecules such as those containing complex carbohydrates, which characterise cell surfaces and which are known to have a bearing on cell recognition phenomena (e.g. see p. 8). Cells from different tissues will adhere non-specifically to some extent and there is an hierarchy of cohesiveness of tissues which indicates a range of adhesive strengths which overlaps from one tissue to another (p. 4). The surfaces of cells of different tissues within the same individual differ in the relative proportions of particular blood group (p. 24) and histocompatibility antigens (p. 25) and in addition, some determinants ("differentiation antigens") seem to be confined to particular tissues (p. 25).

It is possible that these determinants, or molecules of a similar type, may represent the "words" of a "language" by which cells can discriminate between their own kind and those from another source.

Recently Roseman (1970) put forward a theory of cell adhesion which agrees well with our knowledge of the nature of cell surface antigenic determinants and cell surface enzyme activity (Roth, McGuire & Roseman, 1971a). This model would seem to be the most acceptable so far proposed. In Roseman's model, intercellular bonds are formed between glycosyltransferases,
the enzymes which elaborate the complex carbohydrates, and their specific acceptor substrates. The enzymes and substrates are considered to be located on the surfaces of adjacent cells.

Heinmets (1965) had earlier suggested that specificity in cellular interaction is based on enzyme action (see p. 41), but Heinmets made no postulates about the nature of the enzymes. An important distinction between the two ideas is that in Heinmets' model the recognition step takes place within the nucleus, where the enzyme reaction products act as inducers or repressors, while in Roseman's model the enzymes "decode" the surface display of the neighbouring cell by their own stereospecificity.

There is considerable evidence in favour of the Roseman hypothesis. Chick embryo neural retinal cells will separate from liver cells in mixtures of the two disaggregated tissues, but this property is diminished if terminal \( \beta \)-galactoside residues are removed enzymically from the carbohydrates of the retinal cell surfaces (Roth, McGuire & Roseman, 1971b). Further evidence is supplied by the work of Cox & Geiser (1968, 1968a), described on p. 11, in which it was shown that free L-fucose and D-mannose altered the growth behaviour and morphology of non-confluent fibroblasts so that they resembled fibroblasts which had attained confluence. The implication of these observations is that the free sugars in solution saturated the acceptor sites which would normally react with terminal sugars on the surfaces of neighbouring cells. Roth & White (1973) presented evidence that galactose becomes attached to acceptors on the surfaces of Balb/C 3T3 cells (which exhibit contact inhibition of growth) only when the cells are in contact.
Several lines of evidence indicate that glycosylation is carried out by surface-bound enzymes and the authors consider these enzymes to act on substrates on the surfaces of neighbouring cells, ("transglycosylation", see below).

A major failing of the theory is that it does not account for the importance of calcium in cell adhesion (see p. 32). Indeed it is specifically stated that adhesion would be strongly inhibited by calcium in some cases, as the activity of some glycosyltransferases is inhibited in its presence. The major function of calcium could well be concerned with the stabilization of intercellular aggregation-enhancing factors. In this context it is interesting that Armstrong (1966) found Mg$^{++}$ ions to be more potent than Ca$^{++}$ in the enhancement of cell aggregation. Some glycosyltransferases require Mg$^{++}$ as a cofactor (Roseman, 1970).

As originally stated by Roseman (1970) his hypothesis does not account for the enhancement of cell aggregation by cell exudates (see p. 34) and no mention is made of intercellular material which is not surface-bound. The model can be easily modified to include an intercellular cement material carrying specific complex carbohydrate groups which link the glycosyltransferases displayed on neighbouring cell surfaces. Support for the theory that surface-bound proteins are involved in cell adhesion in conjunction with intercellular material is supplied by Pessac and Defendi (1972a, b). These authors identified a non-specific aggregation enhancing factor of vertebrate cells as hyaluronic acid, (which accords with the use of hyaluronidase as a dissociating agent, Kemp, 1969). By the judicious use of hyaluronidase and trypsin, it
was deduced that intercellular hyaluronic acid acts as a link between protein components of the cell membranes. After trypsinization of their surfaces, cells can regenerate the essential membrane-bound protein only if they are maintained at temperatures conducive to normal metabolism (c.f. p. 24).

During the synthesis of proteins and nucleic acids, relatively simple, non-specific enzyme systems build up the polymer, the details of which are determined directly by the genetic material which acts as a template. By contrast, oligosaccharide chains are not synthesized by a template mechanism; instead, a specific complex of glycosyltransferases adds one monosaccharide at a time to the non-reducing end of the chain, the structure of the chain being determined by the specificities of the glycosyltransferases. The latter are specific in terms of the type of monosaccharide transferred, the structure of the acceptor molecule and the linkage of the transferred glycosyl unit to the acceptor molecule. An important point is that the product of each glycosyltransferase reaction becomes the acceptor for the next enzyme in the sequence, a property that has been termed "cooperative sequential specificity" (Roseman, 1970). Since the same set of genes codes for the ABO blood group substances present as glycolipid on cell surfaces, or as glycoprotein in secretions, it would seem that a single multiglycosyltransferase system is responsible for the completion of glycolipids and glycoproteins (cf Roseman, 1970).

Synthesis of the oligosaccharide groups occurs within the channels of the endoplasmic reticulum and Golgi apparatus, the glycosyltransferases being bound to the walls of these structures. The enzymes receive nucleotide sugars from the cytoplasm and transfer the glycosyl unit by some mechanism
not yet understood, to the acceptor group on the other side of the membrane. For synthesis of complete oligosaccharide chains optimal conditions are required for the operation of each enzyme in the system i.e. optimal pH, sugar nucleotide and cofactor concentrations. Since such conditions are unlikely to prevail at all times, according to Roseman (1970), a prediction of his hypothesis is that oligosaccharide chains should frequently be incomplete. This is in fact a familiar feature (Spiro 1969 a, b.)

On the basis of his studies on selective adhesion of differentiated cell types (see p. 39), Steinberg (1964) observed that the selective mechanism must have been modified in very small steps throughout Metazoan evolution while always remaining functional. This echoes Townes and Holtfreter's (1955) suggestion that bonds arise de novo in the course of differentiation while pre-existing bonds are retained, or else the early, indiscriminate bond type becomes modified, but not so much as to exclude indiscriminate adhesions. This line of reasoning fits very well within the Roseman model. Differentiation of the cell surface could be considered as arising by the inclusion of a new and different enzyme within the multiglycosyltransferase system, or alternatively the activity of a particular enzyme could become altered due to a change in conditions within or outside the cell. Specific adhesions could be mediated by surface-bound enzymes relevant to the completion of the new oligosaccharides while non-specific adhesions could occur through incomplete chains common to both cell types.

Roseman (1970) points out that since all the glycosyltransferases catalyze bimolecular reactions, the level of one substrate can effectively regulate the binding constant of the other substrate i.e. the internal level of
a sugar nucleotide could regulate the binding of an enzyme in the cell surface, to the acceptor substrate on another cell. With the exception of the stalyltransferases, the glycosyltransferases require divalent cations for activity. Many require Mn$^{++}$ or Mg$^{++}$. Roseman (1970) suggests that cell adhesion could be regulated by divalent ions or by feedback control by the nucleotide products of the reaction.

Another possible means by which cell adhesion could be regulated is by modification of the specificity of the glycosyltransferases. A galactosyltransferase present in milk normally catalyzes the transfer of galactose to N-acetylglucosamine as acceptor on a glycoprotein, but in the presence of lactalbumin, the same transferase utilizes glucose as an acceptor so that lactose is formed instead (Spiro 1969a). This point is not considered by Roseman, but could be relevant to the action of aggregation enhancing factors (see also p. 52).

Roseman extends his hypothesis to the theory of "intercellular modification". In brief the theory is that a glycosyltransferase bound to the surface of a cell could carry out its more conventional function of extending the acceptor chain by adding on the specific monosaccharide which the enzyme normally handles. This process would convert the acceptor to a product to which the glycosyltransferase would bind poorly, or not at all. The result would be a loss of adhesive bonds, or even dissociation of the tissue. On the other hand, if the next enzyme in a particular multiglycosyltransferase system is also present on the surface, a new bond could form and the net result could be increased adhesiveness. Intercellular modification could therefore result in either gain, or loss of adhesiveness depending on the nature of the surface.
enzymes and the number of each species present. Normal cells growing actively in monolayer culture carry on their surfaces glycolipids with short, incomplete oligosaccharide chains. These chains are completed only after confluence has been attained (see Heath, 1971). Roth & White (1972) have presented evidence that the completion of these chains occurs by intercellular modification or "transglycosylation" (p. 52). This mechanism may also explain the behavior of pigment cells during the development of colour patterns in the Axolotl (p. 6).

In summary, the Rossmann hypothesis of cell adhesion provides a conceptual framework which covers the whole field of cell adhesion. The theory deals with molecules which are known to be present on cell surfaces and which are known to be abnormal in cancerous conditions in which cell affinities are altered (see p. 50). It allows scope for practically unlimited differentiation of the cell surface and for control of cell affinities from both within and outside the cell. As suggested by Roth & White (1972), the concept of intercellular modification of surface determinants may also offer an approach towards the understanding of some embryonic inductions.

Despite the comprehensiveness of the theory, objections may still be raised concerning its general applicability. Differentiated cells display characteristic densities and ratios of blood group antigens, and cancer of certain organs is statistically correlateable with particular blood groups (p. 22). However, persons with the Bombay gene are apparently unaffected by the complete absence of ABH antigens from their bodies. It may be that the enzymes responsible for the details of the glycosphingolipid blood group antigens have a more important function in the synthesis of
other cell surface determinants more relevant to cellular interactions.

Judging by the work of Warren, Critchley & MacPherson (1972), changes in membrane glycoproteins may be more critical in malignancy than those in glycolipids.

Recent work is providing increasing support for the statement of Smith & Good (1969) quoted in the opening paragraph of the introduction, that the first step in the recognition of elements of the environment by a cell involves the fitting together of complementary stereochemically related molecules at the surface of the cell. The tissues of the Small Eye mouse may provide a basis for future critical experiments to test the hypothesis that specificity in cell recognition phenomena is determined by glycosyltransferases bound to the cell surface, acting in conjunction with their specific substrates and products located on the surfaces of other cells.
Mice which are heterozygous at the Small Eye locus have been shown to have characteristic abnormalities in the structure and composition of the lens capsule. Its ultrastructure indicates a defect in glycosylation of collagen, and biochemical analyses reveal shortened carbohydrate chains and a gross increase in the content of sialic acid in the capsule. It is deduced that other basement membranes which appear abnormal in histological section probably have the same type of defect and the suggestion is made that Sey homozygotes die because of similar defects in Reichert's membrane. The general breakdown in eye development in Sey mice could also be due to defective basement membranes. A fault early in development is indicated and it is suggested there may be a breakdown in lens induction due to abnormalities in the properties of the basement membranes of the embryonic ectoderm or optic vesicle, or in other extracellular material synthesized at this stage. Charge anomalies in the capsule could lead to cataract through an upset in the ionic content of the lens.

The abnormalities in the basement membranes are probably due to a defect in the glycosylation of collagen. The hypothesis is advanced that this defect could result from a deficiency in the functioning of the glycosyltransferases. This may be a general failing, or a fault in a
specific glycosyltransferase, possibly one which competes with a sialyltransferase. This deficiency may be related to the availability of a substrate or cofactor. A hypothesis is put forward to explain the apparent effect of maternal age and genotype on the expression of the Sey phenotype in young mice. It is suggested that competition for a limited supply of a factor relevant to glycosylation takes place between a foetus and a mother who is still maturing. The effect of maternal age may be connected with changes in the glycosylation of collagen which are known to occur with advancing age.

There is no reliable indication of an abnormality in the surface-bound carbohydrates of Sey lens epithelial cells, but the behaviour of these cells in culture indicates an abnormality in the relationship of the cells to one another, or to their substrata. The wide variability of cell electrophoresis data with Sey cells, in contrast to normal cells, suggests a defect in the capacity of mutant cells to replace charged material on their surfaces.

In the context of the Roseman hypothesis of cell adhesion (Roseman, 1970), the abnormal adhesions between Sey eye tissues and the invasive tendencies of Sey cells could be explained in terms of hypothetical defects in complex carbohydrates and glycosyltransferases bound to the cell surfaces. Alternatively the abnormal behaviour of Sey lens epithelium may be a response to contact with, or close proximity to collagenous material which it secretes and which has been shown to be abnormal in composition, structure and charge. A more complete explanation of the basis of cellular invasiveness in the eyes of the Small Eye mouse must await a
closer analysis of cell behaviour in culture, and a detailed examination of the enzymes and cofactors relevant to glycosylation.

This work represents one of the very few examples in which an inherited morphological defect has been described in biochemical terms (H. Grøneberg, personal communication).
REFERENCES


Ugolev, A.M. (1965). Membrane (contact) digestion. Physiol. Revs., 45: 555-


Winzler, R.J. (1970b). Glycoprotein antigens isolated from RBC. 
In Aminoff, D. (ed), Blood and Tissue Antigens, 

Wolpert, L. and Gingell, D. (1968). Cell surface membranes and 
Press, pp. 169-199.

on the fate of transfused lymphocytes. J. exp. Med., 
129: 351.

Wourms, J.P. (1965). Reaggregation of completely dispersed amoeboid 
blastomeres during the normal development of annual 

Wright, S. (1934). An analysis of variability in number of digits in an 

of chick blastoderm cells. I. Electrophoretic mobility 
and pH-mobility relationships. J. Cell Physiol., 78: 
225-234.

Zwaan, J. and Williams, R.M. (1967). Cataracts and abnormal 
proliferation of the lens epithelium in mice carrying 