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THE PERMEABILITY OF INTERCELLULAR JUNCTIONS FORMED BETWEEN

ANIMAL CELLS

Malcolm Edwin Finbow

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy at the University of Glasgow. Research work completed at the Department of Biochemistry, University of Glasgow in August, 1976 and thesis completed at the Division of Biology, California Institute of Technology, Pasadena, USA, in March 1979.
To my family both English and Scots
Acknowledgements

I am deeply indebted to my wife without whose loyal support and constant encouragement this thesis would not have been possible. I must also thank my three secretaries, Mrs. I. Finbow (my better half), Mrs. L. Cooper and Mrs. S. Esat for the typing at various stages the two forms of this thesis. My thanks are due to my supervisor, Doctor J.D. Pitts for his never ending advice and invaluable criticism throughout my stay at Glasgow and subsequent post at the California Institute of Technology. I am also indebted to Doctors B.S. Yancey (California Institute of Technology) and R.G. Johnson (University of Minnesota) and Professor J.D. Sheridan (University of Minnesota) for helpful discussions at various times. I am grateful to Professor Jean-Paul Revel (California Institute of Technology) for allowing me time and consideration in completing my thesis in his laboratory. I am indebted to Professors R.M.S. Smellie and A.R. Williamson for allowing me to engage in a research studentship programme sponsored by the Medical Research Council (London, England) in the Department of Biochemistry, University of Glasgow and also for their support and helpful criticism of my work. I must acknowledge the many members of the Department of Biochemistry, University of Glasgow, notably Doctor R. Eason, for their help and assistance during my stay at Glasgow. Also, I thank the staff of the 'Wellcome Tissue Culture Unit', especially Mr. P. Ferry and Mrs. S. Rankine, for their technical assistance. Finally, I am
indebted to my two sons, Challum and Gavin, for showing me that
the products of embryogenesis are more important than the process.

Part of the work given in Chapters 3 and 5 have appeared elsewhere
in review form titled 'Junctional Communication and its Consequences'
by J.D. Pitts and M.E. Finbow, Chapter 3 in 'Intercellular
Communication' edited by W.C. De Mello and published by Plenum
Press, N.Y., 1977. Also, part of the literature survey presented
in Chapter 1 will appear in a review titled 'The Roles of Intercellular
Junctions' by M.E. Finbow and B.S. Yancey in 'The Cell Surface'
edited by P. Knox and to be published by CRC Press, Florida, USA.
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Abbreviations and Nomenclature

The abbreviations and nomenclature which are employed in this thesis are those specified by the 'Biochemical Journal, Policy of the Journal and Instructions to Authors, London, 1978'. Other commonly used abbreviations not specified by this publication are taken from the 'Pocket Edition' of the 'Oxford Dictionary of Current English', Clarendon Press, Oxford, England, 1969. All other abbreviations are specified where appropriate in the text.
SUMMARY OF THESIS

1. The literature concerning the structure of intercellular junctions is reviewed. Particular attention is paid to the gap junction which is generally believed to be the membrane specialization responsible for intercellular communication. The permeability of 'communicating junctions' and the modulation of junctional communication are critically reviewed. The possible roles of junctional communication between cells are discussed.

2. The permeability of junctions formed between animal cells in culture has been examined by following the intercellular movement (or lack of movement) of a selection of endogenous molecules between cells in contact.

3. Using modifications of established methods it was shown that intermediate metabolites derived from choline (phosphoryl choline and CDP-choline) were readily exchanged between cells in contact if the cells formed permeable intercellular junctions. However, phospholipids derived from choline were not exchanged between cells joined by such junctions.

4. By a new approach, dependent upon the cell type specific rate of 2-deoxy-glucose-6-phosphate loss from cells, it was shown that this sugar phosphate is exchanged between cells joined by permeable intercellular junctions but not between cells which are unable to form these junctions. It was also shown that these junctions might
be permeable to intermediate metabolites, but not macromolecules (glycoproteins), derived from glucosamine.

5. The ability of cells to incorporate labelled formate was used as a measure of their folate content. This allowed the development of a novel method which showed that cellular folates are transferred from untreated cells to folate starved cells if the two cell types are joined by permeable intercellular junctions. However, the rate of transfer of cellular folates through these junctions appeared to be much slower than for other molecules. Using cells which had a lesion in folate metabolism, it was shown that this slow rate of transfer could be due to the impermeability of intercellular junctions to the pentaglutamate derivative of folate (which is the predominant form of folate in mammalian cells).

6. The permeability of intercellular junctions to amino acids was examined by using a cell type which was auxotrophic for proline. It was shown that these cells lost their dependence on exogenous proline for growth when cultured with junction forming cells but not with non-junction forming cells. The ability of wild-type, junction forming cells to support the growth of the auxotrophic cells was shown not to be due to an extracellular pathway of proline transfer.

7. The ability of different cell types to form permeable intercellular junctions was measured using two different methods. The two methods gave comparable results and showed that different cell types can vary greatly in their ability to allow junctional exchange of
metabolites.

8. The significance of the observations made in the course of this work are critically discussed and related to other published work on junctional communication. It is proposed that populations of cells which are joined by gap junctions will tend to form 'functional syncyctia' and this property of gap junctions may have an important role in the evolution of animal tissues.

9. The properties of two mutant cell types apparently lacking thymidine kinase were examined. These cells showed characteristic reduced ability to incorporate thymidine at low exogenous thymidine concentrations (10^{-6} M) but exhibited wild-type phenotype at higher (above 10^{-3} M) thymidine concentrations.
Chapter 1  INTRODUCTION
1.1. The Cell Theory and Cell Interactions

"The cell concept is the concept of life, its origin, its nature and its continuity"

Karling, 1940

The discovery by Euclid in the 3rd Century B.C. that curved surfaces of glass could magnify objects to many times their actual size led to the invention a couple of millennia later of two optical instruments which had a profound impact on the scientific world of the 17th Century. One of these instruments, the telescope, allowed astronomers to look at far distant celestial bodies. In contrast the other instrument, the microscope, made it possible for biologists to study the minute structural detail of living organisms.

Robert Hooke (1665) carried out the first microscopic examination of living things on plant tissues. He discovered that these tissues were composed of many millions of homogeneous units which he called cells because they reminded him of the dwelling places of monks in a monastery. Throughout the following two centuries many scientific workers confirmed and extended Hooke's discovery and this led to the formulation of the cell theory by a number of authors in the mid-19th Century (for a historical account of the rise of the cell theory see Karling, 1940). This theory brought the science of biology under a single, unifying concept by
proposing that all living things are composed of cells and products of cells. The theory states that the cell is the structural and metabolic unit of the organism and growth, or the replacement of lost cells, is achieved by the division of a pre-existing cell into two new daughter cells (omnis cellula a cellula). The cell theory has proved to be a fundamental concept of biology.

For the unicelled organisms, such as bacteria, the cell is by definition a complete unit of life capable of growth and generating new organisms independently from other cells of the same species. Although for many of these species of simple life forms there are periods of 'sexual' interaction between the cells for the exchange of genetic material. In contrast to these primitive creatures the cell of the multicelled organism has a dual existence; that of its own cell-cycle and that of the shared life-cycle of the organism. This dual existence must be integrated by a mutual interdependence of the cells on one another if the organism is to be a composite whole. Perhaps the highlight of the cell theory as stated by Dutrochet (1824) is in its recognition that the unity of the multicelled organism is due to the interrelations and interactions between the composite cells (Karling, 1940).

One type of cell-cell interaction is communication. Two forms of intercellular communication that have been known for some time are signalling by hormones and transmission of electrical impulses between excitable cells at chemical synapses. A more recently
discovered form of communication is the movement of molecules and ions directly between cells whose surfaces have come into contact with one another. This direct form of communication implies that the permeability properties of these 'junctional' surfaces is somewhat different from their 'non-junctional' surfaces. Indeed electron microscopy studies have revealed several types of specialized structures at points of contact between animal cells. These differentiations have been termed collectively intercellular junctions. Four classes of intercellular junctions have now been found between animal cells and these are the desmosome, the tight junction, the septate junction and the gap junction. It would seem likely that one or more of these membrane specialisations is responsible for this direct form of communication.

The following sections of Chapter 1 review the literature concerning the distribution, structure and functions of these junctions in order to evaluate their role, if any, in intercellular communication. Also, the literature concerning the types of substances which are believed to pass directly between animal cells will be reviewed to assess whether all classes of cellular substances pass between cells or if there are restrictions. Finally, the evidence for the possible functions of this form of intercellular communication will be reviewed.
1.2. The Desmosome

The desmosome has a wide distribution being found in both vertebrates and invertebrates. Desmosomes are especially common in tissues subject to mechanical stress such as squamous epithelia (for review see McNutt and Weinstein, 1973; Gilula, 1974a; Staehelin, 1974).

In thin sectioned preparations of vertebrate tissues the junctions appear as disc shaped regions in the two plasma membranes. The membranes lie parallel to one another and are separated by a gap of 25 to 35 nm. This gap, or interspace, is filled with a filamentous material containing glycoproteins (Rambourg, 1969) and the space is permeable to a number of tracers such as lanthanum hydroxide (Revel and Karnovsky, 1967). The glycoproteins are believed to form a cement between the two membranes (Staehelin, 1974). The cytoplasmic sides of the desmosomes are often associated with tonofilaments or, in the case of cardiac muscle, with microfilaments (McNutt and Weinstein, 1973) and these filaments often interconnect with other desmosomes to form a cytoskeletal network throughout the cell (Gilula, 1974a).

Desmosome-rich fractions have been isolated from calf nose epithelium (Skerrow and Maltosty, 1974a). Such fractions contain mostly protein with small amounts of lipid and carbohydrate. The proteins are resolved into seven major bands ranging in mol. wt. from 60,000 to 230,000 and a number of minor bands by
electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) and reducing agents (Skerrow and Maltosty, 1974b). The large number of bands found on these fractions suggests that the desmosome is a complex structure (Skerrow and Maltosty, 1974b).

The association of desmosomes with cytoskeletal elements and their abundance in tissues requiring mechanical strength suggest that the role of these junctions is to hold cells firmly together thus giving shape and rigidity to the tissue (McNutt and Weinstein, 1973; Gilula, 1974b; Staehelin, 1974). Whether this junction has other roles, apart from anchoring cells together, is not known.

1.3. The Tight Junction

The tight junction is, by and large, restricted to the vertebrates and is a ubiquitous structure between epithelial cells lining lumenal cavities where it forms a band of intercellular contacts around the apical ends of these cells (Farquhar and Palade, 1963). The myelin sheath around nerve fibres and the cells in the morula and bastula stages of the developing amphibian embryo are also rich in tight junctions (Shinowara and Revel, manuscript in preparation; Smith et al., 1976).

The junctions are characterised in thin sectioned preparations as
very close appositions of the two plasma membranes with no visually detectable space between the membranes (Farquhar and Palade, 1963). This lack of interspace was confirmed by Revel and Karnovsky (1967) who found that lanthanum hydroxide could not penetrate between the membranes at tight junctions though the tracer penetrated between the membranes at other junctions. In freeze fractured preparations, the junctions are seen to be composed of intramembranous particles from both membranes (Kreutziger, 1968) which form a meshwork of interconnecting and anastomosing fibrils immediately below the apical ends of the cells (Friend and Gilula, 1972).

Several studies suggest that the role of these junctions is to act as a barrier of diffusion of molecules by forming a gasket seal. Sheets of epithelial cells joined by these junctions are impermeable to molecules such as haemoglobin (Farquhar and Palade, 1963) and horse radish peroxidase (Revel and Karnovsky, 1967). The movement of lanthanum hydroxide through extracellular spaces of various tissues is stopped by regions where tight junctions are present (Goodenough and Revel, 1970). Tight junctions may also impede the movement of small inorganic ions as the magnitude of the electrical resistance across various epithelia correlates very well with the extent of tight junctions between the cells (Frümter and Diamond, 1972; Claude and Goodenough, 1973). This transepithelium resistance can be lost by wounding one of the cells in the epithelium and the restoration of the resistance accompanies the formation of new tight junctions between the cells.
that have migrated into the wounded area (Hudspeth, 1975).

Thus, tight junctions serve as barriers of diffusion forming a gasket seal between the plasma membranes of adjacent cells.

1.4. The Septate Junction

This class of intercellular junction is restricted mainly to the invertebrates and has been found in a variety of invertebrate organisms such as *Hydra* (Wood, 1959), mussels (Gilula *et al.*, 1970) and insects (Bullivant and Loewenstein, 1968). Septate junctions can extend for many microns in length between two cells forming a belt at their apical regions (Hudspeth and Revel, 1971). The junction appears in thin section as two adjacent membranes joined by septa spaced at regular intervals and lying perpendicular to the membranes. Freeze fractured preparations show the junction to consist of parallel rows of particles and these intramembranous particles have been demonstrated to correspond to the intercellular septa seen in thin section (Gilula *et al.*, 1970).

A number of physiological roles have been assigned to septate junctions. Wood (1959) initially proposed that these junctions may serve as sites of intercellular adhesion. With the discovery of electrical coupling (see section 1.6.2.) and the subsequent finding of these junctions between electrically coupled cells, a
number of workers suggested that septate junctions are the morphological basis of coupling (Weiner et al. 1964; Gilula et al. 1970; Bullivant and Loewenstein, 1968). However, it has been subsequently found that invertebrate cells are joined by gap junctions as well as septate junctions (Hudspeth and Revel, 1971; Rose, 1971; Gilula and Satir, 1971). There is now good evidence that gap junctions mediate electrical coupling in vertebrate tissues (see section 1.6.2.) and they probably serve the same function in invertebrate tissues (Hudspeth and Revel, 1971). As yet, no coupled invertebrate cells which possess only one of these two types of intercellular junctions have been reported. Therefore it is not possible to exclude this function for septate junctions.

There is now an increasing amount of evidence that septate junctions have an occluding role. Their location at the apical end of the cells (Hudspeth and Revel, 1971) is analogous to the location of the tight junction which is known to be an occluding junction in vertebrate tissues (Farquhar and Palade, 1963). Although the interspace of these junctions can be infiltrated by lanthanum hydroxide (Hudspeth and Revel, 1971) they appear to prevent the extracellular movement of such tracers as horse radish peroxidase (see discussion of Hand and Globel, 1972). Lord and DiBonna (1977) have recently shown that the septate junctions of the epidermal cells of Planaria, which are located at the outward facing (apical) end of the interspace, become blistered if the whole animal is immersed into a hypertonic solution of mannitol. This suggests
that the septate junctions prevent the paracellular flow of mannitol thus restricting the effects of the hypertonicity to the epidermal cells. An analogous blistering effect occurs with the tight junctions of frog urinary bladder on exposure to hypertonic solutions of urea (DiBonna, 1972).

Szollosi and Marcaillou (1977) have found that insects, as with vertebrates, have a blood-testes barrier and electron microscopic analysis of the cells forming the barrier revealed an abundance of septate junctions and only a very small number of other types of junctions. They also found that while such tracers as horse radish peroxidase and lanthanum hydroxide can penetrate the interspace of these junctions they do not penetrate the entirety of the interspace.

Thus septate junctions have an analogous role to tight junctions forming a permeability barrier and so preventing paracellular flow of substances. The recent finding of these junctions between Sertoli cells of mammalian testes where other intercellular junctions are also found (desmosomes, tight and gap junctions) raises the question as to what other roles these junctions may have (Connell, 1978).
1.5. **The Gap Junction (Nexus)**

1.5.1. **Introduction**

The gap junction is the most widespread of all the four classes of intercellular junctions. The apparent function of the gap junction is to allow the intercellular exchange of ions and small molecules and indeed, the currently accepted structure of the gap junction containing water filled pores which link the cytoplasms of 'coupled' cells is consistent with this notion. The following sections review the distribution and the structure of the gap junction. As so many reports have been published demonstrating the existence of gap junctions in numerous systems only representative studies will be cited. Also, only the major studies will be cited in reviewing the gross structure of the gap junction from different phyla. Finally, the published experimental evidence implicating the gap junction as the morphological basis of the intercellular exchange of substances will be given, where appropriate, in the survey dealing with the permeability of the junctional plasma membranes of coupled cells (section 1.6.).

1.5.2. **Distribution**

Gap junctions have so far been found in every multicelld animal
studied. For example they have been seen in the sponges (Revel and Mann, unpublished results), the mesozoon *Dycemid* (Wang and Revel, manuscript in preparation), the flatworm *Planaria* (plathyhelminthes; Quick and Johnson, 1977), *Hydra* (coelentrates; Hand and Global, 1972), the leech (annelids; Coggeshall, 1974), mussels (molluscs; Gilula and Satir, 1971), *Daphnia* (arthropod; Hudspeth and Revel, 1971), and *Dycoptera* (arthropod; Flower, 1972) and finally vertebrates (Dewey and Barr, 1962).

These junctions have been found between excitable and non-excitable cells of both vertebrates and invertebrates (Revel and Karnovsky, 1967; Pappas *et al.*, 1971; Rose, 1971; Quick and Johnson, 1977). Also gap junctions have been found between the cells of the early developmental stages of the mammalian embryo (*Ducibella et al.*, 1975) and in the larval stages of the insect (Lawrence and Green, 1975). Finally, many cells in tissue culture form these junctions (*Revel et al.*, 1971; Gilula *et al.*, 1972). Gap junctions have been found in so many tissues that it seems reasonable to believe that these organelles are a ubiquitous structure of multicelled animals being formed perhaps between the majority of cell types.

1.5.3. **Gross Structure**

In thin sectioned preparations of stained tissues the gap junction can be seen as a close apposition of the two plasma membranes to give
a pentalaminar or septilaminar profile depending on the staining technique (Dewey and Barr, 1962; Revel and Karnovsky, 1967). A gap of around 2 nm separates the two membranes and this 'gap' gave rise to the junction's name. The gap, or interspace, is permeable to lanthanum hydroxide and this feature distinguishes the gap junction from the tight junction which otherwise has a very similar appearance in stained thin sectioned preparations (Revel and Karnovsky, 1967). In tangential views, the gap junction can be seen to be a hexagonal array of particles spanning the gap (Revel and Karnovsky, 1967).

Gap junctions can be better viewed by freeze fracture techniques. Replicas of freeze-fractured tissue show the gap junction to be composed of a cluster of intra-membranous particles in both membranes which are in register with one another (McNutt and Weinstein, 1970). In P to E-face steps (Branton et al, 1975) occurring at gap junctions it is seen that there is a close apposition of the two membranes confirming the observations from thin sectioned tissues (McNutt and Weinstein, 1970). Also the particles for vertebrate gap junctions partition to the P-face leaving corresponding pits in the E-face. In contrast, for the arthropod form of the gap junction the particles partition to the E-face leaving the corresponding pits in the P-face (Flower, 1972; Gilula, 1972). Often a particle-free halo is seen around the junction (Johnson et al, 1975).

These features seen in thin section and freeze-fracture are common to all gap junctions although there is considerable structural
diversity. For example the vertebrate form of the junction has a particle size of around 7-8 nm with a centre to centre spacing of 9 nm (McNutt and Weinstein, 1973) whereas for the arthropods, the particles are considerably more heterogeneous being at least 11 nm in diameter and are packed much more irregularly (Flower, 1972). Also, there can be a fair degree of variation in the packing and arrangement of the particles between different cell types in the same animal (see Larsen, 1977 for a review). It has been proposed that the centre to centre spacing of the junctional subunits reflects the permeability state of the gap junction (Perrachia and Dulhunty, 1976; see below and section 1.7.).

As well as structural diversity that can occur in the same animal, there is also a considerable variation in the extent of area occupied by gap junctions between different cell types. In general, cells in excitable tissues are joined to a lesser extent by gap junctions than cells in non-excitatory tissues (Friend and Gilula, 1972). For example, in epithelial tissues such as mammalian liver and arthropod hepatopancreas the junctions are large and numerous (Friend and Gilula, 1972; Gilula, 1974a), whereas the rod and cone cells of the vertebrate retina and the muscle cells and neurons of Planaria are joined by small numbers of small junctions (Raviola and Gilula, 1973; Quick and Johnson, 1977). However, some cells of excitable tissues can be quite extensively joined by gap junctions such as mammalian cardiac muscle and intestinal smooth muscle (Revel and Karnovsky, 1967; Friend and Gilula, 1972).
1.5.4. **Chemical Structure**

By virtue of their resistance to detergents such as deoxycholate (Benedetti and Emmelot, 1968) and sarkosyl NL 97 (Goodenough and Stoekenius, 1972), gap junctions have been isolated in reasonably pure form from mouse liver (Goodenough and Stoekenius, 1972; Duguid and Revel, 1975; Culvenor and Evans, 1977; Ehrhart and Chaveau, 1977), rat liver (Gilula, 1974b; Finbow, Yancey, Johnson and Revel, manuscript submitted to Proc. Nat. Acad. Sci. US) calf lens fibre cells (Dunia et al., 1974) and mouse heart (Goodenough, 1978). Unfortunately gap junctions can only be isolated in pure enough form to permit chemical analysis if collagenase and trypsin are used at some stage during the isolation procedure (ex. Goodenough, 1976). This has led to a considerable variation in the reported sizes of the junctional protein(s). Attempts to reduce the extent of proteolysis during isolation by a very limited exposure to exogenous proteases have resulted in a considerable increase in the number of contaminants thus leading to confusing profiles of the proteins when separated by SDS polyacrylamide gel electrophoresis from junctional fractions (Goodenough, 1976; Culvenor and Evans, 1977).

Reported sizes for the junctional protein(s) range from mol. wts. 10,000, the so called connexins (Goodenough, 1974 and 1976; Gilula, 1974b), 18,000 (Goodenough, 1974 and 1976; Duguid and Revel, 1975), 25,000 (Duguid and Revel, 1975; Dunia et al., 1973), 34,000 (Dunia et al., 1973; Goodenough, 1974; Duguid and
Revel, 1975, Ehrhart and Chaveau, 1977) and 38,000 (Culvenor and Evans, 1977). Despite this great variation in the reported sizes it is generally agreed that the gap junction is composed of only one or two proteins.

Recent work carried out by Hertzberg and Gilula (1979) and Finbow et al (manuscript submitted to Proc. Nat. Acad. Sci. USA) has shown that gap junctions isolated in the absence of proteases but using a urea step to remove contaminants show a prominent band at mol. wt. 26,000 and a very diffuse band running between mol. wts. 45,000 to 52,000. These two groups showed the 34,000 component to be uricase. Moreover, Finbow et al showed that mild trypsinization of urea isolated gap junctions resulted in the rapid degradation of the 26,000 component while a component at 38,000 and the component at mol. wts 45,000 to 52,000 showed relatively little change. The disappearance of the 26,000 component paralleled the appearance of the connexin b component already believed to be of junctional origin (Goodenough, 1976). Finbow et al confirmed the 26,000 component and the connexin b component from trypsin treated samples to be of junctional origin using the rat regenerating liver. They showed both these components to have disappeared at times when it was known that gap junctions had transiently disappeared between hepatocytes (Yee and Revel, 1978; section 1.8.5.).

As yet no carbohydrate has been found in gap junctions which is unusual for a membrane protein (Revel et al, 1978). Analysis of the lipid component shows that it constitutes about half the weight in mouse gap junctions isolated using proteases with the major lipids
being phosphatidylcholine and phosphatidylethanolamine and a small amount of neutral lipid (Goodenough and Stoeckenius, 1972).

1.5.5. Fine Structure

A number of reports suggest that the subunits of the gap junction contain water filled pores which link the cytoplasms of contiguous cells. The first indication of the existence of these pores was the finding that the central region of each subunit could be penetrated by colloidal lanthanum during fixation prior to thin sectioning or by colloidal lanthanum during fixation prior to thin sectioning or (Revel and Karnovsky, 1967) or by soluble heavy metal salts (ex. uranyl acetate) during negative staining of plasma membrane fractions (Benedetti and Emmelot, 1968). McNutt and Weinstein (1970) also saw a central dot measuring about 2.5 nm in diameter in the centre of each subunit in well shadowed replicas of freeze-fractured tissues. They thus proposed a model of the gap junction in which each subunit contained a water-filled channel which ran the entire length of the subunit and so joined the interiors of cells. Goodenough and his colleagues (Caspar et al., 1977; Makowski et al., 1977) have carried out a detailed structural analysis of isolated mouse liver gap junctions in an attempt to prove the 'channel hypothesis' of McNutt and Weinstein (1970).

They confirmed that negative stains such as uranyl acetate could penetrate the centre of each subunit and by optical re-enforcement techniques showed that this region measured about 2 nm in diameter (Caspar et al.,
Analysis by X-ray diffraction on isolated gap junctions first pelleted by centrifugation to have the same orientation showed the central region to have a similar electron density as water whereas the perimeter of the subunit had an electron density similar to protein (Makowski et al., 1977). These two results are certainly suggestive of a central pore running through the entirety of each subunit. However, these analyses were carried out on gap junctions purified from plasma membrane fractions which had been treated with proteases (collagenase and sometimes trypsin; Caspar et al., 1977) and thus there remains the possibility that such channels do not occur in native gap junctions.

An earlier study by Perrachia (1973b) has shown that negative stain can also penetrate the centre of the subunits of arthropod gap junctions in homogenised tissues. Optical re-enforcement techniques showed the central region of the subunits from these junctions to measure about 2 nm. The studies on both the vertebrate and arthropod forms show the junctions to have hexagonal symmetry (Caspar et al., 1977; Makowski et al., 1977; Perrachia, 1973b).

The X-ray diffraction studies of Makowski et al., (1977) also showed that protein probably spans through both plasma membranes and the 2 nm gap. Moreover, these studies and the electron microscopic studies of negative stained isolated gap junctions and replicas of freeze-fractured tissues (Caspar et al., 1977) showed that while there was good long range order of the subunits, there was considerable short range disorder. Thus each subunit
has some degree of freedom of movement producing what has been termed a 'liquid crystal' (Caspar et al., 1977; Makowski et al., 1977).

Finally, as mentioned briefly above, there is evidence that a reduction in the centre to centre spacing of the junctional subunits correlates with 'uncoupling' (i.e. loss of permeability to ions and small molecules; Perrachia and Dulhunty, 1976; section 1.7.). There certainly is some variation in tissues of the packing of the subunits (Perrachia, 1977; Goodenough et al., 1978) and a close packing of the particles can be brought about by conditions which are known in some systems to cause uncoupling, for example intracellular increase in free calcium (Rose and Loewenstein, 1976; Perrachia, 1977 and 1978; Perrachia and Dulhunty, 1976). The X-ray analysis of isolated mouse junctions (Makowski et al., 1977) can be interpreted in samples where the packing of the subunits is close (i.e. a reduced lattice constant) to suggest that the putative channels are partially closed in these samples. However, these samples, as opposed to the samples with a higher lattice constant, had been treated with trypsin during their isolation (see table 1, Caspar et al., 1977) which is already known to cause the junctions to form vesicles instead of sheets (Goodenough, 1976; Ehrhart and Chaveau, 1977). Thus the relevance of this interpretation to the in vivo situation is not clear. As will be discussed later (section 1.7.) there are two schools of thought as to whether calcium directly modulates permeability of gap junctions and for the vertebrates whether it can cause uncoupling.
1.6. The Permeability of the Junctions Formed Between Animal Cells

1.6.1. Introduction

Numerous studies have been carried out over the last two or three decades to identify particular substances that may move between cells in contact with one another in order to gain more insight into cellular interactions. Nearly all classes of cellular substances have been examined and they all have been claimed by one study or another to move between contiguous cells. Some of the earlier studies suffered from experimental artefacts or the molecular basis was not fully understood and this led to some confusion. However, a clear picture is now emerging as to what substances can or can not move directly between contiguous cells.

The currently held view is that cells joined by gap junctions exchange between themselves inorganic ions and molecules up to mol. wt. 1,000 to 1,500 and thus cellular macromolecules are not transferred directly between cells. This intercellular exchange is thought to occur by passive diffusion through water filled pores believed to be located in the centre of each subunit (connexon) of the gap junction. The evidence supporting these views is analysed below.
1.6.2. Low Resistance Junctions and Electrical Coupling; The Transfer of Inorganic Ions and Tracer Dyes

The first indication that inorganic ions could move directly between animal cells came from a study by Weidmann (1952) on heart Purkinje fibres. The fibre cells are arranged in series along the fibre axis and Weidmann found that there was a low resistance core along the axis. He suggested that the cells were joined by low resistance membranes that allowed the intercellular movement of ions. The observation of Weidmann was extended in a study by Eurshpan and Potter (1959) on the nerve cord of the crayfish. They found that a depolarizing current pulse injected into the pre-synaptic lateral giant fibre passed with little attenuation to the post-synaptic giant motor fibre; that is, the two neurons were electrically coupled. The injection of the current pulse and the measurement of the subsequent voltage changes were carried out by impaling the neurons with glass micro-electrodes. This electrical transmission did not have the characteristic delay of the chemical synapse although they found the transmission could take place in only one direction (i.e., a rectifying synapse). However, later studies showed that 'electronic synapses' in other excitable systems, including different nerve fibres of crayfish, allow transmission in both directions (Watanabe and Grundfest, 1961; Hagiwara and Morita, 1962; Wilson, 1961).

Electrical coupling was believed to be special property of excitable
cells until Loewenstein and Kanno (1964) found that the cells of *Drosophila* salivary gland, a non-excitable epithelial tissue, were likewise electrically coupled. As with the neural fibres of crayfish (Furshpan and Potter, 1959), there was little attenuation of the injected current pulse in surrounding cells and indeed the resistance through a chain of salivary gland cells was only a little higher than that of the cytoplasm (150 ohms/cm\(^2\) compared to 100 ohms/cm\(^2\)) but, of course, was considerably less than that of the surface membrane (10^4 ohms/cm\(^2\)). They extended their studies by injecting the tracer dye fluorescein (mol. wt. 332) through a micro-electrode impaled into one cell and observed that it spread to surrounding cells that were electrically coupled to the injected cell. This led Loewenstein and Kanno (1964) to hypothesise the existence of 'low resistance junctions', postulated earlier by Weidmann (1952), which form between cells in contact and which allow the movement of inorganic ions and molecules between the cells.

Like gap junctions, low resistance junctions are particularly widespread in multicelled animals which would be expected if the two are synonymous. Electrical coupling has been found between cells from many different phyla such as the porifera (Loewenstein, 1967a), ascahelminthes (DeBell et al, 1963), annelids (Hagiwara and Morita, 1962; Wilson, 1961), molluscs (Potter et al, 1966), echinoderms (Tupper and Saunders, 1972), arthropods (Watanabe and Grundfest, 1962; Loewenstein and Kanno, 1964), tunicates (Miyazahi et al, 1974) and vertebrates (Nagai and Prosser, 1963; Furshpan, 1964). As mentioned
above electrical coupling is found between excitable and non-excitable cells from both invertebrates and vertebrates (Furshpan and Potter, 1959; Loewenstein and Kanno, 1964; Kuffler and Potter, 1964; Furshpan, 1964; Loewenstein et al, 1965; Kuffler et al, 1966) and also between a variety of embryonic cells (Potter et al, 1966; Sheridan, 1968; Tupper and Saunders, 1972; Miyahazi et al, 1974). Many cells in tissue culture have been found to be capable of electrical coupling (Furshpan and Potter, 1968; Hyde et al, 1969; Siegenbeck et al, 1970).

Finally, many tumour and transformed cells are capable of electrical coupling but there are also many examples that are incapable of such coupling (for references see beginning of section 1.8.5.).

Attempts have been carried out to discover what species of inorganic ions can move between electrically coupled cells. By injection of radio-isotopically labelled ions Bennett and his colleagues (Bennett et al, 1967) showed that $\text{Na}^+$, $\text{K}^+$, $\text{Cl}^-$, $\text{I}^-$ and $\text{SO}_4^{2-}$ could all pass between crayfish septate axons. They also found that $\text{Co}^{2+}$ could pass between these axons providing it was injected at a low enough concentration not to cause the axons to 'uncouple' (Politoff et al, 1972; see section 1.7.).

In the vertebrates it has been shown that $\text{Na}^+$, $\text{K}^+$, $\text{Rb}^+$ and tetra-ethyl ammonium ions pass between coupled cells. The studies on the vertebrates employed quite different techniques to those used for arthropods. Weidmann (1966) and Weingart (1974) used bundles of myocardial cell fibres drawn through a small hole between two (or
more) chambers. They then incubated the fibres in a bathing solution and to one of the chambers added the tracer ion to be examined. Weidmann used the radioisotope of potassium $^{42}$K and Weingart used $[	ext{14C}]$-tetraethyl ammonium. After the appropriate length of incubation the fibres were removed, sliced and the slices counted for radioactivity. Both Weidmann and Weingart found that these two ions had moved quite far distances along the bundle of fibres extending into the chamber not containing the added tracer ions.

A novel approach has been used in showing transfer of Na$^+$, K$^+$ and Rb$^+$ between cultured cells (Corsaro and Migeon, 1977a; Ledbetter and Lubin, 1979). In these experiments the differential sensitivity to the cardiac glycoside ouabain of the mouse and human form of the plasma membrane bound Na$^+$/K$^+$ ATPase was utilized. At a low concentration ($10^{-5}$ M) of this inhibitor in the culture medium the human cells (oub$^S$) are unable to grow due to a sodium/potassium imbalance whereas the mouse cells (oub$^R$) are unaffected (Ledbetter and Lubin, 1977). However, the human cells are able to grow at a normal rate in the presence of this inhibitor if they are in contact with the mouse cells. This can be explained by the mouse cells restoring the sodium/potassium balance in the human cells because of the ability of the interacting cells to transfer these two ions between themselves when they are in contact with each other.

Ledbetter and Lubin (1979) have extended this system by using the
radioisotope of rubidium, $^{86}\text{Rb}$ as an analogue of potassium. They found that in mixed cultures of oub$^S$ and oub$^R$ cells in the presence of oubain the mixed cultures contained a higher amount of $^{86}\text{Rb}$ than would be expected. Again this result can be explained by the transfer of $^{86}\text{Rb}$ between the oub$^R$ and oub$^S$ cells. (These results can also be explained by the transfer of the oub$^R$ form of the enzyme but this is probably unlikely as proteins are not normally transferred between cells; see section 1.6.4.).

Finally, a brief report by Turin (1977) shows the transfer of Co$^{2+}$ and AuCl$_3$OH$^-$ between the cells of the early developmental stages of Xenopus embryos. He injected these ions into one cell and after precipitation of the heavy metals with (NH$_4$)$_2$S saw that they had spread to other cells. However, using the same technique, He found that PbEDTA$^{2-}$ did not appear to move from the injected cell to the surrounding cells. The significance of this result is discussed below.

As mentioned above low resistance junctions are also permeable to tracer dyes. The most commonly employed tracer dyes are fluorescein and procion yellow although many other dyes have been used and found to be transferred (Kanno and Loewenstein, 1966). A number of fluorescent tagged amino acids, oligopeptides and oligosaccharides have been found to pass between arthropod and between vertebrate cells (Johnson and Sheridan, 1971; Simpson et al, 1977;
Loewenstein, 1975). Cell to cell passage of these fluorescently labelled molecules will be dealt with in more detail later on in this survey.

Generally it has been found that cells which are electrically coupled can also transfer dyes (Sheridan, 1976) but there are a number of exceptions, notably embryos in the early developmental stages, where only electrical coupling has been found. These studies will be reviewed in section 1.6.8, dealing with the mechanism of intercellular transfer because they have implications in assessing whether there is a common pathway or multiple pathways of intercellular transfer of substances.

Typical examples of where dye transfer has been found along with electrical coupling are between a variety of cultured vertebrate cells (Furshpan and Potter, 1968; Johnson and Sheridan, 1971; Azarnia and Loewenstein, 1971), re-aggregated embryonic *Xenopus* and *Fundulus* cells (Sheridan, 1971a, Bennett et al, 1978), insect salivary gland cells (Kanno and Loewenstein, 1966) and crayfish septate axons (Payton et al, 1969). However, not all dyes are able to be transferred between electrically coupled cells. Imanaga (1974), using the myocardial fibre technique developed by Weidmann (1966) found that whereas the dye procion yellow (mol. wt. 630) was transferred between sheep myocardial fibre cells the dye Chicago sky blue (mol. wt. 1,000) was not transferred. This suggests a size limit on transfer and there is now good evidence that such
a size limit does exist for arthropods and possibly for vertebrates of around this mol. wt. value (see following sections).

In most cases so far examined electrical coupling correlates with the existence of gap junctions between the coupled cells (ex. Barr et al, 1965 and 1968; Asada and Bennett, 1971; Pappas et al, 1971). In some cases these are the only junctions that have been found (Furshpan and Potter, 1968; Sheridan, 1971b; Revel et al, 1971; Johnson and Sheridan, 1971; Gilula et al, 1972). It has also been shown that cells unable to form these junctions are unable to become electrically coupled (Gilula et al, 1972; Azarnia and Loewenstein, 1977; Larsen et al, 1977). Moreover, the establishment of electrical coupling between cells correlates very well with the formation of gap junctions (Sheridan, et al, 1978; Griepp et al, 1978). However, it is possible that in some special circumstances that electrical coupling could occur in the absence of these structures (de Laat et al, 1976; de Laat and Bart, 1976).

Two studies have been carried out to calculate the size of the putative water filled pores occurring in the gap junction by using an estimate of the junctional permeability. In one study Sheridan et al (1978) measured the average area occupied by gap junctions between Novikoff rat hepatoma cells and the junctional conductance. From this data they estimated the diameter of the pore to be about 2 nm. In the other study Weingart (1974) compared the rates of
diffusion of potassium (Weidmann, 1966), procion yellow (Imanaga, 1974) and the tetraethyl ammonium ion (Weingart, 1974) along myocardial fibres with the sizes of these three substances. From this data he calculated the size of the pore to be somewhat greater than 1 nm. Both these studies give surprisingly close values to the estimate obtained by Goodenough and his collaborators (2 nm; Caspar et al, 1977; Makowski et al, 1977) from X-ray analysis and negative staining techniques of isolated mouse liver gap junctions. As will be discussed below an estimate of the pore size of arthropods gap junctions by using tracers has been obtained (Simpson et al, 1977; section 1.6.5.)

1.6.3. RNA, DNA and Nucleotides; the Phenomenon of Metabolic Co-operation

Many studies have now shown that nucleotides can be readily exchanged between a variety of cultured animal cells in contact with one another. As with electrical coupling, the ability to transfer nucleotides correlates with the ability to form gap junctions (see below for references). Thus cells that can form gap junctions can exchange nucleotides but cells that cannot form these junctions do not exchange nucleotides. The studies on nucleotide transfer have been mainly confined to the vertebrates but there is a report that electrically coupled invertebrate cells can also exchange nucleotides (Rieske et al, 1975).
It is generally accepted that macromolecules do not pass through intercellular junctions (cf. Pitts and Simms, 1977) but there are two claims in the literature that nucleic acids do pass. Kolodny (1971) reported that RNA but not nucleotides was transferred between cells and a report by Bendich et al., (1967) claimed to show DNA transfer. The evidence concerning transfer of DNA will first be reviewed.

Bendich et al., (1967) observed that after transformation of BHK cells (a hamster fibroblast cell line) by a viral or chemical agent, the cells formed numerous pseudopodia-like cytoplasmic extensions which often make contact with other cells. These "intercellular bridges" frequently contained DNA as seen by cytochemical staining and autoradiography after first labelling the cells with $[^{3}H]$-thymidine. They therefore suggested that these bridges could act as a pathway for the direct transfer of DNA between cells. This apparent DNA transfer seems to be restricted to transformed cells as they found that BHK cells do not form such DNA containing bridges. Of course this observation is only suggestive and it still awaits to be unequivocably proven.

There is also experimental evidence that cells in culture can uptake DNA added to the culture medium and in some cases express and replicate this internalized DNA (Ottolenghi-Nightingale, 1974, for a review). Whether such DNA transfer occurs to any extent in vivo is not known but if it does it is obviously an indirect
mode of transfer not dependent on cell contact.

Two independent studies have shown that DNA is not transferred between cultured cells. Peterson and Rubin (1970) and Pitts and Simms (1977) labelled fibroblasts with $[^3]H$-thymidine and after chasing out the labelled thymidine nucleotide pools by a period of growth in unlabelled medium, added unlabelled fibroblasts and grew the mixed cultures for up to 24 h. Autoradiographic analysis showed no detectable transfer of DNA between contacting or non-contacting labelled and unlabelled cells. In view of the many studies that have used the ability to mark populations of cells by labelling their DNA with radioactive labelled thymidine and the two above studies, it seems safe to conclude that DNA is not normally transferred between animal cells. Further experimental evidence against transfer of DNA is given below in the studies showing nucleotide transfer.

The experiments of Kolodny (1971 and 1972) appear to show quite conclusively that RNA is transferred between cells but uridine nucleotides are not transferred. For these experiments Kolodny used 3T3 cells (a mouse fibroblast cell line) and established a method by which two populations of cells could be physically separated from one another after being grown together. Cultures of 3T3 cells were first allowed to ingest tantalum particles. These 'heavy' cells were labelled with $[^3]H$-uridine for several h and then unlabelled, 'light' cells were seeded into the cultures
of heavy cells and the mixed cultures grown for several more h. The cells were then suspended and layered on a ficoll gradient. Thus, by centrifugation, Kolodny was able to separate the heavy cells from the light cells; the heavy cells being spun to the bottom of the gradient and the light cells remaining at the top.

Kolodny found that after a 4 h co-culture there was a substantial amount of tritium counts in the RNA of the light cell fraction. He interpreted this result as evidence for the intercellular movement of molecules derived from uridine, i.e. RNA and uridine nucleotides. The amount of labelled RNA appearing in this fraction was unaffected by inhibiting RNA synthesis during the co-culture with actinomycin and thus Kolodny concluded that RNA can be transferred between 3T3 cells but uridine nucleotides can not be transferred. Kolodny did not present evidence on the possible mechanism of this apparent RNA transfer but speculated that intercellular junctions may be involved.

In view of the many recent studies which show conclusively the transfer of nucleotides and not RNA (see below), it appears that Kolodny's interpretation is erroneous. It is possible that the separation technique used by Kolodny may not be effective enough to use in these types of studies. This possibility is supported by Kolodny's (1973) claim to have demonstrated protein transfer as well and there is now abundant evidence showing otherwise (see section 1.6.4.).
The first indication that nucleotides could be freely transferred between animal cells in contact came from a chance observation by Subak-Sharpe et al (1966 and 1969). They found that when mutant BHK cells which lacked hypoxanthine; guanine phosphoribosyltransferase activity (HGPRT-) and thus unable to incorporate hypoxanthine, made contact with wild-type BHK cells in medium containing [3H]-hypoxanthine, the mutant cells became labelled to a far greater extent than mutant cells not in contact with wild-type cells. This phenotypic change also occurred to mutant cells in contact with the wild-type cells through other mutant cells. In fact Pitts (1976) detected a phenotypic change in mutant cells at least 50 cell diameters away from the wild-type cells producing a gradient of incorporation of labelled material (also see Michalke, 1977).

Subak-Sharpe et al (1966 and 1969) termed this contact mediated phenotypic change of the mutant cells metabolic co-operation and proposed that this change could be due to the transfer from the wild-type cells of (a) labelled nucleotides and/or polynucleotides synthesised from these nucleotides, or (b) the wild-type form of the enzyme or the information to synthesise the enzyme (i.e. the mRNA or gene coding for this enzyme).

Pitts (1971) and Cox et al (1970) independently presented evidence against transfer of molecules of the second category. They grew wild-type and HGPRT- fibroblasts together at a density where most
mutant cells were in contact with wild-type cells and, after a prolonged growth period, re-seeded the cultures at low density where most of the cells were no longer in contact with one another. They argued that if the wild-type cells transfer the active enzyme (or the mRNA or gene coding for the enzyme) then the mutant cells would be able to incorporate $[^3\text{H}]$-hypoxanthine after separation from the wild-type cells. However, both Pitts and Cox et al found that incorporation of radioactivity derived from $[^3\text{H}]$-hypoxanthine into the mutant cells stopped immediately after separation.

Although these experiments demonstrate that the mRNA and the DNA coding for the wild-type form of the enzyme HGPRTase are not transferred directly between cells they do not rule out the possible transfer of polynucleotides included in the first category. For example, the labelled material appearing in the mutants could be due to the transfer of labelled rRNA, tRNA or the DNA coding for these two types of RNA.

In contrast to the conclusions of Cox et al, (1970) and Pitts (1971), Ashkenazi and Gartler (1971) suggested that metabolic co-operation was due to the transfer of the wild-type form of the enzyme. They found that HGPRTase activity could be partially restored in HGPRT fibroblasts by adding sonicates of wild-type fibroblasts to the growth medium. Likewise, there was a similar partial restoration of branched chain amino acid decarboxylase activity (BCD) in mutant fibroblasts lacking this enzymic activity when sonicates of wild-type fibroblasts were added to the medium.
There is now substantial evidence that fibroblasts can readily 
uptake from the growth medium added enzymes, notably lysosomal 
enzymes, which retain their activity intracellularly (for a review 
see Neufield, 1974).

Obviously, this is not the basis of metabolic co-operation as cell 
contact is not required for partial restoration. In fact, the 
experiments of Cox et al (1970) and Pitts (1971) show that HGPRTase 
activity is not restored in the mutant cells. Indeed Pitts (1971) 
showed that he could have detected HGPRTase activity in the mutant 
fibroblasts even if it was only present in these cells 10 min after 
separation from the wild-type fibroblasts. Thus the lack of HGPRT-
ase activity in the mutant cells after separation in his experiments 
can not be attributed to a rapid degradation of any transferred 
wild-type HGPRTase.

Since the discovery of metabolic co-operation other mutant 
cultured fibroblasts that have lesions in other salvage enzymes 
have been shown to be likewise phenotypically changed by contact 
with wild-type cells. These include mutants which lack activity 
in the enzymes adenine phosphoribosyltransferase (APRT^−; Burk 
et al, 1968), thymidine kinase (TK^−; Pitts, 1971) and deoxycytidine 
kinease (Goldfarb et al, 1974). Of course the resulting labelled 
polynucleotide in comparable experiments with the TK^− and dCK^− 
mutants is DNA, whereas for the HGPRT^− and APRT^− cells it is both 
DNA and RNA.
The first demonstration that metabolic co-operation was due to nucleotide transfer and not polynucleotide transfer came from an experiment carried out by Pitts (1971) who used mixed cultures of HGPRT− and TK− BHK cells. He grew these two cell types in a 1:1 ratio at a density where most of the cells were in contact with each other and added aminopterin, hypoxanthine and thymidine to the growth medium. Aminopterin inhibits folate metabolism and consequently the cells are unable to synthesise purines and thymidine by de novo pathways and therefore rely on exogeneous sources of these two metabolites. Hence, neither of these two types of mutant are able to grow by themselves in this selective medium. However, Pitts discovered that the two cell types in these mixed cultures grew at their normal rate in this medium. Further, he found that if he varied the ratio of the two cell types from 95:5 for either cell type there was initial cell death followed by a growth period where the two cell types were found to be in a 1:1 ratio, thus demonstrating that the cells were dependent upon each other for growth.

It is difficult to explain this result in terms of polynucleotide transfer because it requires the transfer of whole genomes and their RNA transcripts between the cells which from the earlier studies appears not to occur (Pitts 1971; Cox et al, 1970). However, the results can be easily explained by the transfer of nucleotides between the cells. Thus the HGPRT− cells supply the TK− cells with thymidine nucleotides and the TK− cells supply the HGPRT− cells with
purine nucleotides (Pitts, 1971). This result also demonstrates that nucleotide transfer is bidirectional confirming a similar observation of Burk et al (1968) who used HGPRT\textsuperscript{−} and APRT\textsuperscript{−} fibroblasts in mixed culture.

From knowing the amount of RNA per cell and the average generation time, Pitts (1976) calculated from his earlier experiments the minimum flux of purine nucleotides between the cells to be \(10^6\) nucleotides/cell pair/sec which obviously is a fast rate of transfer.

Sheridan et al (1975 and manuscript in preparation) have also obtained results using metabolic co-operation which likewise can only be explained by nucleotide transfer. They found that wild-type BHK cells in the presence of a high concentration of exogenous hypoxanthine have an expanded purine nucleotide pool derived from hypoxanthine which resulted in a reduction of purine synthesis by \textit{de novo} pathways as shown by a decreased level of formate incorporation. Of course the HGPRT\textsuperscript{−} BHK cells were unaffected in anyway by the high level of hypoxanthine. However, when both cell types were grown together at varying ratios, the activity of the \textit{de novo} pathway of purine synthesis in the mutant cells was also reduced. This result can be explained by an elevated level in the mutant cells of purine nucleotides which have been synthesised from hypoxanthine in the wild-type cells and subsequently transferred to the mutant cells.
The results of Pitts (1971) and Sheridan et al. (1975) raise the intriguing possibility that cells when they are in contact with each other may not be individual units of metabolism, at least with respect to purine nucleotides. The metabolism of purine nucleotides in these experiments appears to be integrated between all the cells in the culture and is not the sole property of each cell. The recent studies discussed above (section 1.6.2.; Corsaro and Migeon, 1977a and Ledbetter and Lubin, 1977b) on the 'rescuing' of oubain sensitive cells by oubain resistant cells corroborate this suggestion because they indicate that the control of intracellular inorganic ion concentrations (Na⁺ and K⁺) may also be shared in populations of electrically coupled cells.

Further evidence of nucleotide transfer being the basis of metabolic co-operation comes from a study by Cox et al. (1976). They enucleated human skin fibroblasts with cytochalasin b to produce karyoplasts (nucleus surrounded by a small amount of cytoplasm and plasma membrane) and cytoplasts (cytoplasm surrounded by plasma membrane). Because cytoplasts do not contain a nucleus they are unable to synthesise RNA (except presumably mitochondrial RNA) and this can be seen, using autoradiography, by their lack of incorporation of [³H]-hypoxanthine into cellular material. However, when HGPRT⁻ human skin fibroblasts came into contact with cytoplasts the fibroblasts were found to have extensively incorporated tritium into cellular material. Because the cytoplasts could not synthesise RNA then the labelled cellular material appearing in the HGPRT⁻ cells must be due
to the transfer from the cytoplasts of labelled purine nucleotides derived from $[^3H]$-hypoxanthine which are subsequently incorporated by the mutant cells into RNA and DNA. Further, the cytoplasts themselves did not become labelled in these situations even though the mutant cells contained labelled RNA.

To resolve the results obtained by Kolodny (1971) with the contrasting results obtained from metabolic co-operation, Pitts and Simms (1977) developed a uridine pre-labelling method suggested by Kolodny's work but instead of analysing the results by a physical separation of the cells, they used autoradiographic procedures that had been employed for the earlier metabolic co-operation studies. They first labelled dilute cultures of BHK cells with $[^3H]$-uridine for several h (termed donor cells) and, after extensively washing the cells fresh, unlabelled BHK cells (termed recipient cells) were added and the mixed cultures grown for several h before processing for autoradiography. During the fixation procedure the cultures were washed with trichloracetic acid (TCA) and therefore the $[^3H]$-uridine nucleotides were removed to leave behind RNA as the only labelled molecules. Pitts and Simms (1977) found extensive transfer of labelled material to recipient cells in contact with the donor cells in a pattern analogous to metabolic co-operation.

When actinomycin was present during the co-culture there was a large reduction in the amount of labelled material found in the recipient cells. As evidence that actinomycin was not somehow interfering
with the transfer process, Simms (1973) showed that TK⁻ BHK cells could still be phenotypically changed by contact with wild-type BHK cells in the presence of this inhibitor. Pitts and Simms argued that if RNA was transferred between the cells then there would be little or no effect on the amount of labelled material appearing in the TCA insoluble material of the recipients in the presence of actinomycin. Therefore, the labelled material appearing in the recipients must be due to the transfer of \(^{3}H\)-uridine nucleotides. The residual amount of labelled material found in the recipients when actinomycin was used could be accounted for by an incomplete inhibition of RNA synthesis.

As further proof of uridine nucleotide transfer and not RNA, Pitts and Simms (1977) grew the labelled donor cells for 24 h (termed chased donors) before culturing with recipients. In the chased donors most of the \(^{3}H\)-uridine nucleotides had become incorporated into stable species of RNA and therefore, in contrast with the 'unchased' donor cells which had an approximately equal number of tritium counts located in uridine nucleotides and RNA, practically all of the tritium counts in these cells were in RNA with very few counts located in uridine nucleotides. They found the recipients in these experiments had very little labelled TCA insoluble material. This of course is the predicted result if uridine nucleotides are transferred between the cells but not RNA. A similar result was reported by Cox et al (1974 a and b) when they found a much reduced amount of labelled material in HGPRT⁻ fibroblasts if the wild-type fibroblasts were pre-labelled with \(^{3}H\)-hypoxanthine
instead of adding the $[^3H]$-hypoxanthine during the co-culture.

A recently published report by Lawrence et al. (1978) suggests that cyclic AMP can also be transferred between cells. They grew primary cultures of mouse myocardial and granulosa cells together. These two cell types respond to nor-adrenaline and follicle stimulating hormone (FSH) respectively with cyclic AMP as the second messenger in both cases. Addition of nor-adrenaline to the medium of the mixed cultures evoked a response in both cell types but only the myocardial cells responded in separate cultures. Likewise addition of FSH brought about a response in both cell types in the mixed cultures where only the granulosa cells responded in separate cultures. These results can be explained by the cyclic AMP synthesised in one of the two types of target cells in response to the appropriate stimulus being transferred to the other target cell type and consequentially evoking a response in both cells. These experiments offer an exciting prospect in our understanding of hormone action at the tissue level.

A preliminary report by Tsien and Weingart (1974) showed the transfer of tritium activity along calf myocardial bundles using the cut end method of Weidmann (1966 and see section 1.6.2.) when one end of the bundle was incubated in the presence of $[^3H]$-cyclic AMP. As a phosphodiesterase inhibitor (Ro 7-2956) was added to the bathing solutions they suggested that cyclic AMP could move between the cells. A more detailed study (Tsien and Weingart, 1976) showed that most of the $[^3H]$-cyclic AMP had in fact been converted to other
purine nucleotides even in the presence of Ro 7-2956. However, there was a slight but reproducible increased twitch tension in the region of the bundle adjacent to the portion incubated in the presence of cyclic AMP thus indicating that despite the rapid conversion to other purine nucleotides, some cyclic AMP reached this test region.

A system which initially supported the cell to cell movement of cyclic AMP has since been found to be open to other interpretations. The toad urinary bladder epithelium is made up of two major cell types, the granular cells (G-cells and comprise about 80%) and the mitochondrial-rich cells (MR-cells and comprise about 15%; Choi, 1963). The epithelium is involved in sodium and water transport and morphological studies have shown the G-cells to be the major transporting cells (DiBonna et al, 1969; Davis et al, 1974). This transport is controlled by the polypeptide hormone vasopressin which is mediated intracellularly by cyclic AMP (Handler et al, 1965). In contrast to the morphological studies, Scott et al (1974) found that only the MR-cells showed a large intracellular increase in cyclic AMP in the presence of oxytocin (which also has antidiuretic activity) when the G- and MR-cells had been separated from each other by first incubating the epithelium with EDTA, followed by centrifuging the resulting cell suspensions on a ficoll gradient. However, Goodman et al (1975) showed that in the intact epithelium there was an equal distribution of cyclic AMP in response to addition of vasopressin. On the basis of the results of Scott et al (1974), they suggested that cyclic AMP is transferred between the cells thus accounting
for the morphological response seen in the G-cells. A later study by Handler and Preston (1976), however, showed that isolated G-cells could synthesise cyclic AMP as well as MR-cells in response to vasopressin if the G-cells were first disrupted by homogenisation. Thus, the results of Scott et al (1974) may be due to experimental artefacts which occur during isolation and therefore this system cannot be taken as direct evidence for the intercellular movement of cyclic AMP.

In summary, there is a large amount of evidence that cells in culture can freely transfer between themselves a variety of purine and pyrimidine nucleotides. However RNA and DNA are probably not transferred in chemically significant amounts. Most of the studies described above were carried out on fibroblast cells in culture. However many other different cell types in culture have now been found to transfer nucleotides. These cells include epithelial cell lines (Pitts 1972; Pitts and Burk, 1976; Michalke, 1977), as well as a number of primary epithelial cells (Fentiman et al, 1976; Lawrence et al, 1978) myocardial cells (Lawrence et al, 1978), myoblasts (Kalderon et al, 1977) and some tumour and transformed cells including embryonal carcinoma cells (Subak-Sharpe et al, 1966 and 1969; Pitts, 1972; Pederson et al, 1976; Corsaro and Migeon, 1977b; Hooper and Slack, 1977; Nicolas et al, 1978). However, not all cells are capable of transferring nucleotides, for example L cells, mast cells and a number of tumour and transformed cells (Pitts, 1972; Gilula et al, 1972; Azarnia and Loewenstein, 1972; Cox et al, 1974b; Fentiman et al, 1976).
Also Pitts (1972) and Cox et al. (1974b) have found a number of cell types which have a reduced ability to transfer nucleotides compared to fibroblasts. In some cases, transformed fibroblasts exhibit a lower capacity to transfer nucleotides (Pitts, 1972; Corsaro and Migeon, 1977b) than the fibroblasts from which they were derived. There are now a number of instances that show the ability to transfer nucleotides is correlated with the ability to form gap junctions and become electrically coupled, (Gilula et al., 1972; Revel et al., 1971; Pitts, 1971; Pederson et al., 1976; Johnson and Sheridan, 1971; Kalderman et al., 1977; Lawrence et al., 1978).

In general it has been found that there is no specificity of nucleotide transfer between different cell types. Thus mammalian cells can exchange nucleotides with amphibian cells and amphibian cells can transfer nucleotides to fish cells (Pitts, Kuluska and Ferry, manuscript in preparation). Similarly, granulosa and myocardial cells exchange nucleotides (Lawrence et al., 1978). However, for certain types of epithelial cells it has been found that they only exchange nucleotides readily with other epithelial cells (Pitts and Burk, 1976; Fentiman et al., 1976; also see Pitts, 1978). Although, over long periods of contact with fibroblasts, they can probably exchange nucleotides with these cells as efficiently as they do with other epithelial cells (Pitts unpublished data but see section 4.2.).

Recently Nicolas et al. (1978) have reported a similar specificity
for multipotent and nullipotent embryocarcinoma (EC) cell lines. They find that the cells of these cell lines are able to communicate by nucleotide exchange very well with themselves but not with fully differentiated cells and to varying degrees with other EC cell lines. This is in contrast to the result of Hooper and Slack (1977) who found that cells of a similar EC cell line exchanged nucleotides efficiently with hamster fibroblasts.

Pitts and Burk (1976) observed that the fibroblasts and epithelial cells used in their study sorted out from one another in a pattern similar to that found earlier by Steinberg and Garrod (1975) for chick embryo liver and limb bud cells. This led Pitts and Burk to suggest that the specificity probably did not lie in the communication mechanism itself (i.e. gap junction) but elsewhere on the cell surface. Nicolas et al (1978) also found that during in vitro differentiation of one of the EC cell lines (PCC3/AL1) used to generate differentiated cells in their study and which exhibited specificity, the EC cells remained separated from the resulting differentiated cells.

The functional significance of this specificity of junctional communication is not clear. Although it could be imagined that such specificity would allow the establishment of 'patterns of communication' in a tissue, all these studies were carried out in tissue culture and thus these different cell types may not normally occur together in vivo situations.
All of the above studies were carried out in vitro but there is evidence that cells in vivo can likewise transfer nucleotides. Pitts (1972) injected HGPRT− polyoma transformed BHK cells into hamsters to form tumours and then injected \(^{3}H\)-hypoxanthine. Autoradiographic analysis of the tumours and host tissues showed a gradient of incorporation of radioactivity along the tumour typical of metabolic co-operation.

Two studies have been carried out on coupled invertebrate cells to discover if they could exchange nucleotides and nucleic acids. In an unpublished study by Loewenstein (but see Loewenstein, 1975) there was lack of transfer of the RNA of bacteriophage F-2 (mol. wt. around \(10^6\)) between insect salivary gland cells.

A more detailed study has been carried out by Rieske et al (1975) on the electrically coupled giant neurons (Retzius cells) of the leech which occur in pairs in each segmental ganglion. They injected \(^{3}H\)-orotic acid and \(^{3}H\)-uridine into one of the Retzius cells and saw by autoradiography that the adjoining Retzius cell also became labelled although to a somewhat lesser extent. In the injected cell the nucleus became labelled first but shortly afterwards the perikaryon also becomes quite heavily labelled. For the non-injected cells the nucleus was always the site of the heaviest incorporation with very little labelling of the perikaryon. This pattern of labelling in the coupled, non-injected Retzius cell is more consistent with the transfer of labelled low molecular weight precursors that the transfer of labelled high molecular weight
products (i.e. RNA). This is because if high molecular weight products were transferred, then it would be expected that the perikaryon areas of the non-injected cell adjacent to the sites of transfer to exhibit equal or higher labelling than its nucleus. These transfer sites appear not to be located in the somata where the nucleus is found as the somata of the Retzius cells were seen not to be in contact (Rieske et al., 1975).

Thus, the pattern of labelling in the coupled, non-injected Retzius cells can be explained by the transfer of low molecular \(^3\text{H}\)-precursors (i.e. nucleotides) which were washed out during autoradiographic processing and therefore only the nucleus was seen to be labelled where the precursors had been incorporated into high molecular weight products (i.e., RNA). Rieske et al. (1975) also injected other \(^3\text{H}\)-metabolites and found transfer of radioactivity to the adjoining Retzius cell (see following sections). They did not find transfer to surrounding glia cells although it is known that the glial cells of the leech are electrically coupled to one another (Kuffler and Potter, 1964). A recent morphological study has also shown that some, as yet, unidentified neurons of the leech central nervous system are joined by gap junctions (Gomer, personal communication).

Finally, it is worth turning attention to the embryonic situation where it was believed that the transfer of macromolecules from mesenchyme tissues to ectodermal tissues could be responsible for embryonic induction (see Tiedmann, 1967). An electron microscopy study using autoradiographic techniques by Kelley (1968) showed
that when *Xenopus* chordamesoderm which had been pre-labelled with $[^{3}{H}]$-uridine, was grown next to presumptive neuroectoderm for several h there was transfer of labelled material. Analysis of the distribution of the labelled material showed the nucleolar regions to be particularly heavily labelled. Kelley interpreted these results as the transfer of labelled RNA which was subsequently rapidly degraded or the transfer of labelled uridine and/or labelled uridine nucleotides.

A later study by Grainger and Wessells (1974) showed conclusively that RNA is not transferred during embryonic induction from the inducer tissue to the responding tissue. They pre-labelled chicken or mouse lung mesenchyme with $[^{15}{N}]$ and $[^{3}{H}]$-nucleosides and then grew this tissue with lung epithelium during which time induction took place. If any species of RNA from the mesenchyme had been transferred to the epithelium then they could be detected by their high density on cesium chloride gradients due to being synthesised from $[^{15}{N}]$-nucleosides. No heavy species of RNA were found in the RNA extracted from the lung epithelium although the RNA was labelled with tritium showing that transfer of nucleosides and/or nucleotides had taken place. By this method Grainger and Wessells could have detected a level of transfer of only 0.01% of the pre-labelled RNA and thus they concluded that RNA is not the signal for embryonic induction.

Whether nucleotides can be transferred directly between two interacting
tissues during embryonic induction still remain unresolved as the above described observations could be accounted for by either direct transfer of labelled nucleotides or an indirect transfer (ex. the culture medium) of labelled nucleosides.

1.6.4. Proteins

Two early reports have claimed to show the transfer of proteins between a variety of animal cells in contact but have subsequently been shown to suffer from artefacts. A study by Kanno and Loewenstein (1966) initially showed the transfer of fluorescein labelled bovine serum albumin (mol. wt. 68,000) between the electrically coupled cells of the Drosophila salivary glands. They injected the labelled protein through micropipettes impaled into one cell and found the fluorescence spread from the injected cell to other surrounding cells suggesting the proteins had been transferred. However, a later study showed that fluorescein labelled albumin is not transferred between the cells of the Chironomus salivary gland (Simpson et al, 1977 and Loewenstein, 1975) and the spread of fluorescence in the earlier experiments was probably caused by released fluorescein (or fluorescein labelled oligopeptides) due to degradation of the injected proteins.

Bennett and his colleagues have also discovered that an earlier study carried out by themselves could suffer from experimental artefacts. They (Reese et al, 1971) found that after micro-injection
of microperoxidase (mol. wt. 1,800) into the lateral giant (septate) axon of crayfish it appeared to spread to the neighbouring axon as seen by histochemical staining techniques. Later experiments indicated that this intercellular movement occurred during the fixation of the tissue and not while the cells were living (Bennett et al., 1973).

As well as transfer of RNA, Kolodny (1973; see section 1.6.3.) has also claimed that 3T3 cells exchange protein. He used the same separation techniques as that used to show RNA transfer. In view of the erroneous results given by this method these experiments must be repeated under much more controlled conditions before they can be accepted as evidence for transfer of proteins.

Evidence against transfer of protein on a gross scale between cells in contact comes from two independent experiments carried out on secondary chick fibroblasts and BHK cells by Peterson and Rubin (1969), and by Pitts and Simms (1977). After labelling the protein of either of these two fibroblasts with $^{3}$H-leucine and seeding unlabelled fibroblasts next to these cells, no transfer of labelled material can be seen to the cells in contact with the pre-labelled cells. The experiments on metabolic co-operation also show that individual proteins such as HGPRTase (Cox et al., 1970 and Pitts, 1971) are not transferred. Cox and his collaborators (Cox et al., 1972) have extended these studies by culturing wild-type human skin fibroblasts with mutant cells which lacked glucose-6-phosphate dehydrogenase activity. After prolonged contact with the wild-type
cells, the dehydrogenase activity could not be detected by cytological staining techniques in the mutant fibroblasts.

Two studies carried out on chimeric mice appear on first sight to give conflicting results on protein transfer. A study by Mintz and Baker (1967) showed that the enzyme isocitrate dehydrogenase was not transferred between cells from a variety of mouse tissues. They made chimeric mice from two parental strains of mice which had different forms of this enzyme that could be readily distinguished from one another by their different electrophoretic mobility. Analysis of the resulting adult tissues showed that in all tissues examined, except skeletal muscle, only the two parental strains of the enzymes were present. However, in skeletal muscle a hybrid form was also present and this difference can be explained by the cells of skeletal muscle being multinucleate and therefore they will have genetic material from both parental strains. Because the hybrid form was not found in tissues where the vast majority of the cells are mononucleate, these experiments show that this enzyme, or the means to synthesise this enzyme (i.e. mRNA or DNA), is not transferred between cells.

A more recent study by Feder (1976) gave contrasting results to the experiments of Mintz and Baker (1967). He made chimeric mice from wild-type parents and mutant parents which lacked β-glucoronidase activity. Histochemical staining of many of the adult tissues for β-glucoronidase activity showed groups of cells to have a normal
amount of enzymic activity and many of the remaining groups of cells to have a considerably higher than background activity of the enzyme. Thus it appears that this activity is transferred between cells.

The discrepancy of these two results can be readily explained. β-glucoronidase is a lysosomal enzyme and there is now substantial experimental evidence that such enzymes can be transferred between cells via the extracellular environment (Neufield, 1974), presumably by secretion of the lysosomal contents into the interstitial fluids (or culture medium) and subsequent uptake into other cells through endocytosis. This form of transfer is obviously not dependent on cell-cell contact.

Thus it appears that proteins are not generally transferred between cells by a direct pathway. However there may be exceptions. For example, it has been proposed by Lasek et al (1977) that because of the long distance between the axon and its cell body for the neurons of the squid giant fibre and the lack of ribosomes in the axon, that the associated glial cells may provide the proteins for the axons and, indeed, they present some indirect experimental evidence to support this hypothesis.

Further evidence against transfer of protein between electrically coupled invertebrate neurons will become apparent in the following section.
1.6.5. Amino Acids and Oligopeptides

Johnson and Sheridan (1971) first presented evidence that fluorescently labelled amino acids could be transferred between electrically coupled cells. They injected by electrophysiological techniques the dansylated derivatives of aspartate and glutamate (mol. wt. 366 and 380 respectively) into Novikoff hepatoma cells and saw the spread of the tagged amino acids to surrounding cells in contact. They found that these cells could also form gap junctions.

Work carried out over the last few years in Loewenstein’s laboratory has shown that a variety of fluorescently labelled amino acids and oligopeptides of ranging sizes up to mol. wt. 1624 can be transferred between the cells of the Chironomus salivary gland (Simpson et al., 1977; Loewenstein, 1975 and 1978). These fluorescent probes were synthesised in order to test the size limit of the permeability of the junctional membranes between these cells under normal conditions and in increasing concentrations of intracellular calcium which is thought to uncouple cells (see section 1.7.). For these studies Loewenstein and his collaborators injected the fluorescent labelled probes labelled with fluorescein isothiocyanate, lissamine rhodamine B and dansyl groups into one cell by electrophysiological techniques and followed the spread or lack of spread of fluorescence to surrounding cells. This work has now been extended to cultured rat liver cells where preliminary results indicate a cut-off size of 900 daltons (Flagg-Newton, unpublished results but see Loewenstein, 1978) which
is in agreement with the results of Imanaga (1974) discussed above (section 1.6.2.).

To ensure that transfer of fluorescence to surrounding cells was not due to enzymatic breakdown of the labelled oligopeptides causing the release of the fluorescent dye, Simpson et al (1977) incubated a number of the synthesised labelled oligopeptides for up to 12 h in a homogenate of the salivary glands. They found no breakdown by chromatographic analysis of these labelled oligopeptides. Moreover, the precise cut-off point also argues against transfer being due to release of the labelling dyes.

Assuming these labelled oligopeptides to have a spherical shape, Simpson et al (1977) calculated the effective pore size to be between 1.0 nm to 1.3 nm. This is reasonably close to the estimate of 2 nm obtained by Perrachia (1973b) on negatively stained gap junctions from another arthropod, the crayfish.

Van Venrooij et al (1975) have also found that the dansylated derivatives of a variety of amino acids are able to spread to surrounding cells after injection into one cell in the Drosophila salivary gland. However, from an analysis of the rate of spread when expressed in a theoretical model of the salivary gland (Van Venrooij et al, 1974), they argue that the non-junctional membrane of the cells is as permeable to these dansylated amino acids as the junctional membranes. This is in direct contradiction to the findings of other workers and is discussed in further detail below (section 1.6.8.).
Another study conducted on the arthropods has been carried out by Hermann et al (1975). They injected $[^3\text{H}]$-glycine into the lateral giant axon of crayfish by ionotopheretic techniques and, after several h, fixed the tissue in either formalin or gluturaldehyde before processing for autoradiography. They saw that labelled material had moved from the injected axon to surrounding lateral giant axons and their somata. However, the associated glial cells were not labelled indicating a direct intercellular pathway of movement. The amount of labelling was greatly increased in the injected and surrounding neurons when gluturaldehyde was used as the fixative. This is because gluturaldehyde fixed both the protein and free amino acids whereas formaldehyde only fixed the protein. Indeed, when formaldehyde was used there was practically no labelled material in the axons but there was still a substantial amount of labelled material in the somata indicating that this is the major site of protein synthesis in these neurons. Because the only pathway of transfer of $[^3\text{H}]$-molecules open to the somata was first interaxonal movement of the labelled molecules and because the axons appeared to contain little labelled proteins in comparison to the somata, Hermann et al (1975) concluded that the labelled material that had moved between the axons was $[^3\text{H}]$-glycine and not $[^3\text{H}]$-protein.

This study confirms an earlier investigation on the crayfish motor neuron carried out by Droz et al (1974). They found that after injection of $[^3\text{H}]$-lysine into the axon of this neuron there was some transfer of labelled material to surrounding coupled neurons and glial cells. Incubation of the ganglion containing these neurons in
an excess of cold lysine appeared not to qualitatively effect the extent of labelling in the non-injected cells but considerably reduced the background labelling thus suggesting that transfer was by a direct pathway. Although Droz et al found transfer to glial cells and Hermann et al (1975) did not find such transfer their different results can possibly be reconciled by the finding that many smaller axons of the abdominal nerve cord of crayfish, where both these two types of neurons are found, form gap junctions with glial cells (Perrachia and Dulhunty, 1976). Such junctions have not yet been reported between glial cells and lateral giant axons despite a number of electron microscopic studies on this ganglion (Pappas et al, 1971; Perrachia, 1973a and b; Perrachia and Dulhunty, 1976).

Finally, a similar study carried out by the same research group as Hermann et al (1975) has been conducted on the Retzius cells of leech (Rieske et al, 1975; also see section 1.6.3.). They find that after injection of either $^3\text{H}$-glycine or $^3\text{H}$-lysine into one Retzius cell there was rapid transfer of labelled material to its neighbouring Retzius cell. However, when puromycin (mol. wt. 471) was injected into one of the pair of Retzius cells followed by injection of the labelled amino acid into either cell, there was little incorporation of label in the cell first injected with puromycin and, in a few cases, little incorporation in either cell. As puromycin is a potent inhibitor of protein synthesis, these results suggest that the transferred labelled species were low molecular weight protein precursors (i.e. glycine and lysine) and not proteins. The
experiments where there was little incorporation of label in either cell can be explained by their being sufficient transfer of puromycin between the two cells that protein synthesis had effectively been inhibited in both cells. The transfer appeared to be by a direct intercellular pathway as there was little incorporation of label by surrounding glial cells and neurons and yet if \(^{3}\text{H}\)-glycine was injected into the central neuropil of the ganglion all the cell became labelled to a similar extent.

1.6.6. Transfer of Carbohydrates and Their Derivatives

A number of studies have shown that sugars maybe transferred between coupled cells. Bennett and Dunham (1970) and also Hermann et al (1975) have presented evidence for the transfer of sucrose and glucosamine respectively across the septa between lateral giant axons of crayfish. These two research groups used the same techniques as reviewed earlier to demonstrate transfer of ions (Bennett et al., 1967; see section 1.6.2.) and glycine (see above). Likewise, Rieske et al. (1975) using the techniques described above with puromycin have demonstrated the transfer of low molecular weight glycoprotein precursors of \(^{3}\text{H}\)-fucose and \(^{3}\text{H}\)-glucosamine between the Retzius cells of leech. Finally in the invertebrates Loewenstein and his collaborators (Simpson et al. but see Loewenstein, 1975) have shown transfer of fluorescent labelled oligosaccharides up to mol. wt. 1,500 between the cells of Chironomus salivary gland.
Two studies have been carried out which indicate that mammalian cells in culture may transfer sugars between themselves. Simms (1973) has extended his study on uridine nucleotide transfer (Pitts and Simms, 1977) by labelling cultures of BHK cells with \(^3H\)-glucose before adding unlabelled BHK cells to these cultures. Autoradiographic analysis showed transfer of labelled material from the 'donor' BHK cells to the 'recipient' BHK cells in contact with the donors. Again if he grew the donor BHK cells for 24 h before culturing with the recipient BHK cells there was a marked reduction in the amount of transfer of labelled material suggesting the transferred labelled molecules were low mol. wt. precursors and not high mol. wt. products (see section 1.6.3.). However, as Simms (1973) points out the results cannot be taken as evidence for the transfer of sugar phosphates because the \(^3H\)-glucose is likely to have been converted to other metabolites such as nucleotides which are known to be transferred.

The second study was carried out by Kohen and Kohen (1977). They injected by piezoelectric or microelectrophoresis techniques glucose-6-phosphate into cells in culture and monitored the resulting change in the NAD\(^+\)/NADH\(^+\) caused by the oxidation of the injected glucose-6-phosphate by measuring the increase in blue fluorescence emission. When NCTC 8739 cells (mouse embryo cell line) were used they found a change in the blue fluorescence in the injected cell and in a neighbouring cell in contact. Indeed there was no apparent delay in the spread of this change of fluorescence indicating that the injected glucose-6-phosphate equilibrated very rapidly between the
pair of cells.

As Kohen and Kohen (1977) point out the transferred molecules could also be catabolites of glucose-6-phosphate, for example lactate. As well as these molecules other candidates for transfer not mentioned by them are the cofactors NAD$^+$ and NADH$^+$. Under certain conditions they also find that pairs of L cells (a cell type that has been shown to be unable to form junctions of any type or electrically and metabolically couple; Gilula et al, 1972) are able to share the response to injected glucose-6-phosphate when under the same conditions they do not transfer fluorescein. These conditions are after prolonged growth in atractylate which is an inhibitor of extramitochondrial-mitochondrial ADP translocation. Therefore they suggest that transfer may also occur by non-junctional membranes. This anomalous result does make it rather uncertain though as to what is the explanation of the response being shared by the neighbouring cells. They did not mention whether the response in the neighbouring L cell was the same or less than the response in the injected L cell. It appeared to be about the equal for the NCTC 8739 cells. Thus it is not possible from their report to assess the relative amount of transfer through junctional versus non-junctional membranes.

1.6.7. Phospholipids and Their Precursors

Only one study has been carried out to discover if phospholipids or
their precursors can move directly between cells. This was carried out by Peterson and Rubin (1970) on secondary chick embryo fibroblasts and they employed very similar techniques as those used much later by Pitts and Simms (1977). Peterson and Rubin first labelled cultures of fibroblasts with $[^3H]$-choline and then grew the cultures for 24 h so that most of the radioactivity would be located in phospholipids (TCA insoluble material) with very little tritium counts in the intracellular pools of phosphoryl choline (TCA soluble). These labelled cells were then co-cultured with unlabelled fibroblasts and after several h growth the cells were fixed, washed in TCA (thus removing phosphoryl choline) and processed for autoradiography. They found extensive transfer of labelled material from the 'chased donor' cells to the 'recipient' cells in contact with the 'chased donors'. From a quantitative analysis they showed that the chased donor cells lose as much as 90% of their labelled acid insoluble material to surrounding recipient cells in contact when at the time of mixing these cells had at least 70% of the tritium counts located in phospholipids. They thus conclude that phospholipids are readily exchanged between cells when they come into contact with one another. Although they suggested that phosphoryl choline is not transferred, they did not present evidence against the transfer of this metabolite.

The transfer of phospholipid introduces a different aspect intercellular communication because these molecules are insoluble in water. Thus, the transfer of these molecules cannot take place through water filled pores and must occur by some other mechanism.
(Peterson and Rubin, 1970). They did not detect the transfer of proteins between these fibroblasts and this therefore suggests that the mechanism of transfer is specific for phospholipids and probably does not involve the transfer of segments of membranes as these would presumably contain proteins. Part of the present study is to further investigate if phospholipids and/or its precursors (phosphoryl choline and CDP-choline) move between contacting cells.

1.6.8. The Mechanism of Intercellular Exchange of Substances

In summary it appears that many small molecules and inorganic ions can freely pass between coupled animal cells but large molecules cannot pass. The passage of biologically unrelated substances, the size specificity of transfer and the apparent common pathway of transfer are all consistent with the hypothesis of intercellular movement of substances by passive diffusion through water filled pores. Also the dependence on the ability to form gap junctions to become coupled and the structure of this junction suggest that these hypothetical pores occur at the gap junction. Thus it seems reasonable to conclude that in the majority of cases coupled cells can be defined as those cells joined by gap junctions. However, there are a number of studies which are not consistent with this conclusion and have been interpreted as evidence for other mechanisms of cell to cell transfer.
For example, there are now a number of cases, notably from embryonic systems, where electrically coupled cells suspected or known to be joined by gap junctions have been found to be unable to transfer dyes such as fluorescein. These include cells from cleavage to blastula stages of *Xenopus* embryos (Slack and Palmer, 1969), cells from the 32 cell stage and earlier cleavage stages of *Asterias* embryos (Tupper and Saunders, 1972) and re-aggregated cells from blastula stages of *Fundulus* (Bennett et al, 1972). For the *Xenopus* and the *Fundulus* embryos it is known that the cells are joined by gap junctions (Bennett and Trinkaus, 1970; Bennett and Gilula, 1974; Sanders and Dicaprio, 1976).

Moreover, Baker and Warner (1972) have found that EGTA injected into one blastomere of early cleavage *Xenopus* embryos only prevents further cleavage in that blastomere and not surrounding blastomeres suggesting that EGTA can not pass between the blastomeres. Also, in a brief report Turin (1977) has found that whereas some ions did pass between *Xenopus* cells from early stages, a higher molecular weight ion (PbEDTA−) did not pass.

Two other examples of where electrical coupling has been found in the apparent absence of dye transfer are the intersegmental synapses of the medial and giant fibres of the earthworm (Mulloney, 1970) and a number of segregant hybrids of human fibroblasts fused with 1C-1D cells (a malignant subline of L cells; Azarnia and Loewenstein, 1977).
For the embryonic systems, one of the interpretations is that the embryonic junctions have qualitatively different permeability properties from their adult counterparts (cf. Bennett, 1973; Wolpert, 1978). A conclusion drawn by Politoff (1977) from many of these studies is that electrical coupling and intercellular movement of substances are separate phenomena. However there are alternative explanations of the apparent absence of dye transfer between electrically coupled cells.

In contrast to the other studies, Sheridan (1971a) found fluorescein transfer between re-aggregated *Xenopus* embryo cells although he used cells from later stages of development (gastrualae and neuralae). In his recent review Sheridan (1976) points out a number of complications in interpreting the apparent lack of injected tracer dye in the other studies. For example the large size of cells from early embryonic stages will dilute the injected tracer dye making it difficult to observe any intercellular transfer. This could be further compounded if there is a paucity of junctional elements between these cells. As yet no morphometric studies have been carried out on the extent of gap junctions between early embryonic cells. However, the studies that have been carried out do not suggest that early embryos are particularly rich in these structures although it seems likely that gap junctions are a common feature of embryos (Bennett and Gilula, 1974; Ducibella et al, 1975; Sanders and DiCaprio, 1976; Smith et al, 1976; Hogan and Trinkaus, 1977).
Supporting Sheridan's arguments are the recent findings of Bennett and his collaborators (Bennett et al., 1978; Spray et al., 1978). In a re-evaluation of their earlier finding (Bennett et al., 1972) on the killifish Fundulus they do find intercellular transfer of a newly developed dye lucifer yellow (Stewart, 1978) between re-aggregated cells of these embryos (Bennett et al., 1978). They (Spray et al., 1978) have also found transfer of this dye between cells of Ambystoma embryos from 32 cell stage to morula. Furthermore, Turin found transfer between Xenopus cells from early developmental stages of an ion that has similar mol. wt. to that of fluorescein (AuCl₂OH⁻; mol. wt. 321 compared to 330) which had been seen in the earlier study of Slack and Palmer (1969) not to pass between these cells. However, Turin (1977) did not detect transfer of a larger ion (PbEDTA⁻, mol. wt. 498). Turin did not report if this ion could be transferred between adult cells so it is conceivable that it is bound in the cytoplasm of the injected cell and is thus not available for transfer. Moreover, if transfer occurs by passive diffusion, then it would be expected that larger ions pass much slower than smaller ions and so a longer period of time after injection would be needed in order to detect any transfer of PbEDTA⁻ (for example see the recent study of Brink and Barr, 1978).

With regard to the apparent lack of transfer of EGTA between early cleavage cells of Xenopus embryos (Baker and Warner, 1972), Sheridan (1976) points out that a study by de Laat and Bluemink (1973) shows
that the two blastomeres after the first cleavage of the Xenopus embryo remain joined by a mid-piece until the second cleavage. Thus, he argues that if EGTA does not appear to be transferred when there is a possibility of the blastomeres still having cytoplasmic continuity then these experiments can not be taken as good evidence of the 'impermeability' of embryonic junctions to molecules larger than inorganic ions such as $K^+$. 

Turning now to the non-embryonic examples, Larsen et al (1977) carried out a companion morphological study on the 'fully' communicating segregant hybrids (i.e. transfer dyes and electrically coupled) and on the 'partially' communicating segregant hybrids (i.e. do not or only occasionally transfer dyes but are generally capable of electrically coupling) isolated by Azarnia and Loewenstein (1977) after fusing human fibroblasts with 1C-1D cells. Although they did not carry out a comprehensive quantitative analysis it is reasonable to conclude from their study that the fully communicating segregants are joined by many more gap junctions than the partially communicating segregants. This conclusion provides further support for Sheridan's arguments (Sheridan, 1976). That is, fewer junctional elements may be required to detect electrical coupling than are required to detect dye transfer. Indeed, Loewenstein et al (1978) have recently claimed to be able to detect accretion of new junctional elements in forming junctions by quantal increases in junctional conductance.
The other non-embryonic system where the absence of dye transfer has been reported in the presence of electrical coupling is the intersegmental synapses between lateral and medial giant fibres of the earthworm (Mulloney, 1970). However, Mulloney did find transfer of procion yellow between the two electrically coupled lateral giant fibres. Also, Brink and Dewey (1978) have recently shown extensive transfer of a variety of fluorescein dyes across the septa of the earthworm median giant fibre. As discussed above, this apparent difference in permeability of these related synapses may not necessarily be a qualitative difference but a quantitative difference; that is a smaller number of junctional elements forming the intersegmental synapses. Again the large size of these neurons may compound any quantitative difference.

Politoff (1977) also cites a study by Fain (1975) as showing lack of dye transfer in the presence of electrical coupling. In this study Fain (1975) injected procion yellow into the red rod photoreceptor cells from the retina of *Bufo marinus* as a means of morphological identification. These cells are joined by quite extensive gap junctions and also, albeit to a lesser degree, to other photoreceptor cells (Fain et al, 1975). Moreover, it has recently been shown that there is good electrical coupling between the red rod cells (Leeper et al, 1978). In the limited number of preparations after fixation suitable for morphological studies, Fain (1975) found that the injected procion yellow was located only in the injected red rod but on one occasion (1 out of 6) he saw that the procion
yellow was also located in surrounding contiguous cells. He interpreted these results as an artefact of fixation but, of course, there is no reason to believe that such transfer did not occur when the cells were viable. Moreover these cells are reasonably large (about 100 μm in length; Fain, 1975) which could add to the problem of detection of dye transfer. Obviously, this system needs to be studied under much more rigorous conditions.

In the light of these studies it would seem that there may not be differences in the permeability of embryonic and adult junctions. Therefore it is not possible to make the statement that under normal conditions electrical coupling can occur in the absence of dye transfer between cells if the cells are joined by gap junctions. As will be discussed later (section 1.7.) there is some evidence that intracellular levels of calcium can modulate the permeability of gap junctions. Thus, under abnormal conditions where the intracellular level of free calcium is high it is conceivable that electrical coupling could occur in the absence of dye coupling.

A related study to those just reviewed above is that of Van Venrooij et al, (1975) on Drosophila salivary gland. They claim that the permeability of the junctional and non-junctional plasma membranes of the salivary cells to tracer molecules such as fluorescein and a variety of dansylated amino acids are very similar. Thus, they conclude that intercellular transfer of these molecules does not take place through specialised junctions but by simple leakage from one cell to the next cell.
In contradiction to the conclusion reached by Van Venrooij et al. (1975), Brink and Barr (1978) have presented evidence that the intercellular junctions across the septa of the earthworm median giant axon are several orders of magnitude more permeable to fluorescein and a variety of halogen derivatives of fluorescein than the plasma membrane. Many of the cell surface structures interpreted by them as gap junctions (nexuses) are rather dubious and could well be other intercellular junctions (septate junctions; see figure 3c of Brink and Barr, 1978). Thus, their calculation of the total area occupied by gap junctions necessary to compute their permeability could be greatly overestimated. However, this would mean that gap junctions are even more permeable to these tracers than Brink and Barr (1978) calculated.

Furthermore, the conclusion of Van Venrooij et al. (1975) is not consistent with the results obtained by others in different arthropod systems. Simpson et al. (1977) found a precise cut-off point for the size of molecules that could be transferred between the cells of Chironomus salivary gland and that would not be expected to occur if the mechanism of transfer was by simple leakage from one cell to the next. Moreover, when one cell was injected with a fluorescently labelled oligopeptide below this cut-off point and also with a differently fluorescently labelled oligopeptide above this cut-off point, they saw that only the smaller of the two tracers moved between the cells.

Finally, the results of Hermann et al. (1975) showed that after
injection of $[^3H]$-glycine or $[^3H]$-glucosamine into one lateral giant axon of crayfish, the labelled material only spread to other giant axons and not to associated glial cells although there are many glial processes infiltrating the fibrillar septa between the axons (Pappas et al, 1971). This absence of transfer to glial cells is again not consistent with the claim if Van Verooi\textit{j et al} (1975).

Although there may be some transfer of these tracer molecules by simple leakage between salivary gland cells of Drosophila, the inconsistency of the conclusion drawn by Van Verooi\textit{j et al} (1975) with the observations of others on similar systems, (i.e. other arthropod species) places an element of doubt in their calculations of the permeability of the junctional versus the non-junctional plasma membranes. However, their study does raise an argument against using fluorescent tracers for studying permeability instead of endogenous molecules.

Other systems which are contrary to the channel hypothesis are 'rectifying' electrotonic synapses. That is, where electrical current only passes efficiently in one direction. There are now a number of such synapses almost all in excitable tissues between certain neurons (Furshpan and Potter, 1959; Auerbach and Bennett, 1969; Ringham, 1975).

The anatomical and physiological basis of junctional rectifications is still not fully understood despite its discovery some 20 years ago.
The almost exclusive location of rectification to particular neurons suggests that cell coupling may have been adapted to some specialised physiological function between these neurons.

However, a morphological analysis by Hanna et al. (1978) suggested that the gap junctions at one of these rectifying synapses (lateral giant axon and motor neuron of crayfish; Furshpan and Potter, 1959) were structurally similar to other gap junctions. They also found that fluorescein was unable to pass between these two neurons in either direction.

There is one report of junctional rectification in non-excitable tissues. Slack and Warner (1975) found that in a few pairs of electrically coupled endoderm cells isolated from late blastulas of *Xenopus* embryos, injected current could only flow efficiently in one direction. This rectification did not appear to be caused by a leak in one cell suggesting that the junctional plasma membranes could discriminate between current flow in one direction and in the opposite direction. Junction rectification does not appear to be a general property of coupled endoderm cells as it was only found in a limited number of pairs of cells (3 out of 17). The functional significance of this limited distribution of junctional rectification in *Xenopus* embryos is unknown but Slack and Warner (1975) speculate that it may have some importance in spacial patterning.

Finally, Politoff (1977) in his recent review calculates that after
taking into consideration the bound water layer which would be expected
to occur in any protein lined water filled channel, the diameter of
the putative channels at gap junctions open for intercellular
movement of molecules is smaller (0.8 nm maximum) than the size of
substances known to move between cells (ex. procion yellow, about
0.9 nm; Politoff, 1977). His calculation is based on the structural
diameter of these putative pores being between 1 and 2 nm.

The most recent estimations of the 'structural' diameter of the putative
pores at gap junctions were found from negative stained preparations
(and also X-ray diffraction) and show the diameter for both vertebrate
and arthropod forms to be about 2 nm (Casper, et al, 1977; Makowski
et al, 1977; Perrachia, 1973b). As this technique relies on the
penetration of a heavy metal salt solution in water, usually uranyl
acetate, it seems reasonable to believe that the diameter obtained
from these preparations is fairly close to the 'effective' pore size.

Politoff (1977) further suggests that assuming water filled channels
of a diameter (1.2 nm) sufficient to allow the intercellular movement
of procion yellow, the rate of diffusion would be too slow to account
for the amount of transfer seen across the septa between the lateral
giant axons of crayfish (Payton et al, 1969). Unfortunately, he does
not present his calculations or all the assumptions involved in his
arriving at this conclusion. Thus it is not possible to fully analyse
his conclusion. However, he does mention that the morphometric study
necessary for his calculation was taken from a morphological study
on crayfish lateral giant axons carried out by Pappas et al (1971). It is difficult to see how an estimate of the extent of the average area occupied by gap junctions between lateral giant axons can be made from this study as little quantitative data was given and all of this was based on a thin-section analysis which can not give a precise estimate of the frequency or size of gap junctions between cells (cf. Yee and Revel, 1978, for a quantitative analysis).

Nevertheless, Politoff does raise the interesting point of a bound water layer which must be taken into account in estimating the effective pore size of the putative channels at gap junctions.

Because of his objections to the channel hypothesis, Politoff (1977) has suggested alternative mechanisms of electrical coupling and molecular exchange. He points out that proteins are possible semiconductors and so any protemaceous structures that link the cytoplasms of contiguous cells will electrically couple the cells.

For the transfer of molecules Politoff (1977) has suggested that there could be mutual exocytosis and endocytosis at the junctional membranes. A similar mechanism was proposed by Subak-Sharpe (1969) to explain metabolic co-operation before it was known what the molecular basis of this phenomenon was. This mechanism of transfer is not consistent with the results of the many studies on intercellular transfer. It cannot account for the lack of transfer of cellular macromolecules and the precise cut-off size. Although this mechanism may operate between certain cell types, it cannot be a universal mechanism.
Finally, one of the interpretations of experiments carried out by Goldfarb et al (1975) is that transfer of nucleotides in metabolic co-operation is a multi-component system, for example separate 'transferases' for each nucleotide. They isolated mutant PyBHK cells (mec") that were able to allow intercellular exchange of purine nucleotides but appeared to be unable to allow exchange of pyrimidine nucleotides. However, a later study (Wright et al, 1976) showed that these mec" cells could exchange pyrimidine nucleotides if they had first been treated with cyclic AMP which had caused them to change their morphology to a more fibroblastic form. They suggested that their earlier observations could be due to a level of transfer of pyrimidine nucleotides that had been too low to detect but, by growing the mec" cells in the presence of cyclic AMP, there may have been an increased number of junctional elements resulting in a greater extent of transfer of these nucleotides. In contrast to this study, Cox et al (1974a) found no apparent effect on the level of metabolic co-operation between Hela cells and human fibroblasts when the morphology of the Hela cells had similarly been altered by treating with cyclic AMP or hydrocortisone. However, Cox et al (1974a) did not carry out a quantitative analysis whereas Wright et al (1976) did carry out such an analysis. Therefore it is possible that there was a slight increase in metabolic co-operation between the Hela cells and fibroblasts after an induced change in morphology.

One other point that must also be taken into consideration is that any specific transfer system cannot readily account for the transfer
of biologically unrelated molecules (ex. tracer dyes).

In conclusion, the majority of the studies which appear contrary to the channel hypothesis can, to varying degrees, be reconciled within the framework of this hypothesis. Nevertheless, our knowledge of the permeability of these putative channels is still surprisingly scant. For example, the only endogenous molecules known for certain to pass between coupled vertebrate cells are the nucleotides. This aspect will be discussed further when the aims of this present study are outlined (section 1.9.).
1.7. The Control of Junctional Permeability

There are now numerous reported instances of cells being able to quickly and reversibly break junctional communication, or uncouple, under certain conditions. Many of these reports are by Loewenstein and his colleagues on the Chironomus salivary gland where they show that conditions expected to cause an increase in the intracellular concentration of free calcium is paralleled with uncoupling of the cells. The original observation of uncoupling led Loewenstein (1967b) to formulate the calcium hypothesis in which he proposes that junctional permeability is directly modulated by the intracellular concentration of free calcium.

Examples of treatments used by Loewenstein and his colleagues which they found to cause uncoupling of insect salivary gland cells were direct injection through micropipettes of calcium (Loewenstein et al., 1967; Deleze and Loewenstein, 1976), poisoning with cyanide or dinitrophenol (Politoff et al., 1969), substituting extracellular Na\(^+\) with Li\(^+\) (Rose and Loewenstein, 1971), exposure to medium lacking Ca\(^{2+}\) and Mg\(^{2+}\) (Rose and Loewenstein, 1971), and finally exposure to Ca\(^{2+}\) containing medium in the presence of calcium ionophore or after puncturing the cells with a small glass rod (Rose and Loewenstein, 1975; Oliveira-Castro and Loewenstein, 1971). By injecting the salivary cells with aequorin, a protein which fluoresces in the presence of calcium, Rose and Loewenstein (1976) showed that these treatments, when they could be examined by this technique, all resulted in a
rise in the intracellular concentration of free calcium. Moreover they have shown by this technique that calcium injected into the centre of the cell did not cause immediate uncoupling but only caused uncoupling when the calcium had spread to the peri-junctional regions (Rose and Loewenstein, 1975). This is consistent with the contention that calcium could act directly on the junctional elements (Loewenstein, 1967b). The use of aequorin also allowed an estimate of the calcium concentration necessary to cause uncoupling (see below).

Rose et al (1977) have recently extended the use of the fluorescent oligopeptide probes described earlier (section 1.6.) in examining the role of calcium in modulating junctional permeability. They found that as the free intracellular calcium concentration in Chironomus salivary gland cells was increased from $10^{-7}$ M to $5 \times 10^{-5}$ M as estimated with aequorin, there was a progressive decrease in the size of the fluorescent probe which could be transferred until the cells were only capable of electrical coupling and finally, at the highest calcium concentration, the cells became totally uncoupled. Thus, it is possible that there may be a graded change in the junctional permeability; that is a gradual decrease in the diameter of the pores formed at gap junctions. They also suggest that such a result would be obtained if there are channels which have both different permeability properties and different sensitivity to calcium. Alternatively, such a result can also be accounted by a gradual loss in the number of fully functional junctional elements (Deleze and Loewenstein, 1976).
Finally, an observation from the aequorin studies consistent with the calcium hypothesis is the finding by Rose and Loewenstein (1976) that injected calcium does not appear to traverse the junctional membrane of *Chironomus* salivary gland cells. They showed this by injecting two adjacent cells with aequorin followed by injection of one of the cells with calcium. The aequorin fluorescence though, only occurred in the calcium injected cell indicating that calcium cannot pass between the salivary gland cells in detectable amounts.

Although non-junctional membrane depolarization generally precedes uncoupling in the experiments conducted on the *Chironomus* salivary gland cells, Rose and Loewenstein (1975) present evidence against this event being the cause of uncoupling. They showed that salivary cells which were voltage clamped close to their resting membrane potentials could be uncoupled by calcium injection. Moreover, salivary cells still maintained electrical coupling for some time after membrane depolarization caused by incubation of the gland in a medium containing a high concentration of $K^+$.  

Insect salivary gland cells are not the only arthropod cells that can become uncoupled by intracellular increases in free calcium. For example cultured insect cells, TN cells, can be readily uncoupled in the presence of calcium ionophore (Epstein and Gilula, 1975). Crayfish septate axons can be uncoupled by treatment with EDTA, lowered temperatures, substitution for chloride with propionate in the bathing medium and exposure to DNP (Payton *et al.*, 1969; Asada *et al.*, 1969).
1971; Perrachia and Dulhunty, 1976). Injection of calcium or sodium as well as calcium ionophore have all recently been found to uncouple the electrical synapses of neurons in the buccal ganglion of the mollusc Navonax if the (Baux et al, 1978) ganglion had first been incubated for 90 min in the presence of cyanide.

At first sight the evidence of the calcium hypothesis for at least insect salivary gland cells appears quite strong. However, Sheridan (1978) has recently reviewed this evidence and shows that there are some discrepancies which need to be resolved before this hypothesis can be accepted as proven dogma. For example, the experiments of Rose and Loewenstein (1976) against membrane depolarization being the primary cause of uncoupling is not consistent with the earlier observation of Oliveira de Castro and Loewenstein (1971) on Chironomus salivary gland cells. In this earlier study it was found that perforating the cell membrane with a sufficiently large hole to cause complete membrane depolarization also resulted in transient uncoupling. Such uncoupling could be prevented if the membrane potential of an immediate neighbouring cell was held by current injection. This suggests a direct relationship between uncoupling and the depolarization of the cell membrane. This point was not discussed in the later study by Rose and Loewenstein (1976).

Sheridan (1978) also points out a recent review by Blinks et al (1976) in which these authors state that there is no satisfactory method in calculating the free intracellular concentration of calcium by measuring the response of injected aequorin. Thus, Sheridan puts
forward a number of reasons to expect that the free calcium concentration estimated by Rose and Loewenstein (1976) using the aequorin technique would be much higher than they calculated. Moreover, these same reasons make it difficult to interpret the experiment carried out by Rose and Loewenstein (1976) where they attempt to show the lack of intracellular transfer of calcium by the aequorin technique.

A number of studies have attempted to investigate the morphological basis of uncoupling between arthropod cells. Two studies (Bullivant and Loewenstein, 1968; Rose, 1971) concentrated on structural changes on septate junctions (section 1.4.) as it was believed at the time that these junctions were the basis of coupling. Two other studies have been carried out on crayfish septate axons to examine changes in the gap junctions in relation to uncoupling. The septate axons occur as pairs separated by a fibrillar network of connective tissue and glial cell processes, the so-called septa. The axons, however, make contact with each other and form gap junctions at small openings in the septa. In a semiquantitative study Pappas et al (1971; also see Asada and Bennett, 1971) found that conditions which resulted in increased septal resistance caused a loss of contact and hence gap junctions between the axons at these openings. Thus they suggested that uncoupling between these axons is caused by a loss of gap junctions.

In contrast to the interpretations of Pappas et al (1971), Perrachia and Dulhunty (1976) suggest that uncoupling of crayfish septate axons is simply caused by a structural change of the gap junctions. They found
that an increase in septal resistance was correlated with the subunits, or connexons of the gap junction becoming aggregated with one another to form a tightly packed and regular crystalline structure. This subtle change could be seen both by thin section and freeze-fracture techniques although for the latter gap junctions in other axons of the crayfish abdominal ganglion were studied and not those between the septate axons. They therefore suggested that this contracted form of the gap junction is impermeable and hence represents the 'uncoupled state'. However, Perrachia and Dulhunty (1976) did not carry out a morphometric analysis and thus could not rule out the possibility that uncoupling is caused by loss of gap junctions.

In summary for the arthropods, whilst intracellular increases in free calcium can cause uncoupling it is not yet clear whether calcium directly modulates junctional permeability or if it is some other calcium sensitive mechanism (e.g. membrane depolarization) which is the primary cause of uncoupling. Moreover, the morphological basis of uncoupling between arthropod cells is not understood.

For the vertebrate there are two opposing schools of thought on calcium modulating junctional permeability. Observations consistent with a role for calcium are the findings that a number of systems can become uncoupled by direct intracellular injection of calcium. For example such uncoupling has been found to occur for cardiac Purkinje cells (De Mello, 1975b), phytohaemagglutinin stimulated lymphocytes (Oliveira-Castro et al, 1973), pancreatic acinar cells (Iwatsuki and Peterson, 1977) and re-aggregated blastomeres of Xenopus embryos.
Injection of Na\(^+\) also causes uncoupling between Purkinje cells and liver hepatocytes and it is believed that Na\(^+\) injection causes an increase in intracellular free calcium (De Mello, 1975a, 1977). Electrical uncoupling also occurs in the presence of DNP between macroblastomeres of newt embryos (Ito et al., 1974).

As with the arthropod systems, Perrachia (1977) has shown that anoxic conditions caused by incubation in DNP or lack of O\(_2\) results in a contraction of the connexons of the gap junctions of rat stomach epithelia. However, it was not shown in this system if the epithelial cells uncoupled. Perrachia (1978) has recently shown a similar contraction of gap junctions from isolated bovine lens fibre plasma membranes when the membranes were incubated in a buffer containing a free calcium concentration of 5 x 10\(^{-7}\) M. Such changes do not appear to be fixation artefacts or due to artefacts of infiltration of glycerol prior to freeze-fracture as replicas of chick ciliary epithelium, prepared for freeze-fracture by rapid freezing techniques, show similar changes in gap junctional structure after 5 min incubation of the tissue in DNP (Raviola et al., 1978).

In direct contradiction to the observations of Loewenstein et al. (1978), Turin and Warner (1977) found no detectable loss of electrical coupling between Xenopus blastomeres when one blastomere was injected with calcium. However, they found uncoupling could occur if the intracellular pH was lowered (see below). In the same set of
experiments Epstein and Gilula (1975) carried out on insect TN cells, they found no loss of electrical coupling between mouse 3T3 cells in the prolonged presence of the calcium ionophore at five times the concentration which caused almost immediate uncoupling between the TN cells. Similarly, mouse myocardial cells still beat synchronously (an indication of electrical coupling; section 1.8.4.) in the presence of this elevated level of the ionophore. In metabolic co-operation experiments Cox et al (1974a and b) found that there was no qualitative effect in the transfer of nucleotides between cultured human fibroblasts in the presence of DNP although there was a large reduction in the incorporation of these nucleotides into RNA. Unpublished experiments of Epstein (see Sheridan, 1978) show that calcium ionophore and metabolic poisons do not cause uncoupling between Novikoff hepatoma cells.

Thus, a role for calcium in modulating junctional permeability between coupled vertebrate cells has not been established. The contrasting experiments may simply be due to differences in the systems used although this is clearly not the case for the blastomeres of Xenopus embryos. However, a number of recent studies show that uncoupling between vertebrate cells can be caused by a number of other conditions. For example, lowering of the intracellular pH causes loss of electrical coupling between re-aggregated Xenopus blastomeres and pancreatic acinar cells (Turin and Warner, 1977; Iwatsuki and Peterson, 1978a). As lowering of the intracellular pH can be caused by injection of calcium due to an exchange for protons by mitochondria (Meech and Thomas, 1977),
both Turin and Warner (1977) and Meech and Thomas (1977) speculate that this may be the primary cause of the uncoupling in the insect salivary gland cells. It has recently been shown that there is a conformational change indicative of quaternary symmetry instead of the hexagonal symmetry (section 1.5.5.) of the gap junctions in isolated bovine lens fibre plasma membrane when the membranes are incubated in low pH (<6.5) buffers (Perrachia and Perrachia, 1978).

External application of acetylcholine has been found to cause uncoupling between pancreatic acinar cells and lacrimal acinar cells (Iwatsuki and Peterson, 1978b and c). As the pancreas is a target tissue for acetylcholine, such uncoupling is important in our understanding the response of tissues to hormones. However, uncoupling by hormonal stimulation does not occur between mouse myocardial cells and granulosa cells in culture (Lawrence et al, 1978). Thus, uncoupling by hormones of their target tissues may be limited to certain cases (see section 1.8.2. for a further discussion of these results).

A brief report by Spray et al (1978) shows that re-aggregated _Ambyostoma_ blastomeres can be electrically uncoupled by changing the membrane potential in either direction of one of the blastomeres. This uncoupling can also be seen by a lack of the intercellular spread of lucifer yellow when injected into one of the blastomeres. This form of uncoupling may be difficult to generate in well coupled systems as coupling tends to make populations of cells isopotential with one another. Thus such uncoupling would be expected to be limited to cells
of low communication capacities such as vertebrate blastomeres (section 1.6.8.). Nevertheless, the cause of such uncoupling is interesting and may provide some information on the mechanism of junction formation as the blastomeres quickly re-couple on restoration of an equal membrane potential between them.

Finally, the cardiac glycoside ouabain may cause uncoupling. Ledbetter and Lubin (1978), continuing measurements of co-operativity using oub^R and oub^S cells (section 1.6.2.), have found that the index of co-operativity markedly fell if the ouabain concentration was increased from that normally used in these experiments even though the concentration was well below the level which affected the oub^R cells. This appeared to be a general effect as it occurred for many different cell types in culture. This result could be explained if the oub^R and oub^S cells became uncoupled. Alternatively, such a result can be explained by the non-junctional plasma membranes becoming more permeable to K^+ in the presence of the oub^S cells.

In summary, there exists mechanisms by which coupled cells can rapidly but reversibly break junctional communication with one another. As yet the mechanisms of uncoupling are far from understood. Although it is becoming accepted that the contracted form of the gap junction represents the 'uncoupled state' and is thus impermeable to ions and metabolites (Perrachia, 1977 and 1978; Raviola et al, 1978), there is no direct evidence to support this contention. Indeed it could be argued for the vertebrates that the contracted form of the gap junction
represents a stage in its breakdown. Both anoxia and ischaemia (Fawcett, 1978; Yancey et al., 1978 and manuscript submitted to J. Ultr. Res.) causes the formation of cytoplasmic 'annular gap junctions' which is currently believed to be one mechanism of junctional breakdown (Larsen and Hai-Nan, 1978). Similarly incubation of intestinal smooth muscle in DNP results in loss of gap junctions between the muscle cells (Daniel et al., 1976). Thus, the contraction of the gap junction possibly caused by an increase in intracellular free calcium may signal this mechanism of turnover. Such a mechanism may be important in ensuring that communication is broken between healthy cells and dead or dying cells in order to prevent further cell death (Rose and Loewenstein, 1976). Apart from acting as a possible safety mechanism in unhealthy tissues, another role uncoupling may have is in allowing discrete events to occur in cells at particular instances such as in embryonic development (Spray et al., 1978).

1.8. The Physiological Roles of Cell Coupling

1.8.1. Introduction

The proposed roles of cell coupling can be grouped into two functional categories. One of these categories is the maintenance of homeostasis and the other is the intercellular transmission of signals (Sheridan, 1976). For some of these proposed roles the evidence is at most
only suggestive (ex. pathways for morphogens and growth control signals). However, for the other roles (i.e. homeostatic functions) the evidence is much more substantial if the premise that cell coupling through gap junctions allows for the rapid intercellular transfer of small molecules and ions is accepted. One role that has been well established for cell coupling is that it acts as an electrotonic synapse in excitable tissues and, as mentioned earlier (section 1.6.2.), it was in this context that cell coupling was first discovered.

In the following sections these physiological roles will be reviewed in turn. Because many of the homeostatic roles were proposed on the basis of the above premise, it will be assumed for the purpose of this survey that it holds true. The validity of this premise will be discussed in section 1.9.

1.8.2. Co-ordination of Cellular Activities

As has been suggested by many research workers in the field this form of intercellular communication will tend to reduce differences in internal concentrations of ions and metabolites between coupled cells (cf. Sheridan, 1976; Pitts and Finbow, 1977). This will not only stabilize the internal compositions of cells but also co-ordinate the activities of cells within a population. The stability resulting from cell coupling may be of importance in maintaining optimum ionic concentration for enzymic activities (VanVenrooij et al., 1975) and optimum concentration of certain metabolites which are critical for growth (Socolar, 1973 and see section 1.8.5.).
One aspect of cellular activities that has been suggested to be co-ordinated by cell coupling is metabolism (Pitts and Finbow, 1977). They argue that if cells can readily share their metabolites then they will in consequence integrate their metabolism. Thus, the cell will lose its metabolic identity and the population of coupled cells assumes the status of the unit of metabolism. There is some experimental evidence to support the notion that coupled cells will integrate their metabolism. Sheridan et al (1975 and manuscript in preparation; see section 1.6.3.) have shown in a metabolic co-operation system in culture that coupled cells are able to influence the activities of each other's metabolic pathways which lead to the synthesis of purine nucleotides. One obvious advantage of integration of metabolism is that the metabolic resources of all the cells in a tissue will be used equally. In other words, the resources of one cell will not be over taxed while the resources of another cell in the same population will not be under taxed.

Another activity that may be co-ordinated by cell coupling is the response of a target tissue to hormones by the sharing of intracellular second messengers (ex. cyclic AMP; Pitts, 1972). The recent experiments of Lawrence et al (1978 and see section 1.6.3.) strongly support this proposed role. By using two different target cell types which responded to different hormones albeit via the same intracellular second messenger (cyclic AMP), they showed that when only one of the hormones was added to mixed cultures both cell types responded. This not only suggests that cyclic AMP is transferred between cells
but further suggests that there is sufficient transfer to evoke a full response in a target cell which has not itself received hormonal stimulation at least in this particular system.

Co-ordination of the response to hormones will only occur, of course, if the target cells are coupled. Whilst it seems likely that gap junctions are common features of target tissues there is recent evidence that external stimuli can uncouple target cells. Iwasuki and Peterson (1978b and c) have shown that the acinar cells of rat and mouse pancreas and rat lanchrimal glands can become uncoupled by acetyl choline. This may be restricted to acetyl choline as nor-adrenaline does not uncouple myocardial cells and FSH does not uncouple granulosa cells from myocardial cells (Lawrence et al, 1978). Nevertheless, uncoupling could be of importance in tissues of mixed target cell types which respond to different hormones but via the same second messenger.

1.8.3. Cell Coupling as a Transport System

The capacity of cell coupling to reduce internal differences in metabolic compositions between cells means that it could act as a transport system moving nutrients from cells situated close to blood capillaries to less favourably situated cells (Bennett, 1973), or from yolk cells to other cells in the developing embryo before the establishment of a blood sinus system (Sheridan, 1976). There is some evidence for this
stemming from the prediction that highly cellular, avascular tissues should be particularly rich in gap junctions. Three examples of such tissues are the vertebrate lens, the mammalian Graafian follicle and the vertebrate enamel organ. The cells in all three tissues are extensively joined by gap junctions (Dunia et al, 1973; Albertini et al, 1975; Garant, 1972). Moreover, there is experimental evidence that the oocyte in preantral follicles may be dependent for its growth on a supply of nutrients from the surrounding granulosa cells (Eppig, 1977). The oocyte-cumulus complex will be discussed at the end of this study (section 5.5.).

1.8.4. The Electrotonic Synapse

An established function of cell coupling is to act as an intercellular pathway of transmission of electrotonic signals in excitable tissues (for reviews see Pappas and Waxman, 1972; Bennett, 1972 and 1973). It is a particularly widespread function of cell coupling and is used extensively in the nervous systems of both invertebrates and vertebrates (Bennett, 1972 and 1973; Sotelo, 1976) as well as in smooth muscle and heart (Dewey and Barr, 1962; Dreifuss et al, 1966). In almost all cases the transmission is bidirectional and this feature plus the absence of a synaptic delay, distinguishes the electrotonic synapse from the chemical synapse (Bennett, 1972). As discussed earlier (section 1.6.8.), there are a few examples of rectifying electronic synapses (i.e. current can only pass in one direction; ex. Furshpan
One system that has received much attention is the synchronization of beating of heart cells in culture when they come into contact. Here there is a good correlation between synchronization, electrical coupling and formation of gap junctions (Goshima, 1970; Griepp et. al, 1978). Further, synchronization of beating between two heart cells can be mediated by mutual contact with non-excitable cells such as Hela cells and FL amniotic cells (Goshima and Tonomura, 1969).

1.8.5. **Cell Coupling and Endogenous Cellular Growth Control**

The discovery by Loewenstein and Kanno (1966) that some types of tumour cells did not form low resistance junctions whereas the normal cells in the tissues from which these tumours derived showed good electrical coupling, led Loewenstein (1968) to propose that cell coupling was directly involved in general cellular growth control. At the time this seemed quite a plausible function as the need for cell coupling in non-excitable tissues was not understood. Loewenstein (1978) has recently elaborated on his hypothesis and indeed three other growth control models based on cell coupling have since been proposed.

The models of Loewenstein (1968, 1978), Burton (1971) and Socular
(1973) stem from the contention that there is a causal relationship between loss of cell coupling and uncontrolled growth. Although Sheridan's model (1976) also predicts such a relationship, he proposed his model from the possibility that changes in the intracellular level of cyclic nucleotides during the G1 phase of the cell-cycle (Sheppard, 1975), where it is known there is a growth restriction point (Pardee, 1974), are used as growth control signals.

The growth control models of Loewenstein (1968, 1978), Burton (1971) and Sheridan (1976) are very similar. They propose that each cell is capable of synthesising junctional permeant growth control substances (ex. cyclic nucleotides) either in a pulse-like fashion at particular points in the cell-cycle (ex. G1; Loewenstein, 1968, 1978; Sheridan, 1976), or in a sinusoidal fashion throughout all the phases of growth (Burton, 1971). These substances are rapidly degraded and if their intracellular concentration should fall below a certain threshold concentration in a particular cell, then this cell will cease to grow. Thus, growth within a population of densely packed coupled cells will cease as the concentrations of these substances in the cells which are synthesising them at any one time will be diluted out to below threshold values by junctional transfer to non-synthesising cells. Hence, for uncoupled cells the growth control substances will not be diluted, so giving rise to uncontrolled or cancerous growth. These three models thus use the dilution aspect of junctional communication for a cell to estimate its surrounding local cell density.
Secular's model (1973) has much in common with these three other models. However, instead of making use of particular growth control substances he proposes that cellular growth could be controlled by limiting the concentration of certain metabolites necessary for growth. Cell coupling would serve to dilute out any local build up in the concentration of these metabolites that might occur and which would lead to non-essential growth.

Since the original observations of Loewenstein and his colleagues on tumour cells, many studies have now shown that whereas some types of malignant cells are totally incapable of forming permeable junctions, (Loewenstein and Kanno, 1966 and 1967; Jamakosmanovic and Loewenstein, 1968; Kanno and Matsui, 1968; Borek et al, 1969; Azarnia and Loewenstein, 1971; Cox et al, 1974b; Fentiman et al, 1976; Corsaro and Migeon, 1977), other malignant cells still have the ability to readily form these junctions (Furshpan and Potter, 1968; Borek et al, 1968; Sheridan, 1970; Johnson and Sheridan, 1971; Pitts, 1972; Flaxman and Cavato, 1973; Shen et al, 1976; Azarnia and Loewenstein, 1976; Fentiman et al, 1976; Corsaro and Migeon, 1977). Thus it would appear that there is no direct link between loss of the ability to form permeable junctions and the onset of cancerous growth. This may simply be that there are many causes of cancer and loss of the ability to form permeable junctions is one of them. If this is so then it infers that growth control mechanisms based on cell coupling only operate in the particular cell types where loss of coupling results in their transformation to cancerous growth. However, the above
four models do take into account 'communicating' cancer cells and thus still can be considered as general growth control mechanisms. Moreover, it appears that communicating cancer cells do have a reduced ability to communicate in comparison with the untransformed cell types from which they were derived (Pitts, 1972; Corsaro and Migeon, 1976; Loewenstein, 1978).

Apart from the observation that transformation is associated with a change in the ability to form permeable junctions, the only other evidence supporting the contention of this being a causal relationship comes from a recent study carried out by Azarnia and Loewenstein (1977). They hybridized normal human skin fibroblasts with the highly malignant 1C-1D cells. These latter cells are a subline of L cells which have been shown by many to be totally incapable of forming permeable junctions (Gilula et al, 1972; Pitts, 1971; Pitts and Simms, 1977; Ledbetter and Lubin, 1979; Kaplan and Kaplan, 1977). The basis of Azarnia and Loewenstein's study comes from a series of experiments carried out by Harris and his colleagues (Klein et al, 1971; Bregula et al, 1971; Wiener et al, 1971). They found that hybrids of normal and malignant cells exhibited normal growth properties but as the hybrids lost the chromosomes of the normal cells to form more genetically stable segregants, they often reverted back to malignancy depending on the number of normal chromosomes that had been lost. Thus, Azarnia and Loewenstein (1977) argued that if there is a causal relationship, it would be expected that segregants which retain a normal or near normal capacity to
form permeable junctions will exhibit normal growth properties, whereas segregants which have totally lost the ability to form permeable junctions will be highly malignant. Similarly, segregants which show an intermediate ability to form these junctions will exhibit an intermediate level of malignancy. Therefore, Azarnia and Loewenstein examined the communication properties of each segregant hybrid by electrical coupling and injected fluorescein and the growth properties by measuring tumorigenicity in mice and density at confluence when grown as monolayer cultures.

Indeed, they did find a good correlation between loss of the ability to form permeable junctions and the reversion to malignancy. As noted by Pitts (1978), the study was not sufficiently detailed to be able to pin-point loss of a particular group of chromosomes derived from the human cells. While experiments of this type may elucidate the role of permeable junctions in growth control in this particular system, all that can be concluded at present from the study of Azarnia and Loewenstein is that their results are consistent with a causal relationship but are not direct evidence supporting such a relationship.

An anomaly of Azarnia and Loewenstein's study is that they used 1C-ID cells and not the parental L cells themselves as the abnormal cell. L cells only exhibit low tumorigenicity as also do the fully communicating BHK cells (Klein et al, 1971; Jarrett and Macpherson, 1968). Indeed, when L cells are fused with the highly malignant Ehrlich ascites cells the resultant hybrids exhibit the low
tumorigenicity of the L cells (Klein et al, 1971). Contrary to the hypothesis of a causal relationship, this result suggests that the normal growth pattern of hybrids of normal and malignant cells may not be dependent on the communication properties of the normal cells. However it is conceivable that these two cell types are able to complement any communication defects they may have and thus their hybrids are readily able to form permeable junctions.

Further evidence against a causal relationship comes from a study by Hulser and Webb (1973). They found a number of rat liver epithelial cell lines that exhibited near normal in vitro growth properties (i.e. comparitively low cell density at confluence) and yet were not electrically coupled. Moreover, two of the cell lines were tested for tumorigenicity in neonatal rats and found to be incapable of raising tumours. Although this might be interpreted as direct evidence against a causal relationship, it can be argued that loss of the ability to communicate can sometimes be a possible but not a sufficient step for the onset of cancerous growth. Thus for cancerous growth to be expressed by non-communicating cells there may also be a need for another alteration, ex. a change in cell shape (Folkman and Moscona, 1978). If transformation to malignancy is a two or multiple step phenomenon of which loss of the ability to communicate is one of the possible steps, then it can be further argued that the hybrids of L cells and Ehrlich ascites cells express the low tumorigenicity of the L cells because genes expressed by the L cell chromosomes are dominant to the other alterations required for cancerous growth. For example
the hybrids may take on the shape of the L cells.

There are two other studies related to permeable junctions and cancer but are not concerned with the four growth control models. Stoker et al (1967) found that the growth of polyoma transformed BHK cells could be arrested if they were seeded into non-dividing confluent cultures of BHK cells. However, the growth was not arrested if they were seeded into cultures of confluent but still dividing BHK cells. As PyBHK cells can readily form permeable junctions (Subak-Sharpe et al, 1966 and 1969; Furshpan and Potter, 1968), Stoker et al (1968) speculated that these junctions maybe somehow involved in the growth arrest of the PyBHK cells. In contrast to this study, Todaro and Green (1964) found that adding SV40 virus to non-dividing confluent cultures of 3T3 cells resulted in rapid growth of the 3T3 cells which had been transformed although there was still a large proportion of normal 3T3 cells still present in the culture. Again 3T3 cells and their SV40 transformed derivative form permeable junctions (Pitts, 1971). Thus, supposing the mechanism of growth arrest in Stoker et al's study involves permeable junctions, it appears not to be a universal mechanism. As yet the mechanism of this growth arrest is still not known.

The second study was carried out on embryonal carcinoma cells and mouse embryos by Mintz and Ihlmensee (1975). They found that making chimeric mice by injecting EC cells (OTT 6050) into pre-implantation mouse embryos resulted in adult mice which although most of their tissues contained cells derived from the EC cells, were quite normal.
As mentioned earlier (section 1.6.3.) EC cells do form permeable junctions (Hooper and Slack, 1977; Nicolas et al, 1978) and so it is possible that the reversion to normal growth of the EC cells is dependent on them forming these junctions with the cells of the pre-implantation embryo.

In summary, it is too early yet to make any definite conclusions on the relationship of permeable junctions to cancer. However, from the studies that have been carried out to date there is no more reason to believe that loss of the ability to form permeable junctions results in cancerous growth than any other associated change which occurs on transformation. Indeed, there is some indirect evidence against a causal relationship between these two phenomena. The problem in attempting to study a possible involvement of permeable junctions in cellular growth control by examining cancer is that cancer is a multiplicity of diseases making general assumptions difficult and, moreover, is an abnormal state where the natural growth mechanisms have been overridden. Thus some attention should be paid to normal growing systems.

With this in mind Sheridan (1976) has predicted from his model that populations of asynchronously growing cells in normal tissues should be joined by few junctions to prevent dilution of growth control substances. Whereas cells that have left these populations to the non-dividing regions of tissues should become extensively joined by permeable junctions to prevent further growth. Sheridan (1976) cites
a system which is consistent with this prediction. McNutt et al (1971) do find that the rapidly dividing cells of the basal regions from the squamous epithelium of human cervix are joined by few gap junctions. However the cells that have left this region to non-dividing regions and which are destined for keratinization are extensively joined by gap junctions.

In contrast to the study of McNutt et al (1971), Orwin et al (1973) find that the rapidly dividing cells of the basal region from the cortex of the sheep wool follicle are as extensively joined by gap junctions as the non-dividing cells that have left this region. A morphometric analysis by thin section shows that gap junctions occupy several percent of the surface of both the dividing and non-dividing cortex cells which is one of the highest values so far reported. In an embryonic system it has been found that the rapidly dividing mesenchymal cells in the progress zone of the developing chick limb bud are as extensively joined by gap junctions as the non-dividing mesenchymal cells which have left this zone (Kelley and Fallon, 1978). However, it is possible that the gap junctions present in the progress zone may be there for morphogenetic reasons (ex. transmission of morphogenetic signals; section 1.8.6.). Although this possibility tends to argue against the generality of growth control mechanisms based on cell coupling. Thus there appears to be no clear cut difference in the distribution of gap junctions joining dividing and non-dividing cells.

A model system for the study of growth control which has received a
considerable amount of attention in the last 50 years is the mammalian regenerating liver. Three studies have analysed the communication properties of the hepatocytes of regenerating liver. An early study by Loewenstein and Penn (1967) showed there to be no detectable difference in the electrical communication properties of rat liver hepatocytes 2 days, 3 days and 1 week after partial hepatectomy. However, two recent morphometric studies (Yee and Revel, 1978; Yancey et al, 1979) showed there to be a cycle of disappearance and reappearance of gap junctions between rat hepatocytes. Between 24 and 28 h after partial hepatectomy, hepatocytes precipitously lost nearly all of their gap junctions but by 36 h the gap junctions began to reappear and reach near normal levels by 44 h. This has now been confirmed biochemically by the loss of the polypeptides (the tryptic digested polypeptide of 10,000 daltons and native 26,000 dalton polypeptide; section 1.5.4.) from gap junction rich fractions (Finbow et al, manuscript in preparation).

It is possible that this cycle of disappearance and reappearance of gap junctions is involved in some growth control mechanism similar to those proposed by the four models. However, the cycle occurs concurrently with the first wave of mitosis and it could be in response to this that gap junctions are transiently lost. Arguing against mitosis being the cause is that cells in mitosis have been found neither to electrically uncouple (O'Lague et al, 1970) or lose gap junctions (Merk and McNutt, 1972) with surrounding interphase cells. Moreover, all the hepatocytes lose gap junctions at a time when only
a very small percentage of them are engaged in mitosis (Bucher, 1963).

As well as a transient loss of gap junctions, the network of tight junctions surrounding the bile caniculi also becomes dramatically altered between the 28 to 36 h period. The tight junctions are not lost but become much looser and many large macular tight junctions are commonly found some distance away from the peri-canicular regions of the plasma membrane (Yee and Revel, 1978; Yancey, unpublished observations). This suggests that there may be an overall re-organisation of the plasma membrane during this period. Thus gap junctions may not be lost in order to allow for further proliferation of the hepatocytes but as a consequence of this re-organisation. It might be expected that tight junctions would not be lost as it would result in leakage of harmful bile salts into the sinusoidal spaces of the liver.

Finally, there is direct evidence that growth control through cell coupling can operate in vitro. In the earlier discussed system of mixed cultures of HGPRT<sup>+</sup> and TK<sup>-</sup> BHK cells (section 1.6.3.) it was shown by Pitts (1971) that in the presence of aminopterin, the two cell types became interdependent upon one another producing a self-regulating population. This interdependency was due to the junctional exchange of nucleotides between the two types. Although Pitts (1978) has recently speculated that similar interdependencies may operate in vivo, he points out that it requires a level of differentiation not hitherto recognised. Moreover, such interdependencies will not
control growth \textit{per se} but only the ratio of one cell type to another and of course, the mechanisms of growth control will inherently control the ratio of the various cell types in a tissue to one another.

In summary, there is no compelling reason to believe that cell coupling has a direct role in endogenous cellular growth control. As other roles can be assigned to cell coupling in non-excitabl e tissues, the hypothesis of it being directly involved in growth control has lost much of its impetus. Cell coupling could, though, co-ordinate the response of a tissue to initiate growth due to an external stimulus as discussed earlier (section 1.8.2.).

\subsection*{1.8.6. Cell Coupling and the Transmission of Morphogens}

As with growth control, it is attractive to believe that cell coupling may have a role in embryonic development (Furshpan and Potter, 1968). There are now a number of developing systems which are suggestive of the intercellular movement of morphogenetic signals through permeable junctions but there is still a lack of direct experimental evidence to support this proposed role (Wolpert, 1978).

Wolpert (1969) has pointed out that most embryonic fields are of the order of no more 100 cell diameters (about 1 mm) and that it takes the order of several h to set up gradients of positional information. With this in mind Crick (1970) calculated that such a gradient of
positional information could be established by the diffusion of a low molecular weight morphogen but this was based on there being facilitated diffusion across the plasma membrane and, of course, cell coupling is believed to occur by passive diffusion. However, Wolpert (1978) has recently pointed out that such diffusion of morphogens through junctions is still plausible if the ratio of area occupied by gap junctions to the total contact area is no less than 1 in 100. As yet, no morphometric measure of the extent of gap junctions has been carried out in embryonic tissues at the time positional information is specified. Such a value does not seem unreasonable from morphometric studies that have been carried out on adult tissues (Revel et al, 1971; Orwin et al, 1973; Yee and Revel, 1978). Although as reviewed earlier (section 1.6.8.), for cells of the early cleavage stages of vertebrate embryos it appears that their junctional communication capacities are much less than those of adult cells and possibly embryonic cells of later stages. This being so, it infers that the earliest morphogenetic events are not mediated by intercellular transfer of morphogens through permeable junctions.

Developing systems which are consistent with positional information being specified by a gradient of a diffusible morphogen include the inhibition of head formation in hydra in regions that are close to the head (Wolpert et al, 1972), the polarity of epidermal cells in the insect Rhodnius (Lawrence et al, 1972) and the specification of digits in the vertebrate limb (Tickle et al, 1975). Thus, this possible function of cell coupling might be widespread in multicelalled animals.
It must be stressed however, that there are alternative mechanisms of specifying positional information which are not dependent on the type of intercellular communication afforded by cell coupling.

A number of studies have been carried out in an attempt to correlate possible changes in communication that could occur at the same time as various developmental events. Dixon and Cronly-Dillon (1972 and 1974) found that gap junctions disappear from the central portion of the amphibian retina at about the same time axial polarity is specified. Likewise Eley and Shelton (1976) found a disappearance of gap junctions prior to organization of the cells into clusters to form ommatidia in the compound eye of locust. Both these groups of workers speculate that the change in gap junctions has some causal relationship to the resulting differentiation.

An electrophysiological study by Blackshaw and Warner (1976) has shown for three different amphibians that the cells of somitic mesoderm are electrically coupled but shortly before somite formation, the cells destined to join the new somite at the intersomite border become uncoupled from the cells remaining in the unsegmented mesoderm. As with the studies on vertebrate retina and the insect compound eye, the significance of this event is unknown but Blackshaw and Warner (1976) suggest it may act as a trigger for somite formation.

Fallon and Kelley (1977) have recently shown an abundance of gap junctions between cells of the apical ectoderm ridge from embryos
from several different species of birds and mammals. This tissue is believed to specify the proximal-distal axis of the vertebrate limb by inducing a zone of cells in the underlying mesoderm to proliferate. This zone is kept near constant in size and it appears that the proximal-distal axis is specified by counting the number of times the cells divide in this zone (Summerbell et al., 1973). The mesenchymal cells in this zone and in more basal regions have been found to be joined by gap junctions (Kelley and Fallon, 1978). Fallon and Kelley also report that the other ectodermal cells of the developing limb have few gap junctions thus suggesting a special functional role for these junctions between the ridge cells in limb morphogenesis. It is not known what the morphogenetic signal is which is released by the apical ectoderm ridge cells. This signal may not only just specify the size of the zone of proliferating mesoderm cells but also activate the 'zone of polarising activity' which specifies positional information along the anterior-posterior axis (Tickle et al., 1975). Fallon and Kelley (1977) suggest that the abundance of gap junctions in this tissue is to integrate its function by sharing metabolites and ions. In this sense, the gap junctions would have homeostatic functions rather than signalling functions.

Saxen et al. (1976) have presented evidence that embryonic induction may be dependent on cell contact. They grew mouse metanephric mesenchyme and salivary mesenchyme or spinal cord on opposite sides of nucleopore filters of various pore sizes. The ability of the last two tissues to induce tubule formation in the metanephric mesenchyme correlated
very well with the ability of the tissues to send processes through
the nucleopore filter and make contact with the metanephric mesenchyme.
However, there is also evidence that induction is signalled by the
extracellular matrix and it is quite possible that the results of
Saxen et al (1976) can be explained on this basis (Hay, 1976).

It is tempting to speculate that cell coupling could be intimately
involved but, as pointed out by Wolpert (1978), there may be several
different mechanisms of specifying positional information. The studies
carried out to date are only suggestive of cell coupling having a
direct role in morphogenesis. However, this form of intercellular
communication can account for many of the morphogenetic processes
occurring in development (Wolpert, 1978).

1.9. Summary of the Literature Survey and Aim of this Present Study

Cell coupling and its morphological correlate, the gap junction, are
very widespread in multicelled animals. They have been found in both
simple and higher animals. This apparent ubiquity suggests they have
some important roles in the tissues of these animals. It is possible
that this direct form of intercellular communication is involved in
a variety of homeostatic functions such as co-ordination of cell
activities and the transmission of signals including electrical impulses.

A large number of studies have been conducted on the permeability
of junctional plasma membrane between coupled cells. For the vertebrates it has been shown that inorganic ions, nucleotides, a variety of fluorescent tracer dyes and fluorescent labelled amino acids are all transferred between coupled cells. There is also some indication that sugar phosphates and phospholipids can be transferred. However, cellular macromolecules (RNA, DNA and proteins) are not directly transferred between cells. With arthropod tissue it has been shown that coupled cells can exchange a variety of fluorescently labelled amino acids, oligopeptides and oligosaccharides up to mol. wt. 1500, as well as inorganic ions and sugars. For the annelids there is evidence suggesting the transfer of amino acids, nucleotides, sugars and a variety of fluorescein anions between electrically coupled cells. By far the majority of these studies are consistent with the concept of a common pathway and it is now commonly accepted that the gap junction provides this pathway. Moreover, the apparent general permeability of junctional plasma membranes between coupled cells suggests that there are water filled pores spanning these membranes and so linking the cytoplasms of coupled cells. There is now evidence that gap junctions contain such pores which have a diameter which can account for the known permeability.

The existence of such pores predicts that all small molecules and ions can move between coupled cells. Although this appears to be true from looking at the above cited substances, for the vertebrates the only classes of endogenous substances that are definately known to pass between coupled cell are the nucleotides and the small inorganic ions. It has been argued (Van Venrooij et al, 1975), that fluorescent labelled amino acids and tracer dyes do not reflect the natural
permeability of cell junctions. If this is the case then our knowledge of junctional permeability is somewhat scant. Furthermore, it might be thought that the junctional membranes need only be permeable to ions and nucleotides. Such permeability would account for electrical coupling in excitable tissues and co-ordination of the response to hormones through second messenger sharing (ex. cyclic AMP) as well as the co-ordination of metabolism by the sharing of the energetically important metabolites (ex. ATP). Moreover, even if it is assumed that all metabolites are transferred between coupled cells, there is only information available on the rate of transfer of one class of metabolite (namely the nucleotides). Obviously, in trying to decide what roles cell coupling might have, it is important to know the rate of intercellular movement of substances. If there is only slow leakage of metabolites (other than nucleotides) then this would argue against cell coupling having much importance in general homeostasis.

This study therefore attempted to further define the permeability of cell junctions joining coupled cells. A range of very different metabolites was chosen in order to establish whether there is any specificity of the permeability of these junctions. These metabolites include the phospholipid precursors phosphoryl choline and CDP-choline, the sugar phosphates of 2-deoxy-glucose and glucosamine, the amino acid proline and its precursors and the vitamin derived cofactor tetrahydrofolate. BHK cells were chosen for this study because the permeability properties of their cell junctions have been well documented. Also, it was known how extensively these cells are
joined by gap junctions (Revel et al., 1971) and thus it might be possible from the results to make reasonable calculations of how fast the metabolites are transferred between cells in vivo. The L cell was used as a control in these studies because of its well characterised inability to form intercellular junctions permeable to ions and nucleotides (Gilula et al., 1972).
Chapter 2 MATERIALS AND METHODS
2.1. **Materials**

2.1.1. **Chemicals**

Hypoxanthine, thymidine, 5-bromo-2-deoxyuridine, glycine, pyruvate, (sodium salt, type II), choline chloride, D-pantothenate (hemicalcium salt), pyridoxyl-HCl, riboflavin, folic acid, niacinamide and inositol were all obtained from Sigma, St. Louis, Missouri, U.S.A. Reagent grade chemicals were used throughout and the reagents were not further purified.

Thiamine-HCl and N.N'-methylene-bis-acrylamide (bis-MSB) were obtained from British Drug Houses, Ltd., London.

Xylene, Toluene, Triton X-100 and 2, 5-diphenyloxazole (PPO) were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England.

2.1.2. **Radiochemicals**

All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., England. These radiochemicals were 5-bromo-2'-deoxy-[5-^3H]-uridine (2,000 mCi/mmole), methyl-[^3H]-choline (10,000 mCi/mmole), 2-deoxy-D-[1-^3H]-glucose (20,000 mCi/mmole), deoxy-[8-^3H]-guanosine (4,000 mCi/mmole), [C-^3H] folic acid (2,500 mCi/mmole), [14C] formic acid (50 mCi/mmole), [^3H] formic acid (200
mCi/mmole), L-[6-3H]-fucose (12,000 mCi/mmole), D-[6-3H]-glucosamine (10,000 mCi/mmole), [G-3H]-hypoxanthine (1,000 mCi/mmole), [methyl-3H]-thymidine (21,000 mCi/mmole), and [3-3H]-uridine (26,000 mCi/mmole). All radiochemicals were greater than 97% pure radiochemically on dispatch.

2.1.3. **Tissue Culture Cells**

The following tissue culture cells were used:

(i) BHK-21/C13 cell line (Syrian hamster fibroblasts; Macpherson and Stoker, 1962).

(ii) BHK-HGPRT<sup>-</sup> (TG2) cell line (as in normal BHK cells but lack hypoxanthine-guanine phosphoribosyl transferase; Marin and Littlefield, 1968).

(iii) BHK-TK<sup>-</sup> (B1) cell line (as in normal BHK cells but lack thymidine kinase; Marin and Littlefield, 1968).

(iv) PyBHK cell line (polyoma transformed BHK cells; Stoker and Macpherson, 1964).

(v) 2601 PyBHK-TK<sup>-</sup> (polyoma transformed BHK cells which lack thymidine kinase; Slack, personal communication).
(vi) L (clone 929) cell line (mouse fibroblast; Sanford et al, 1948).

(vii) L-HGPRT<sup>−</sup> cell line (as in normal L cells but lacks hypoxanthine-guanine phosphorybosyl transferase; Littlefield, 1966a).

(viii) 3T3 cell line (Swiss mouse fibroblast; Todaro and Green, 1963).

(ix) Hela cell line (human epithelial cervical carcinoma; Gey et al, 1952).

(x) Int407 cell line (human embryonic intestine epithelia; Henle and Deinhardt, 1957).

(xi) Chang cell line (human liver epithelia; Chang, 1954).

(xii) BSC-1 cell line (African green monkey kidney epithelia; Hopps et al, 1963).

(xiii) CHO-K1 cell line (derivative of the CHO cell line from hamster ovary requiring proline; Kao and Puck, 1968).

(xiv) CHO-K1/AuxBl cell line (derivative of CHO-K1 requiring thymidine, glycine and a purine; McBurney and Whitmore, 1974a).

(xv) HTC cell line (rat hepatoma; Thompson et al, 1966).

(xvi) H35 cell line (rat hepatoma; Pitot et al, 1964).
(xvii) 4629 P10 cell line (murine splenic B-lymphocytes; Prekumar-Reddy et al., 1976).

(xviii) BRL cell line (Buffalo rat liver epithelia, isolated by Puck and obtained from Dr. R. R. Burk).

(xix) Xenopus cell line (Xenopus laevis kidney epithelia; obtained from Dr. K. Jones, Edinburgh).

Further information on many of these cell lines can be obtained from the Flow Laboratories Catalogue (1976).

2.1.4. Cell Culture Medium

Stock solutions of amino acids and vitamins and also foetal calf serum were all obtained from Bio-Cult Laboratories, Glasgow. Trypsin (E.C. 3.4.4.4.) was obtained from Difco Laboratories, Surrey and penicillin and streptomycin were obtained from Glaxo Laboratories Ltd., Middlesex, England.

The following types of tissue culture media were used:

(i) \( EFC_{10} \)-contained Eagle's medium + foetal calf serum (9:1).

(ii) \( cdEFC_{10} \)-as in \( EFC_{10} \) without choline chloride.
(iii) \textit{pyrEFC}_{10}^-\textit{ as in EFC}_{10} without glucose but + pyruvate (25mM).

(iv) \textit{fdEFC}_{10} - as in \textit{ghtEFC}_{10} without folic acid.

(v) \textit{ghtEFC}_{10} - as in \textit{EFC}_{10} except using dialysed serum and supplemented with glycine (100\textmu M), hypoxanthine (37\textmu M) and thymidine (21\textmu M).

(vi) \textit{htEFC}_{10} \textit{NEAA} - as in \textit{EFC}_{10} plus non-essential amino acids and supplemented with hypoxanthine (37\textmu M) and thymidine (21\textmu M).

(vii) \textit{pdEFC}_{10} - as in \textit{EFC}_{10} except using dialysed serum.

(viii) \textit{psEFC}_{10} - as in \textit{pdEFC}_{10} except supplemented with proline (11.5\textmu g/ml).

Eagle's medium has the composition shown in the Flow Laboratory Catalogue (1976). Dialysed serum was prepared by dialysing against water (3 changes) for 24 h at 5^\circ C and sterilising by passage through a millipore filter (0.22\textmu m pore size).

2.1.5. Solutions

(i) Amfix - solution contained 20% (v/v) Amfix (May and Baker, Dagenham) in water.
(ii) Balanced Salt Solution (BSS) - composition shown in table 1.

(iii) Developer D19b - composition shown in table 2.

(iv) Formol saline - solution contained 0.08N NaCl, 0.2N Na₂SO₄ in 4% (v/v) formaldehyde in water.

(v) Giemsa stain - contained 0.75% Giemsa (w/v; Gurr, London) in glycerol/methanol (1:9, v/v).

(vi) Triton X-100/Xylene scintillant - 1:1 (v/v) contained 0.5% (w/v) PPO and 0.05% (w/v) bis MSB.

(vii) Toluene scintillant - contained 0.5% (w/v) PPO.

(viii) Trichloracetic Acid (TCA) solution - contained 5% (w/v) TCA in water.

(ix) Coulter Counter Fluid - composition shown in table 4.

Table 1  Balanced Salt Solution (BSS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>6.8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 g</td>
</tr>
<tr>
<td>CaCl₂·6H₂O</td>
<td>0.393 g</td>
</tr>
</tbody>
</table>
Table 2  Developer D19b

Na$_2$SO$_3$·7H$_2$O  144 g
Na$_2$CO$_3$  48 g
KBr  4 g
Hydroquinone  8.8 g
Metol (Kodak)  2.2 g

Total volume  1 litre

Table 3  Trypsin Solution

Trypsin (Difco)  2.5 g
NaCl  6 g
Sodium citrate  2.96 g
Phenol red  0.015 g
Solution adjusted to pH 7.8
Total volume  1 litre

Table 4  Coulter Counter Fluid

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.12 M</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.05 M</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.0037 M</td>
</tr>
</tbody>
</table>
2.2. Methods

2.2.1. Maintenance of Cell Cultures

All cell cultures were grown as monolayers incubated at 37°C in an atmosphere of 95% air and 5% CO₂. Cell cultures growing on plastic tissue culture dishes were kept in a humidified incubator continually flushed with air and 5% CO₂. Cell cultures growing in glass bottles (4 oz and 8 oz medical flats or Roux flats) were flushed with air containing 5% CO₂ before being sealed.

 Cultures of all cell lines were maintained in 4 oz medical flats (containing 10 ml of medium) or 8 oz medical flats (containing 15 ml of medium). These stock cultures were sub-cultured every 3 to 5 days by trypsinizing the monolayers to yield a cell suspension (see below) and culturing 1/10 of the suspension in new bottles containing the appropriate medium. Monthly checks for mycoplasma contamination were made on cell suspensions from these stock cultures. After 10 to 15 times of sub-culturing the cells were discarded and new cultures started from cells stored in liquid Nitrogen.

2.2.2. Preparation of Cell Suspensions by Trypsinization
The growth medium of cell cultures was discarded and the monolayers briefly washed with a sterile solution of trypsin (table 2) prewarmed to 37°C (1 ml trypsin/50 mm culture dish, 2 ml/4 oz medical flat, 3 ml/8 oz medical flat and 5 ml/Roux flat). The trypsin washing was discarded and the same volume of fresh trypsin added. The cultures were then incubated at 37°C until the cells began to detach. The trypsin was carefully removed and fresh medium, pre-warmed to 37°C, was added (1 ml medium/50 mm culture dish, 5 ml/4 oz medical flat, 8 ml/8 oz medical flat and 15 ml/Roux flat). The cells were dispersed into a uniform suspension by pipetting up and down several times.

The cell density of the suspensions was found by counting portions of the suspensions with either an 'Improved Neubauer' haemocytometer or a Coulter Counter (Coulter Electronics Inc.; Series B model). For counting with a Coulter Counter the cell suspension was first diluted 1 to 50 (v/v) with counting fluid (Materials section 2.1.5.).

2.2.3. Preparation of Cells for Experimental Purposes

Cell suspensions derived from the stock cultures were seeded into Roux flats (5 x 10^6 cells/Roux flat; each Roux flat has 120 cm^2 culture surface) and 50 ml of the appropriate culture medium added. The cells were grown for 2 days and then harvested by
trypsinizing (section 2.2.2.) and suspended in the appropriate medium. After measuring the cell density the suspensions were used immediately for experimental purposes.

2.2.4. Preparation of 10 day Folate Starved BHK Cells

Growing cultures of BHK cells were established in Roux flats as described in the above section. The culture medium used was ghtEFC\textsubscript{10} (materials section 2.1.4.) and after 2 days growth this medium was removed from the cultures. The monolayers of cells were washed 4x with BSS (20 ml/wash/Roux flat, pre-warmed to 37°C) to remove ghtEFC\textsubscript{10}. The cultures were trypsinized and suspended in folate deficient medium (fdEFC\textsubscript{10}; see materials section 2.1.4.). The suspensions were seeded into new Roux flats (5 x 10\textsuperscript{6} cells/Roux flat) and 50 ml of fdEFC\textsubscript{10} added to each Roux flat. The cells were grown at 37°C and sub-cultured every third day in fdEFC\textsubscript{10} (5 x 10\textsuperscript{6} cells/Roux flat). After 10 days growth in fdEFC\textsubscript{10} the cells were trypsinized, suspended in fdEFC\textsubscript{10} and used for experimental purposes. For following the time course of folate depletion (section 3.4.2.), cultures were trypsinized and suspended in fdEFC\textsubscript{10} after shorter periods of growth in fdEFC\textsubscript{10}, as indicated.

2.2.5. Preparation of Glass Coverslips for Cell Culture
Glass coverslips (Chance Glass Co.; No. 1, 13 mm diameter) were cleaned by boiling in 1N NaOH for 10 min and then washed free of the alkali by continuous rinsing under tap water for 3 h. The cleaned and washed coverslips were rinsed 3x with de-ionised water and 2x with methylated spirits before drying on filter paper in a warm oven. The dried coverslips were transferred to a glass petri dish and sterilised in a hot oven at 160°C for 6 h. The coverslips were transferred from the storage dish to the culture dishes using aseptic techniques. Generally, 8 coverslips were used in each 50 mm culture dish.

2.2.6. Preparation of Cells for Acid Insoluble Autoradiography

The radioactive medium was removed from dishes containing tritium labelled cell cultures growing on coverslips (details of the labelling procedures are given in the appropriate figure legends) and the cultures washed 2x with ice cold BSS (3 ml/wash/dish). The cultures were then fixed by adding 5 ml of 10% formol saline (materials, section 2.1.5.). The fixation was carried out at 5°C for 90 min to 2 h. Coverslips were then transferred to porcelain racks (A.H. Thomas Inc., Philadelphia, USA). The racks were immersed 3x in a bath of ice cold 5% TCA (volume of bath approximately 100 ml) for 3 min each time, 2x in ice cold distilled water (approximately 100 ml) for 3 min each time and then briefly dipped 2x into methylated spirits. The methylated spirit wash was
omitted for cells labelled with \([{}^{3}\text{H} -\text{methyl}]\)-choline. The coverslips were air dried at room temperature and then mounted, cells uppermost, on microscope slides (McFarlane Robson Ltd., Glasgow) with DePex (Gurr, London). Microscope slides were stored in methylated spirits and air dried before using.

2.2.7. Preparation of Autoradiographs

Ilford L4 Nuclear Research Gel (5 g) was melted at 47°C and diluted to 15 ml by adding distilled water at 47°C. The microscope slides were dipped into the diluted emulsion and drained to remove excess emulsion. The coated slides were dried in a stream of cold air and placed into light-tight slide boxes containing activated silica gel. The boxes were stored at room temperature for the required period of exposure.

After the exposure period, the autoradiographs were developed in D19b (table 2) at 20°C for 5 min, without agitation, fixed with Amfix (diluted 1 to 4 with distilled water) for 4 min at 20°C and rinsed in tap water for 1 min. The autoradiographs were stained with a solution of Giesma (materials, section 2.1.5.), freshly diluted 1 to 20 with distilled water, for 1 min and then rinsed under tap water for 10 min. The stained autoradiographs were air dried and then covered with extra coverslips mounted with DePex. The period of exposure varied depending whether the autoradiographs
were to be used for grain counting studies (short exposure) or be photographed (long exposure). Exposure times are given in the figure and table legends. Photographic records of autoradiographs were made with Ilford Pan F 35 mm roll film using a Leitz Orthomat photomicroscope.

2.2.8. Counting of Autoradiographic Grains over Tritium Labelled Cells

Autoradiographic grains over cells were counted using a Leitz Orthomat light microscope with a x100 oil immersion plan objective and x10 wide angle eyepieces. In experiments using the pre-labelling technique of Pitts and Simms (1977; sections 3.2. and 4.2.) the extent of transfer of labelled cellular material was estimated by counting grains over recipient cells directly in contact with donor cells. Autoradiographic background in these experiments was found by counting the grains over 50 recipient cells not in contact with labelled cells. This background is composed of the photographic background and any small amount of incorporation due to medium mediated transfer of labelled material (Pitts and Simms, 1977).

In the experiments to examine the transfer of folates, only the grains above isolated cells (i.e. cells not in contact with other cells) were counted. This procedure eliminates confusion caused
by the transfer through intercellular junctions of labelled material
derived from \(^{3}H\)-formate when the cells are in contact with one
another and therefore, gives a direct measure of each individual
cell's ability to incorporate formate. An estimate of the
autoradiographic background in these experiments was found by
counting the number of grains in a cell free high power microscope
field and dividing by the number of cells which in confluent areas
filled the microscope field.

2.2.9. **Measurement of the Incorporation of Radioactivity into
Cell Fractions**

Cell cultures growing in 50 mm dishes were labelled with the
appropriate \(^{3}H\)-precursor; details of the labelling procedures
are given in the figure and table legends. The labelled medium
was removed and the monolayers of cells washed 3x with fresh medium
pre-warmed to \(37^\circ\mathrm{C}\) (3 ml/wash/dish). The first wash removes more
than 95\% of the radioactivity originally present in the medium. The
incorporation of radioactivity into acid soluble and acid insoluble
fractions was measured either immediately or after a further
period of growth. If the cells were to be grown after labelling
then fresh medium was added to the culture dishes (3 ml/dish).
The incorporation of radioactivity into acid soluble and acid
insoluble fractions was measured as follows. After removal of the
final medium wash, the monolayers were washed 2x with ice cold BSS
(3 ml/wash/dish). In experiments where the radioactivity in the growth medium was measured, the 2 BSS washings were combined with the medium washes (total volume of 6 ml from each culture dish) and a 2 ml sample of the mixture was placed in a scintillation vial to estimate its radioactivity (see below). After washing with BSS, the cultures were extracted with ice cold 5% TCA for 5 min (2 ml/dish). The TCA extract (acid soluble fraction) was quantitatively transferred to a scintillation vial if the incorporation of radioactivity into acid soluble material was to be measured; otherwise it was discarded. The extracted cultures were washed 2x with ice cold water (3 ml/wash/dish) and then solubilized with 0.1N NaOH (2 ml/dish) for 10 min at room temperature. The alkali extract (acid insoluble fraction) was quantitatively transferred to a scintillation vial. The measurement of counts of radioactivity is described below. Extraction of cultures with chloroform/diethyl ether is described in table 4.

2.2.10. Measurement of Radioactivity by Liquid Scintillation Counting

Aqueous samples were counted in triton/xylene scintillant (section 2.1.5.). 0.1N NaOH extracts and medium/BSS mixtures were first acidified by adding 0.3 ml 1N HCl to each 2 ml sample. Triton/xylene scintillant (15 ml/sample) was added and the resulting opaque solution mixed until it became clear. For the chloroform/
methanol extracts and samples from the chromatographic analysis of the growth medium and TCA extracts of cells labelled with 2-deoxy-[^3H]-glucose, a toluene based scintillant was used (section 2.1.5.). For each 2 ml sample of chloroform/diethyl ether extract 10 ml of toluene scintillant was added and for each 1 cm^2 piece of chromatograph paper 5 ml of toluene scintillant was added. The chromatograph paper was thoroughly dried by transferring each sample to a scintillation vial and placing in a warm oven for 1 h before adding the scintillant.

All samples were counted in a Nuclear Chicago Isocap 300 liquid scintillation counter. The samples were placed into the counter 30 min before counting to reduce light and temperature induced fluorescence of the scintillant. The efficiency of counting, calculated from the channels ratio (i.e. quench correction), varied by no more than 5% between different samples using the same counting system. Where estimates of disintegrations/min (DPM) were required the count/min (CPM) for each sample was converted to DPM by using a quench correction obtained from a graph of efficiencies plotted against channels ratios of quenched standards. The procedure is described in the Isocap 300 manual.
2.2.11. Estimation of Protein

Protein in alkali (0.1 N NaOH) extracts from cell cultures grown in culture dishes was estimated by the method of Lowry et al. (1951). 1 ml of the extract was added to 3.5 ml of a freshly made solution containing 0.1 N NaOH, 2% Na₂CO₃ (w/v; anhydrous salt), 0.5% CuSO₄·5H₂O (w/v) and 1% sodium potassium tartrate (w/v) and the mixture allowed to stand for at least 10 min. 0.5 ml of 1 N Folin-Ciocalteu's phenol reagent (Sigma, St. Louis, Missouri, USA) was quickly added and the mixture was immediately shaken by vortexing. The absorbance at 750 nm was read against a reagent blank on a Unicam SP-600 spectrophotometer after 30 min. Bovine serum albumin (Sigma) was used as the protein standard.

2.2.12. Statistical Analysis of the Histograms of counts of Autoradiographic Grains from the Co-culture Experiments Examining the Transfer of Cellular Folates

The data presented in figures 9 and 10 were analysed by the method of least squares on the assumption that the individual bands were of Gaussian form (Fraser and Suzuki, 1973). The estimated values of peak height, A₀, the abscissa, X, and the range at half peak height, X₁/₂, were used in the equation where X₀ is the X-coordinate of the peak -
\[ F = A_0 \ln 2 \left[ \frac{2(x - x_o)}{X_1/2} \right]^2 \]

- as the initial parameter values of the Gaussian distributions.

These values were found by a visual examination of the data. The sum of the squares of the difference \( S \) between the computed values and the data points was minimised by the Gauss-Newton method (Fraser and Suzuki; 1973) to compute new parameters for the distributions. The new parameters were now used and progress was followed by determining the root mean square of the deviations, \( \sigma = (S/n)^{1/2} \). After final minimisation of \( S \) values (i.e. after 40 iteration cycles), the unbiased estimate of standard deviation is stated by

\[ \sigma = \frac{S_{\text{min}}}{n - m}^{1/2} \]

where \( n \) = number of data points, and \( m \) = number of parameters.

The residual error for each data point was found by subtracting the observed value (\( y \)) from the calculated value (\( F \)). The residual error curve, or error plot, provides visual evidence of the fit of the generated curves. If the distributions are not Gaussian then the error curves will diverge from the abscissa. If any bands have not been included in the original parameter estimates, then this will be seen by a divergence of the error curve from the abscissa where bands have not been included. However, because the data are in the form of histograms and not smooth curves the resultant discontinuities in the error curves could obscure small bands not included (see Fraser and Suzuki, 1973). Counting the autoradiographic grains over a larger number of cells would provide a smoother curve.
Chapter 3  A STUDY ATTEMPTING TO DEFINE
THE PERMEABILITY OF INTERCELLULAR JUNCTIONS
FORMED BETWEEN VERTEBRATE CELLS IN CULTURE
3.1. **Introduction**

The following sections in this chapter give the results of a study to investigate the permeability of junctions formed between cells in culture.

In most cases BHK cells were used as the 'junction competent' cell (i.e. known to form junctions permeable to nucleotides and ions; Pitts and Simms, 1973; Furshpan and Potter, 1968) and L cells were used as a control being unable to form permeable junctions (Gilula *et al.*, 1972). Generally the methods that were employed were those that were currently in use in Doctor J.D. Pitts' laboratory. For examining possible intercellular transfer through junctions of molecules derived from choline and glucosamine it was possible to directly extend the pre-labelling technique of Pitts and Simms (1977; section 1.6.3.) used to demonstrate intercellular transfer of uridine nucleotides through junctions. However, for examining the transfer of other metabolites such as 2-deoxy-glucose-6-phosphate, cellular folates and proline (and its precursors) it was necessary to devise new approaches.
3.2. **Study on the Transfer of $[^3H]$-molecules Derived from $[^3H]$-methyl-$\beta$-choline Between BHK Cells**

3.2.1. **Introduction**

As discussed in Chapter 1 (section 1.6.7.) Peterson and Rubin (1970) presented evidence which suggested that phospholipid could be freely exchanged between chick embryo secondary fibroblasts in culture when they came into contact with one another. They further suggested that the phospholipid precursor phosphoryl choline was not transferred. Because of the water insolubility of phospholipid and the current view at the time when this study was conducted of molecular exchange occurring through water filled intercellular channels, Peterson and Rubin (1970) speculated that there must be some form of dynamic interaction between the membranes of contacting cells.

For our better understanding of both the permeability of intercellular junctions and the structural integrity of the plasma membranes of contiguous cells, it is important to be able to completely distinguish between transfer of phospholipid and phosphoryl choline. If phosphoryl choline cannot be transferred between contacting cells then it would argue strongly against the existence of aqueous channels joining the cytoplasms of contiguous cells. The study presented here attempts to define what molecules labelled with
[\textsuperscript{3}H-methyl]-choline can pass between cells joined by intercellular junctions using similar techniques to those employed by Peterson and Rubin (1970) and later used by Pitts and Simms (1977) to distinguish between RNA and uridine nucleotide transfer (section 1.6.3.).

3.2.2. Preliminary Studies

The study by Peterson and Rubin (1970) showed that cultured fibroblast cells use choline almost exclusively for the synthesis of lipid. An earlier study by Plagemann (1968) on choline metabolism in Novikoff hepatoma cells in suspension culture gave an identical result. Plagemann (1968) also showed that choline is rapidly taken up by Novikoff cells from the culture medium and converted to phosphoryl choline and CDP-choline; these intermediates being the precursors to a number of different lipids (Kennedy, 1956). He also found that the intracellular pools of phosphoryl choline and CDP-choline are not washed out from the Novikoff cells by either BSS or phosphate buffered saline.

In both these studies it was found that after growing either cell type for a few h in the presence of [\textsuperscript{3}H-methyl]-choline that most of the radioactivity in the acid insoluble fraction was located in phosphatidyl choline with trace amounts in lysophosphatidyl choline and sphingomyelin.
To discover if choline metabolism is the same in BHK and L cells in order that they may be used in this study, cultures of BHK and L cells were labelled with $[^3\text{H}]$-methyl-choline for 3 h and washed several times with BSS. The radioactivity in the acid soluble fraction, acid insoluble fraction and chloroform/diethyl ether soluble fraction after TCA extraction was measured. As EFC$_{10}$ is supplemented with choline a modified growth medium was used during the labelling period which contained no added choline (cdEFC$_{10}$; section 2.1.4.). The results are shown in table 4.

For both BHK and L cells practically all the radioactivity present in the acid insoluble fraction is soluble in chloroform/diethyl ether (99.5% for BHK cells and 99.3% for L cells). This is the expected result if choline is used almost exclusively for lipid synthesis in these two cell types. Also there is a sizeable amount of radioactivity in the acid soluble fraction of BHK and L cells. The radioactivity in this fraction is most likely to be located mainly in phosphoryl choline with a trace amount located in CDP-choline (Plagemann, 1968). Likewise most of the radioactivity in the acid insoluble fraction is presumably found in phosphatidyl choline.

One of the methods employed by Pitts and Simms (1977) to distinguish between RNA and uridine nucleotide transfer in BHK cells was to establish two types of $[^3\text{H}]$-uridine labelled donor cells. One type was freshly labelled with $[^3\text{H}]$-uridine (termed unchased donors)
which contained approximately equal amounts of radioactivity in RNA and uridine nucleotides. The other type was grown in unlabelled medium for 24 h after labelling the cells with $[^3\text{H}]$-uridine (termed chased donors) and therefore contained nearly all of the radioactivity in RNA only. Culturing these two types of labelled donor cells with unlabelled BHK cells (termed recipients) allowed Pitts and Simms (1977) to demonstrate that only uridine nucleotides are transferred between BHK cells in contact with one another; that is there was only extensive transfer of labelled material to the recipients when unchased donor cells were used. It should be possible to extend these methods for cells labelled with $[^3\text{H}\text{-methyl}]$-choline. Peterson and Rubin (1970) have previously shown that this is possible for secondary chick embryo fibroblasts. In fact it was because they saw extensive transfer from chased donor cells labelled with $[^3\text{H}\text{-methyl}]$-choline of labelled material to recipients that they proposed that phospholipids moved between cells but phosphoryl choline did not.

To show that growing BHK cells in unlabelled medium for 24 h after labelling with $[^3\text{H}\text{-methyl}]$-choline results in the radioactivity being found mainly in phospholipid (acid insoluble fraction) with little or no radioactivity in phosphoryl choline (acid soluble fraction), cultures of BHK cells in dishes were labelled for 3 h with $[^3\text{H}\text{-methyl}]$-choline in complete medium. After labelling the cultures were washed free of the labelling medium and then grown
Sample cultures were removed at the required time points and the amount of radioactivity in the growth medium, acid soluble fraction and acid insoluble fraction measured. The results of this experiment are shown in figure 1.

The amount of radioactivity in the acid soluble fraction falls by 86.9% over the 24 h with the bulk of the fall occurring in the first 4 h. There is an initial rise in the amount of radioactivity in the acid insoluble fraction, presumably due to the acid soluble precursors (phosphoryl choline and CDP-choline) being incorporated into acid insoluble products (phospholipids). However, after this rise the radioactivity in this fraction falls over the remaining 22 h of culture to reach a value of 47.9% of the amount originally present. The overall fall in the amount of radioactivity in the cells is due to loss from the cells of labelled material to the growth medium (results not shown). Because of this quite extensive loss the medium was changed in the cultures after 4 h and 20 h of growth. The mechanism of the loss of labelled cellular material was not investigated. However, there are two possible routes by which labelled material could be lost. The first route is simply the reversal of the pathway of incorporation thus leading to the release of $[^3H]$-methyl-choline. This has been found to occur for BHK cells labelled with $[^3H]$-uridine or 2-deoxy-$[^3H]$-glucose (Pitts and Simms, 1977; section 3.3.2). The second route is by an exchange system of serum phospholipids with cell membrane phospholipids mediated by proteins in the serum added to the growth medium (Peterson and Rubin, 1969).
In summary there is a large fall in the number of tritium counts in the acid soluble fraction when the cells are grown for 24 h after labelling. Therefore it should be possible to discover which molecules derived from $[^3\text{H}-\text{methyl}]$-choline are transferred directly between BHK cells, if any, using the same techniques employed by Pitts and Simms (1977).

3.2.3. The Transfer of Labelled Molecules derived from $[^3\text{H}-\text{methyl}]$-choline

An experiment was now carried out to discover if labelled molecules derived from $[^3\text{H}-\text{methyl}]$-choline could be transferred directly between BHK cells in contact. L cells were also used in part of this experiment in order to test the requirement on the formation of intercellular junctions for any possible transfer; these cells are unable to form such junctions (Gilula et al., 1972).

For this experiment sparse cultures of BHK and L cells (donors) growing on glass coverslips were labelled for 3 h with $[^3\text{H}-\text{methyl}]$-choline in complete Eagle's Minimal Essential Medium (EFC). The labelling medium was removed by washing the cells with fresh EFC then to half of the labelled donor BHK cultures and to all of the labelled donor L cell cultures, freshly trypsinized suspensions of recipient BHK cells were added at a ratio of approximately 5 unlabelled cells to 1 labelled cell. The mixed cultures were grown for 3 h before processing for autoradiography. The other half of labelled donor BHK cell cultures
were grown for 24 h (i.e. chased donors) with medium changes at 4 and 20 h, before co-culturing with recipients for the 3 h period and processing for autoradiography. Autoradiographs were either exposed for short periods (1 to 7 days) to allow quantitation of any transfer by grain counting, or for long periods (3 to 6 weeks) to allow photographic records to be made. Photographs of typical microscope fields of the various parts of the experiment are shown in figure 2 and the results of quantitation of the extent of transfer by grain counting are shown in table 5.

Figure 2a shows that there has been extensive transfer of $[^3\text{H}]$-molecules derived from $[^3\text{H-methyl}]$-choline from donor BHK cells to recipient BHK cells in contact with the donor cell. The recipients not in contact have a slightly higher grain density than background showing that there has been a limited amount of medium mediated transfer of labelled material. In this particular example, it is seen that there has been transfer of $[^3\text{H}]$-molecules down a chain of recipient BHK cells from one heavily labelled donor cell, producing a gradient of incorporation. This situation is directly analogous to what has been found for uridine nucleotide transfer (Pitts and Simms, 1977) and metabolic co-operation (Subak-Sharpe et al, 1969) in BHK cells. A closer view of the extent and geometry of transfer is shown in figure 2b. It is seen that there is a marked fall in the grain density over the cells at the points of contact between the heavily
labelled donor cell and lightly labelled recipient cells, again analogous to uridine nucleotide transfer and metabolic co-operation.

When the donor BHK cells are first grown in unlabelled medium for 24 h before culturing with freshly trypsinized BHK cells for 3 h, there is a large reduction in the amount of labelled material transferred to the recipient cells in contact with the donors. Indeed, it is difficult to distinguish if the recipients in contact with the donors have more autoradiographic grains over them than the background level. This large reduction is the predicted result if the molecular basis of transfer of labelled material derived from $[^3\text{H-}\text{methyl}]\text{-choline}$ is the cell-cell transfer of phosphoryl choline and/or CDP choline and not the transfer of phospholipids.

To quantify this fall in the amount of transfer from the chased donors to the recipients so that it may be compared to the known changes of the tritium activity in the acid soluble and acid insoluble cellular fractions over the 24 h period after labelling BHK cells with $[^3\text{H-}\text{methyl}]\text{-choline}$, autoradiographic grains over donors and primary recipient (i.e. those recipients directly in contact with the donor BHK cells) were counted. The results are given in table 5.

Accounting for the background grain density (section 2.2.8.) and the different periods of exposure of the autoradiographs it can be seen that there is a fall of 95% in the amount of transfer
of radioactive material from the donor BHK cells to the recipient BHK cells due to growing the donor cells for 24 h in unlabelled medium before co-culturing with recipient BHK cells. This fall compares favourably with the fall (88.0%) in the amount of radioactivity in the acid soluble fraction found to occur over the same period in the earlier experiment (figure 1).

In this earlier experiment the measurements were carried out on whole cultures of BHK cells and of course any comparison should be made on a cell basis. It is possible to calculate the fall in the amount of radioactivity in the acid soluble fraction per cell over the 24 h by knowing an estimate of the average number of times each cell has divided over this period. Such an estimate can be obtained by dividing the fraction of the average amount of radioactivity in the acid insoluble fraction per cell remaining after the 24 h, into the value of the fall in the amount of radioactivity in this fraction per culture. Thus;

Increase in number of cells in 24 h = \[ \frac{\text{CPM in acid insoluble fraction/culture at 24 h}}{\text{CPM in acid insoluble fraction/culture at 0 h}} \times \frac{\text{Average number of grains over chased donors}}{\text{Average number of grains over unchased donors}} \]

From the data given in figure 1 and table 5 the following values can be fitted -
Therefore the percentage fall in the amount of radioactivity in
the acid soluble fraction per cell over 24 h is -

\[
\frac{66,014}{137,760} \times \frac{361.48}{133.77} = 1.3^* \\
\]

This calculated fall in the activity in the acid soluble pool of
90.8% correlates well with the fall in the amount of transfer of
radioactive material (95%) and therefore strongly suggests that
the only \(^3\text{H}\)-molecules derived from \(^3\text{H}\text{-methyl}\)-choline to be
transferred between contacting BHK cells are the precursors
phosphoryl choline and CDP-choline.

Of course there has been a considerable fall (62%) in the amount
of radioactivity per cell in the acid insoluble fraction over the
24 h. However, the results of the quantitation by grain counting
(table 5) are not consistent with the intercellular movement of
tritium labelled acid insoluble material for the following reason.

* This value for the increase in cell numbers compared to a later
  experiment (figure 8) is a little low but can be explained by the
  low density of the cultures (initially 5 x 10^4 cells/50 mm dish)
  and the repeated changes of the culture medium preventing the cells
  from conditioning the medium.
During processing for autoradiography the cultures are extracted with TCA (section 2.2.6.) and therefore the grains formed over the cells are due only to radioactivity in acid insoluble cellular material. If labelled acid insoluble products derived from $[^3\text{H}-\text{methyl}]$-choline are passed directly between BHK cells then the ratio of the number of grains appearing over primary recipient cells to the number of grains appearing over the donors would be unaffected by the amount of radioactivity initially present in the donors. In fact the ratio falls from 13.7% for the unchased experiment to 0.8% for the chased experiment and as described above, this can be wholly explained by changes occurring in the acid soluble fraction.

However, one possibility that cannot be excluded by these experiments is that $[^3\text{H}]$-phospholipids are able to move between BHK cells in the unchased experiment but in the chased experiment the $[^3\text{H}]$-phospholipids have become sequestered during the 24 h before co-culture in the donor cells into membrane structures which prevent their transfer.

A further piece of circumstantial evidence arguing in favour of phosphoryl choline transfer and against phospholipid transfer comes from analysis of the autoradiographs from the unchased BHK experiment. The distribution of grains over both donor and recipient cells is the same with by far the majority of the grains being found over the cytoplasmic regions and only a few being
found over the nuclear regions of the cells. This is consistent to the recipients having the same pathway of incorporation of radioactivity into the acid insoluble fraction as the donors; that is via phosphoryl \(^3\text{H}\)-choline and CDP-\(^3\text{H}\)-choline. Also, if phospholipids were transferred by a cell-cell contact dependant mechanism, then it might be expected that most of the radioactivity in the recipients would be initially located over the plasma membrane. If the majority of the radioactivity is located on the plasma membrane then a much more uniform distribution of grains over the recipient cells would be given but as is seen a very uneven distribution of grains is produced indicating that most of the radioactivity in the recipients is localized in cytoplasmic structures and not in the plasma membrane. Of course there exists the possibility that any transferred \(^3\text{H}\)-phospholipids are rapidly internalised.

The dependence on the formation of intercellular junctions of the transfer of \(^3\text{H}\)-molecules derived from \(^3\text{H}\)-methyl-choline can be seen when L cells are used as donors instead of BHK cells (figure 2). There is little or no transfer above background from L cells to recipient BHK cells in contact with them. Quantitative analysis by grain counting (table 5) confirms this with the primary recipient BHK cells having a grain count no higher than the background level. As mentioned above the L cells do not form intercellular junctions.

Finally, unpublished results of Peterson (Furshpan, personal
communication) suggest the transfer of labelled material derived from \[^{3}H\]-choline observed in his earlier experiments with Rubin (Peterson and Rubin, 1970) may have occurred during fixation. That the results presented above do not suffer from similar fixation artefacts can be seen by the following two reasons.

1) Transfer of labelled materials to recipient BHK cells occurs only with BHK cells as donors and not with L cells. Any fixation artefacts would be expected to be universal and cause approximately equal amounts of transfer from each cell type.

2) Both unchased donor and recipient BHK cells have a very similar distribution of radioactivity and it might be thought that this would be different with most of the radioactivity located in the plasma membranes of the recipients if transfer occurred during fixation.

In summary, when BHK cells come into contact with one another they are able to transfer molecules derived from choline between themselves. These molecules are most likely to be phosphoryl choline and CDP-choline but not phosphatidyl choline. The lack of the ability of L cells to transfer such molecules to BHK cells in contact with them suggests that the route of transfer of these molecules is through permeable intercellular junctions. Further implications of these results are discussed in Chapter 5.
Growing BHK and L cells (section 2.2.3.) were seeded on to separate 50 mm culture dishes (5 x 10^5 cells/dish in 3 ml EFC_{10}) and grown at 37°C for 16 h. The medium was discarded and the cultures washed 3x with cdEFC_{10} pre-warmed to 37°C (3 ml/wash/dish). Fresh cdEFC_{10} containing [^3H-methyl]-choline (10 Ci/mmol; 1 μCi/ml) was added and the cultures grown for a further 3 h. The labelling medium was discarded and the tritium activity in the acid soluble fraction and acid insoluble fraction measured as described in sections 2.2.9. and 2.2.10. Half of the dishes after TCA washing were extracted with 2 ml chloroform/diethyl ether (1:1, v/v) for 2 min at room temperature and the extract measured for tritium activity as described in section 2.2.10. Each estimation is the average of 3 replica cultures which varied by no more than 11% from their respective means. A preliminary experiment of BHK cells cultured on glass coverslips gave similar results.
Table 4 continued/

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DPM/culture dish in TCA soluble fraction</th>
<th>DPM/culture dish in TCA insoluble fraction</th>
<th>DPM/culture dish in chloroform/ether fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK</td>
<td>483,650 (38.7)</td>
<td>765,300 (61.3)</td>
<td>761,400 (61.0)</td>
</tr>
<tr>
<td>L929</td>
<td>836,210 (57.4)</td>
<td>619,510 (42.6)</td>
<td>615,000 (42.4)</td>
</tr>
</tbody>
</table>

* Figures in parantheses are % of total DPM/culture dish.
Growing BHK cells (section 2.2.3.) were seeded on to 50 mm culture dishes (5 x 10^5 cells/dish in 3 ml EFC\textsubscript{10}) and grown at 37°C for 24 h. The medium was discarded and the cultures washed 3x with cdEFC\textsubscript{10} pre-warmed to 37°C (3 ml/wash/dish). Fresh cdEFC\textsubscript{10} containing [\textsuperscript{3}H-methyl]-choline (10 Ci/mmol; 1 μCi/ml) was added and the cultures grown for a further 3 h. The labelled medium was discarded and the cultures washed 3x with EFC\textsubscript{10} pre-warmed to 37°C (3 ml/wash/dish). Fresh medium was added back to the cultures (3 ml EFC\textsubscript{10}/dish) and the cultures again re-incubated at 37°C. Sample cultures were removed as required and the tritium activity in the cellular acid soluble and acid insoluble fraction of each culture measured as described in sections 2.2.9. and 2.2.10. The medium of the cultures used for the 24 h time point was changed at 4 and 20 h after labelling. Each data point is the average of two experiments each containing 3 replica cultures. The replica cultures varied by no more than 8% from their respective means. The error bars show the variation between the two experiments.
Figure 1 continued/

- CPM/50 mm
- Culture dish

- Acid insoluble
- Acid soluble

H of growth after labelling
Figure 2 Autoradiographs of Donor BHK and L Cells Labelled with [3\textsuperscript{H}-methyl]-choline and Co-cultured with Recipient BHK Cells

Unchased donor cultures of BHK and L cells were prepared by growing these cells on glass coverslips in separate 50 mm culture dishes (1 x 10\textsuperscript{5} cells/dish) for 16 h in EFC\textsubscript{10}. The medium was discarded after this growth period and the cultures washed 3x with cdEFC\textsubscript{10} pre-warmed to 37°C (3 ml/wash/dish). 3 ml of fresh cdEFC\textsubscript{10} containing [3\textsuperscript{H}-methyl]-choline (10 Ci/mmol; 5 μCi/ml) was added to each dish and the cultures grown for a further 3 h. After labelling the medium was removed and the cultures washed 3x with EFC\textsubscript{10} pre-warmed to 37°C (3 ml/wash/dish) before adding the unlabelled, recipient BHK cells.

Chased donor BHK cells were prepared in a similar manner except the initial cell density was halved (5 x 10\textsuperscript{4} cells/dish) and after labelling the cultures were grown in unlabelled EFC\textsubscript{10} (3 ml/dish) for 24 h before establishing co-cultures with medium changes at 4 and 20 h. The growth medium was discarded before adding the recipient BHK cells.

The recipient BHK cell cultures were prepared by growing these cells in 50 mm culture dishes (5 x 10\textsuperscript{5} cells/dish for the unchased experiment and 2.5 x 10\textsuperscript{5} cells/dish for the chased experiment) in EFC\textsubscript{10} (3 ml/dish) for 16 h. After this time the cultures were similarly washed and grown in cdEFC\textsubscript{10} (except the medium was unlabelled) before trypsinizing and suspending (section 2.2.2.) each culture in 3 ml of fresh EFC\textsubscript{10}. The recipient cultures to be used in the chased experiment were grown for a further 24 h in EFC\textsubscript{10} (3 ml/dish) before suspending.
Each 3 ml suspension of recipient cells was transferred to separate cultures of donor BHK or L cells and the mixed cultures grown for 3 h before processing for autoradiography (sections 2.2.6. and 2.2.7.). The autoradiographs were exposed for 6 weeks before being developed (section 2.2.7.). The photographs show typical microscopic views from one experiment. A further 4 similar experiments gave identical results.

A/ Mixed cultures of unchased, donor BHK cells and recipient BHK cells-the heavily labelled cell just left of the centre of the field is the donor cell. The magnification is approximately 650 times.

B/ As in A/ except magnification is approximately 2,000 times-note the sharp fall in the grain density at the boundary between the heavily labelled donor BHK cells (left) and the lightly labelled recipient BHK cell (centre).

C/ Mixed culture of chased, donor BHK cells and recipient BHK cells-the doubly arrowed cell is a single, isolated recipient BHK cell. The magnification is approximately 1,700 times.

D/ As in C/ except the magnification is approximately 2,000 times.

E/ Mixed culture of unchased, donor L cells and recipient BHK cells-
the heavily labelled cells are the donor L cells. The magnification is approximately 650 times.

F/ As in E/-the magnification is approximately 650 times.
Figure 2 continued/
Table 5 **Counts of Autoradiographic Grains Over Unchased and Chased Donor BHK and L Cells and Primary Recipient BHK Cells**

Experimental details of growth of donor BHK and L cells and recipient BHK cells, labelling of donor BHK and L cells with $[^3\text{H}]$-methyl-choline and preparation of the mixed donor/recipient cultures are given in the previous figure legend (figure 2). The mixed coverslip cultures were processed for autoradiography as described in sections 2.2.6, 2.2.7, and 2.2.8, and developed after a short exposure. Counting of autoradiographic grains over donor and primary recipient cells (i.e. recipient cells directly in contact with donor cells) and estimation of the background cell density were carried out as described in section 2.2.8. Autoradiographic grains were counted over 300 cells of each type (except for the L cell experiment where grains over 100 cells of each type were counted) on 6 or more coverslips for each part of the same experiment.

<table>
<thead>
<tr>
<th>Experimental details</th>
<th>Counting of autoradiographic grains</th>
<th>Culture preparation</th>
<th>Autoradiography</th>
<th>Background cell density estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth of donor BHK and L cells</td>
<td>Over donor and primary recipient cells</td>
<td>Processed for autoradiography</td>
<td>Developed after a short exposure</td>
<td>Carried out as described in sections 2.2.8</td>
</tr>
<tr>
<td>Cell type</td>
<td>Mean number of grains/cell (mean) ± S.E</td>
<td>Number of days exposure (exp)</td>
<td>Background grain density (bkg)</td>
<td>Adjusted mean for 7 days of exposure, i.e. (mean - bkg) x 7/days of exp</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>BHK primary recipient cells co-cultured with unchased BHK donors</td>
<td>19.17 ±5.42</td>
<td>2</td>
<td>5.06</td>
<td>49.39</td>
</tr>
<tr>
<td>Unchased BHK donors</td>
<td>56.55 ±10.62</td>
<td>1</td>
<td>4.91</td>
<td>361.48</td>
</tr>
<tr>
<td>BHK primary recipient cells co-cultured with chased BHK donors</td>
<td>7.61 ±1.54</td>
<td>7</td>
<td>5.21</td>
<td>2.40</td>
</tr>
<tr>
<td>Chased BHK donors</td>
<td>43.35 ±4.38</td>
<td>2</td>
<td>5.13</td>
<td>133.77</td>
</tr>
<tr>
<td>BHK primary recipient cells co-cultured with unchased L donors</td>
<td>4.70 ±1.70</td>
<td>2</td>
<td>4.83</td>
<td>0</td>
</tr>
<tr>
<td>Unchased L donors</td>
<td>60.67 ±12.83</td>
<td>1</td>
<td>2.61</td>
<td>406.42</td>
</tr>
</tbody>
</table>
3.3. Study on the Transfer of 2-Deoxy-Glucose-6-Phosphate Between Tissue Culture Cells: Preliminary Study on the Transfer of Labelled Metabolites Derived from $[^{3}H]$-glucosamine and $[^{3}H]$-fucose Between BHK Cells

3.3.1. Introduction

Intercellular junctions between arthropod cells are believed to be permeable to sucrose (Bennett and Dunham, 1970), a variety of fluorescently labelled oligosaccharides (Loewenstein, 1975) and low molecular weight derivatives of fucose and glucosamine (Hermann et al, 1975; Rieske et al, 1975). Also a report by Simms (1973) suggested that sugar phosphates are transferred between vertebrate cells. He found that after labelling BHK cells with $[^{3}H]$-glucose they could transfer labelled material to other previously unlabelled BHK cells providing they were in contact with each other. Because the tritium atom on $[^{3}H]$-glucose can be incorporated into many classes of metabolites as well as sugar phosphates, it is unclear if the labelled material appearing in the recipients is due to the transfer of sugar phosphates or the transfer of some other $[^{3}H]$-metabolites or possibly both (Simms, 1973). The recently reported results of experiments carried out on mammalian tissue culture cells by Kohen and Kohen (1977) can also be explained by the transfer of glucose-6-phosphate between the cells. However, their results have alternative explanations (section 1.6.6.). Therefore, whether intercellular junctions joining
vertebrate cells are permeable to sugar phosphates is still an open question.

The results of Simms (1973) show the unsuitability of using glucose as a marker of sugar phosphate transfer due to it being rapidly converted into other cellular molecules. In contrast, the glucose analogue, 2-deoxy-glucose, is only slowly metabolised after its phosphorylation in the C6 position (Kipnis and Cori, 1969). 2-deoxy-glucose is rapidly taken up by cells in culture through the glucose transport system (Kipnis and Cori, 1969) and therefore it should be possible to establish an intracellular pool of 2-deoxy-glucose-6-phosphate labelled with tritium. Possible transfer of this pool through intercellular junctions could be followed by the pre-labelling technique developed by Pitts and Simms (1977). Before beginning this investigation, a number of experiments were carried out to find the best conditions for labelling cells in culture with 2-deoxy-$[^3H]$-glucose and also to examine the stability of the resulting pool of 2-deoxy-$[^3H]$-glucose-6-phosphate.

3.3.2. Preliminary Experiments

Normal tissue culture medium (EFC$^{10}$) contains a high concentration of glucose (25 mM; Flow Catalogue, 1976) and as 2-deoxy-glucose competes for entry into the cell with glucose (Kipnis and Cori,
1969), this medium might only allow poor incorporation of 2-deoxy-[\(^3\text{H}\)]-glucose into cells at concentrations of the \([\(^3\text{H}\)]\)-analogue that are not toxic. Therefore an experiment was carried out to find a suitable modification to the medium that would allow a sufficient incorporation of 2-deoxy-[\(^3\text{H}\)]-glucose at non-toxic concentrations of the analogue. In this experiment the incorporation of tritium into cultures of growing BHK cells was measured after labelling for one h with 2-deoxy-[\(^3\text{H}\)]-glucose in the presence of different concentrations of glucose or pyruvate in the growth medium. Pyruvate is often used as an alternative carbon and energy source to glucose and might not be expected to inhibit 2-deoxy-[\(^3\text{H}\)]-glucose incorporation. The results are shown in figure 3.

Even low concentrations of glucose greatly inhibit the incorporation of 2-deoxy-[\(^3\text{H}\)]-glucose into BHK cells and thus modifying the medium by lowering the glucose concentration is insufficient. Although there is an appreciable inhibition of 2-deoxy-[\(^3\text{H}\)]-glucose incorporation at higher concentrations of pyruvate it is considerably less than in the presence of glucose and there is a sufficient incorporation for experimental purposes at normal medium concentrations of pyruvate. Thus, the medium chosen for labelling cells with 2-deoxy-[\(^3\text{H}\)]-glucose is EFC\(_{10}\) minus glucose but with added pyruvate at a final concentration of 25 mM (pyrEFC\(_{10}\)).

The cause of the inhibition of 2-deoxy-[\(^3\text{H}\)]-glucose incorporation by pyruvate is not understood and the problem was not pursued. However,
possible reasons for this inhibition are, (i) pyruvate is a specific inhibitor of the glucose transport system, (ii) because of gluconeogenesis from pyruvate, there is release from the BHK cells into the medium of glucose which then competes for re-entry with the 2-deoxy-[3H]-glucose.

An analysis of the distribution of tritium between the acid soluble and acid insoluble fractions of cultures of BHK cells after a 1 h labelling with 2-deoxy-[3H]-glucose in pyrEFC10 shows that practically all the tritium is found in the acid soluble fraction (98%; figure 4) immediately after removal of the labelling medium. This is the expected result if 2-deoxy-glucose is phosphorylated but only poorly incorporated into glycoproteins (Renner et al, 1972). Chromatographic analysis of the acid soluble material shows that 91% of the tritium counts are in a material which has a similar R_f value to that of the standard 2-deoxy-[3H]-glucose-6-phosphate and the remainder of the tritium counts is in a material which has a similar R_f value to that of 2-deoxy-[3H]-glucose (figure 5). These results are consistent with 2-deoxy-glucose being phosphorylated and forming a pool inside the cells which is only slowly metabolised.

Although BHK cells may only utilize 2-deoxy-[3H]-glucose-6-phosphate at a very slow rate, it is quite possible that the pool of the phosphorylated analogue could be lost from the cells to the medium by the simple reversal of the pathway of incorporation. Such a situation occurs for uridine nucleotides in BHK cells where the cells
slowly lose uridine to the medium (Ballantyne and Pitts, unpublished results). An experiment was therefore carried out to discover the stability of the 2-deoxy-[\(^3\)H]-glucose-6-phosphate pool in cultures of BHK cells growing in either pyrEFC\(_{10}\) or normal EFC\(_{10}\) (i.e. containing 25 mM glucose). Growing cultures of BHK cells were labelled for 1 h with 2-deoxy-[\(^3\)H]-glucose in pyrEFC\(_{10}\) and after this period the labelled medium was removed with several washes of BSS. Fresh medium, either pyrEFC\(_{10}\) or EFC\(_{10}\), was added to the cultures and the cells were grown for a further 5 h at 37\(^\circ\)C. Sample cultures were removed at hourly intervals and the distribution of tritium counts between the growth medium, cellular acid soluble fraction and cellular acid insoluble fraction measured for each culture. The results are shown in figure 4.

In the presence of either pyrEFC\(_{10}\) or EFC\(_{10}\) the tritium counts in the cellular acid soluble fraction falls over the 5 h period. This fall seems to be solely due to loss of the tritium counts to the growth medium as there is an equal increase of tritium counts in the medium while there is no increase in the tritium counts into the acid insoluble fraction.

Tritium counts appearing in the growth medium could be due to either the loss of 2-deoxy-[\(^3\)H]-glucose-6-phosphate from the cells or loss of 2-deoxy-[\(^3\)H]-glucose formed from the phosphate derivative by phosphatase action. Chromatographic analysis of the medium showed that there is little 2-deoxy-[\(^3\)H]-glucose-6-phosphate in the
medium and practically all the tritium appears to be located in 2-deoxy-glucose. Although this is consistent with the loss of 2-deoxy-[^3]H]-glucose, it does not exclude the possibility that 2-deoxy-[^3]H]-glucose-6-phosphate is lost from the cells, because phosphatases present in the serum added to the growth medium may hydrolyse the phosphate group of any 2-deoxy-[^3]H]-glucose-6-phosphate lost from the cells. However, the phosphate group is not hydrolysed from the standard 2-deoxy-[^3]H]-glucose-6-phosphate during a 5 h incubation in the growth medium at 37°C, therefore making this possibility unlikely (figure 5). Thus, the major pathway of loss of the pool of 2-deoxy-[^3]H]-glucose-6-phosphate from BHK cells is first the removal of the phosphate group presumably by phosphatase action and second, the loss of the resulting 2-deoxy-[^3]H]-glucose from the cells.

In the presence of EFCl0 the rate of fall of radioactivity in the cellular acid soluble fraction is exponential as shown by the straight line given when the logarithm of the tritium counts (expressed as the % CPM of the 0 h CPM) is plotted against the time of culture after removal of the labelling medium (figure 4). The rate of fall has a half-life of 1.35 h. In contrast the fall of the radioactivity in the acid soluble fraction of BHK cells in the presence of pyrEFCl0 show an exponential fall only over the first h, after which the rate of the fall is less and after 3 h the activity in this fraction remains constant. These different results using the two types of medium
can be explained if the $2$-deoxy-$[^3H]^{-}\text{glucose}$ lost from the cells to the medium can re-enter the BHK cells in the presence of pyrEFC$_{10}$, but not in the presence of the high concentration of glucose in the EFC$_{10}$. Thus, for the first 3 h in pyrEFC$_{10}$ tritium counts are lost from the BHK cells to the medium until there is enough $2$-deoxy-$[^3H]^{-}\text{glucose}$ in the medium to establish an equilibrium where the loss and uptake of $2$-deoxy-$[^3H]^{-}\text{glucose}$ are balanced.

The method initially proposed to examine the possible transfer of $2$-deoxy-glucose-$6$-phosphate through junctions between cells in culture was based on the pre-labelling technique using acid soluble autoradiography. That is, donor cells would be pre-labelled with $2$-deoxy-$[^3H]^{-}\text{glucose}$ and after removing the labelling medium, fresh recipient BHK cells would be added and the mixed culture grown for several h. The possible transfer of the $2$-deoxy-$[^3H]^{-}\text{glucose}-6$-phosphate from the donors to the recipients could then be detected by acid soluble autoradiography (see below). Unfortunately, the rapid loss from the BHK cells of the $2$-deoxy-$[^3H]^{-}\text{glucose}-6$-phosphate pool interferes with the proposed approach because in the normal co-culture period of 3 h, over 75% of the tritium counts will have been lost from the cells if they are grown in EFC$_{10}$. Further, the re-absorption of $2$-deoxy-$[^3H]^{-}\text{glucose}$ in pyrEFC$_{10}$ means that if this medium is used in the co-culture there will be a high level of medium transfer of $2$-deoxy-$[^3H]^{-}\text{glucose}$ between the donors and the recipients which would obscure any junctional transfer.
From the results of the experiments shown in figure 4 and the chromatographic analysis (figure 5), the half-life of the radioactivity in the acid soluble fraction of BHK cells growing in EFC\textsubscript{10} appears to be dependant on the equilibrium between 2-deoxy-glucose-6-phosphate and 2-deoxy-\textsuperscript{3}\textsubscript{H}-glucose (i.e. the relative activities of hexokinase and phosphatase), and the permeability of the plasma membrane to 2-deoxy-\textsuperscript{3}\textsubscript{H}-glucose. As these two parameters would be expected to be characteristic properties of a particular cell type, then it is possible that there is some variation of the half-life between different cell types. Preliminary studies showed that this is indeed the case.

The half-life of the radioactivity in the acid soluble fraction of CHO cells (the K1 subline was used in this study which were auxotrophic for proline; Kao and Puck 1968) was found to be about 2 h whereas the radioactivity in L cells has a similar half-life to that in BHK cells (about 1.30 h). The complete experiments measuring the half-life of the pool in these two cell lines are given in the following section.

K1 cells do form permeable intercellular junctions with BHK cells (section 4.1.) and therefore it should be possible to use these differences in the half-life of the pool of tritium counts in the acid soluble fraction between K1, BHK and L cells in a novel approach to examine possible transfer of 2-deoxy-\textsuperscript{3}\textsubscript{H}-glucose-6-phosphate through these junctions. This is described below in the following section.
3.3.3. The Transfer of 2-deoxy-\textsuperscript{3}H\textsuperscript{-}glucose-6-phosphate Between Cultured Cells

If a culture of Kl cells is labelled with 2-deoxy\textsuperscript{3}H\textsuperscript{-}glucose and then a suspension of unlabelled BHK cells is added to establish a 1:1 co-culture of Kl to BHK cells, it follows that if the pool of the 2-deoxy\textsuperscript{3}H\textsuperscript{-}glucose-6-phosphate remains solely inside the Kl cells, the predicted half-life of the pool will be the same as the half-life of the pool in Kl cells cultured alone. However, if the 2-deoxy\textsuperscript{3}H\textsuperscript{-}glucose-6-phosphate pool spreads to the BHK cells by junctional transfer, then because the pool in BHK cells has a shorter half-life, the BHK cells will deplete the Kl cells of 2-deoxy\textsuperscript{3}H\textsuperscript{-}glucose-6-phosphate and the resultant half-life will fall from the characteristic value for Kl cells nearer to that for BHK cells cultured alone. If there is transfer then the involvement of intercellular junctions can be tested using L cells. Thus in mixed cultures of pre-labelled Kl cells and L cells, the pool will remain inside the Kl cells if intercellular junctions are required for transfer. Hence the resultant half-life for the pool in the mixed Kl/L cell culture will be similar to the half-life for the pool in Kl cells cultured separately.

To test this hypothesis an experiment was carried out where growing cultures of Kl, BHK and L cells were seeded in separate dishes at high density (5 x 10\textsuperscript{5} cells/dish) and grown for 16 h
in EFC\textsubscript{10}. Cultures were then labelled with 2-deoxy-\([\textsuperscript{3}H]\)-glucose in pyrEFC\textsubscript{10} for 1 h and after labelling, the medium was removed and the cells washed with EFC\textsubscript{10}. Unlabelled cultures of KI, BHK and L cells were suspended in EFC\textsubscript{10} after trypsinization and these cell suspensions added to the labelled cultures of KI cells; a suspension from one dish being added to one dish of labelled KI cells to give an approximate ratio of labelled to unlabelled cells of 1:1. The mixed cultures and the pure cultures of labelled KI, BHK and L cells were grown in EFC\textsubscript{10} and the tritium counts in the cellular acid soluble fraction measured at hourly intervals over 5 h to allow the calculation of the half-life values for the three cell types and the co-cultures. The results are shown in figure 6. The values of the half-life in the pure cultures are 2.0 h for KI cells and 1.3 h for both BHK and L cells. In the co-culture of pre-labelled KI cells and unlabelled KI cells, the half-life is the same as the value for KI cells in pure culture (2.0 h) showing that the half-life value is not altered by adding freshly trypsinized cells. However, the resultant value of the KI/BHK co-culture is reduced from 2.0 h (the value for KI cells alone) to 1.3 h which is indistinguishable from the value for BHK cells. In contrast, the half-life value of the KI/L cell co-culture is 2.0 h. These results are consistent with the transfer of 2-deoxy-glucose-6-phosphate through permeable intercellular junctions.

Alternative explanations of these results are that BHK cells either
transfer to Kl cells a phosphatase(s) or an activator(s) of phosphatase or somehow alter the permeability properties of the plasma membrane of the Kl cells allowing them to lose 2-deoxy-[^3H]-glucose-6-phosphate. In view of the results obtained in earlier studies (see section 1.6.4.), transfer of phosphatase(s) seems unlikely as an explanation. Possible transfer of an activator of phosphatase cannot be ruled out although this might appear unlikely if 2-deoxy-[^3H]-glucose-6-phosphate is not transferred because the transfer must be through intercellular junctions (as shown by the results of the Kl/L cell co-culture experiment) and so it would be reasonable to presume that intercellular junctions are also permeable to 2-deoxy-[^3H]-glucose-6-phosphate.

The explanation that the BHK cells alter the permeability properties of the Kl cells might be expected to be a general disturbance caused by adding cells to the Kl cultures. However, adding Kl or L cells does not appear to change the permeability properties of the established Kl cells which makes the explanation unlikely. Therefore, the results can be best explained by the transfer of 2-deoxy-glucose-6-phosphate through permeable intercellular junctions.

There is no detectable delay in the fall of the value of the half-life of the pool from 2.0 h to 1.3 h in the Kl/BHK co-culture; i.e. the value after 1 h of the Kl/BHK co-culture is the same as during the remaining 4 h and is not some intermediate value. This suggests that the pool of 2-deoxy-
$[^3H]$-glucose-6-phosphate (originally present only in the Kl cells) is quickly shared between the Kl and BHK cells. Before transfer can take place the BHK cells must first settle on to the monolayers of Kl cells, form contacts and subsequently form intercellular junctions. The time course of BHK cells attaching to the substrate has not been measured but from a qualitative approach most of the cells have attached well within 5 min. As the Kl cultures were near confluent and also the cultures of BHK cells near confluence before being harvested, then the BHK cells will probably make contact and form junctions with Kl cells and other BHK cells when they attach. Pitts and MacKay (unpublished) have detected functional junctions between BHK cells within 5 min after forming contact (this being the earliest possible time point) and so it is probable that within 15 min after establishing the co-culture, sufficient intercellular junctions have been formed to allow the equilibration of the 2-deoxy-$[^3H]$-glucose-6-phosphate pool between the Kl and BHK cells. Such a 15 min delay would not be detectable above experimental variation of the value at the first time point (1 h).

The type of experiment carried out above could readily be used in screening which types of cells are able to form intercellular junctions. Also, it lends itself to investigating the formation of intercellular junctions. The technique could be improved if cell types are found with a larger difference between the half-lives of their 2-deoxy-glucose-6-phosphate pools.
3.3.4. An Attempt to follow Transfer of 2-deoxy-[^3H]-glucose-6-phosphate by Autoradiography

As mentioned earlier (section 3.3.3.) the rapid rate of fall of the tritium counts from cells labelled with 2-deoxy-[^3H]-glucose presents a problem in attempting to investigate the transfer of 2-deoxy-[^3H]-glucose-6-phosphate between cells by an autoradiographic approach. However, a Xenopus cell line (obtained from Dr. K. Jones, Edinburgh University, through Dr. B. E. H. Maden) were found to have a 2-deoxy-[^3H]-glucose-6-phosphate pool with an unusually long half-life (6.4 h; results not shown). These cells are grown at 28°C which may account for the long half-life. Therefore these cells were chosen for an autoradiographic study.

Pitts and Haynes (unpublished results) developed a technique (based on Adams, 1969) for examining thymidine nucleotide pools inside BHK cells after drying [^3H]-thymidine, labelled coverslip cultures at 37°C. The coverslips are then overlayed with a film of photographic emulsion (diluted 1 to 2 with water) on a wire loop. This film is partially dried in air for 30 sec before using when the emulsion is very tacky but holds little moisture. Too much moisture will cause the dried cells to lose their acid soluble pools causing a high general background. The autoradiograph is then exposed and then developed in the usual way (section 2.2.7.).

A number of experiments in which Xenopus cells growing on coverslips
were labelled with 2-deoxy[\(^3\text{H}\)]-glucose (sp. act. 20 mCi/mmol.; 5 μCi/ml) for 1 h in pyrEFC\(_{10}\), washed and then co-cultured with unlabelled Xenopus cells for 3 h in EFC\(_{10}\) before processing for acid soluble autoradiography as described above, showed that there is still a large loss of the tritium counts during this processing technique creating a high background. Although it appeared qualitatively that there was transfer of labelled material from the donors to the recipients, the high background obscured most of the detail and prevented a quantitative analysis. Attempts were made to discover at which point in the processing the tritium counts were lost and it appeared that the drying was a critical step. Preliminary experiments suggest that rapid freezing of the cultures in an ethanol/dry ice bath followed by lyophilisation will overcome these leakage problems.

3.3.5. A Preliminary Study of the Intercellular Transfer of Other Types of Sugars

Glucosamine and fucose have been used extensively for labelling glycoproteins. Studies have shown that these two sugars are used predominantly in the synthesis of glycoproteins and glycolipids and are not metabolised rapidly in other ways (Robinson et al, 1964). Therefore, these two sugars appear to be suitable for examining the transfer of sugar phosphates between cells.
Experiments in which BHK cells or L cells were pre-labelled for 3 h with $[^3\text{H}]-\text{glucosamine}$ or $[^3\text{H}]-\text{fucose}$ and to which fresh, unlabelled BHK cells were added and the mixtures cultured for a further 3 h before processing for autoradiography showed that by 20 days of exposure of the autoradiographs a small number of grains could be seen over both donors and recipients. Preliminary counts of autoradiographic grains for the glucosamine experiments suggested that there was transfer of labelled material from the donor BHK cells, but not from donor L cells, to recipient BHK cells in contact and the amount of transfer could be reduced by growing the donor BHK cells in unlabelled medium for 24 h after labelling and before mixing with the unlabelled BHK cells (see table 6). Thus, this situation is analogous to the transfer of choline phosphates (section 3.2.) and uridine nucleotides (Pitts and Simms, 1977). Analysis of the distribution of tritium radioactivity between acid soluble and acid insoluble fractions of BHK cells after labelling with either $[^3\text{H}]-\text{glucosamine}$ and $[^3\text{H}]-\text{fucose}$ showed that in both cases the counts in the acid soluble fraction fell sharply during the 24 h period after labelling whereas the counts in the acid insoluble fraction only fell by a small amount (table 7). Thus, if the efficiency of incorporation of $[^3\text{H}]-\text{glucosamine}$ and $[^3\text{H}]-\text{fucose}$ can be improved, then these two sugars could be used to further investigate sugar phosphate transfer.

Provisionally, the results for glucosamine suggest that tritium
labelled acid soluble derivatives of \([^3\text{H}]\)-glucosamine (presumably phosphate and nucleotide derivatives of glucosamine, N-acetyl glucosamine, N-acetyl neuramic acid or a mixture of these) are transferred between BHK cells but not acid insoluble derivatives (presumably glycoproteins and glycolipids). The apparent inability of L cells to act as donors of labelled material suggests that transfer is via permeable intercellular junctions.
Figure 3  The Incorporation of Radioactivity Derived from 2-deoxy-[\(^3\)H]-glucose into the Acid Soluble Fraction of Growing BHK Cells in the Presence of Various Concentrations of Exogenous Glucose or Pyruvate

Growing BHK cells (section 2.2.3.) were seeded on to 50 mm culture dishes (5 x 10^5 cells/dish in 3 ml EFC\(_{10}\)) and grown at 37°C for 16 h. The medium was discarded and the cultures washed 3x with BSS pre-warmed to 37°C (3 ml/wash/dish). Fresh medium containing different concentrations of added glucose or pyruvate (concentrations shown in figure) and 2-deoxy-[\(^3\)H]-glucose (20.Ci/mmol; 1 µCi/ml) was added to each dish (3 ml/dish). The cultures were grown for a further 1 h and the amount of radioactivity in the cellular acid soluble fraction then measured (sections 2.2.9. and 2.2.10.). Each data point is the average of 3 replica cultures which varied by no more than 8% from their respective means.
Figure 3 continued/

Concentration (mM) of energy source (top scale pyruvate; bottom scale glucose)
Growing BHK cells (section 2.2.3.) were seeded on to 50 mm culture dishes (5 x 10^5 cells/dish in 3 ml EFC_{10}) and grown at 37°C for 16 h. The medium was discarded and the cultures washed 3x with BSS pre-warmed to 37°C (3 ml/wash/dish). Fresh pyrEFC_{10} containing 2-deoxy-[^3]H-glucose (20 Ci/mmol; 1 μCi/ml) was added to each dish and the cultures grown for a further 1 h. The labelled medium was removed and one half of the cultures washed 3x with EFC_{10} (3 ml/wash/dish) and the other half washed 3x with pyrEFC_{10} (3 ml/wash/dish). The appropriate medium was then added to each of the cultures and they were grown for a further period at 37°C. Sample cultures were removed at hourly intervals (0 h to 5 h) and the radioactivity in the growth medium and in the acid soluble and acid insoluble cell fractions measured (sections 2.2.9. and 2.2.10.). Each data point is the average of two experiments each containing 3 replica cultures. The replica cultures varied by no more than 9% from their respective means and the two experiments varied by no more than 10% from their respective means. The lower graph is plotted as the logarithm_{10} of the percentage acid soluble tritium activity (DPM) of the 0 h time point.
Figure 4 continued/

- BHK cells in EFC$_{10}$
- DPM/50 mm dish $\times 10^{-4}$
- Acid Soluble
- Acid Insoluble
- BHK cells in pyrEFC$_{10}$
- Medium
- Acid soluble
- Acid Insoluble

h of culture after labelling

- Log$_{10}$ % DPM/
- 50 mm dish of
- 0 h
- pyrEFC$_{10}$
- EFC$_{10}$

h of culture after labelling
The standard 2-deoxy-[^3]H]-glucose-6-phosphate was prepared by incubating 2-deoxy-[^3]H]-glucose (20 Ci/mmol; 1 µCi/ml) in 4 mM MgCl$_2$, 20 mM Tris/HCl (pH 8.0) and hexokinase (1 µl/ml incubation mix of a solution of hexokinase at a concentration of 2.5 mg/ml; Sigma, London). The protein from this standard and the culture medium samples was removed by adding one volume of 10% TCA (w/v) to one volume of the standard or medium sample and then centrifuging for 5 min at full speed in an MSE bench centrifuge. The supernatants were collected using a Pasteur pipette, and the TCA from these samples and the TCA extracts of the 2-deoxy-[^3]H]-glucose labelled cultures (growth and labelling of these cultures was carried out as described in figures 4 and 5) was removed by the following procedure. To each sample water saturated diethyl ether was added (1 volume of sample to 8 volumes of ether) in a separatory funnel and shaken. The aqueous phase was removed and the ether extraction repeated a further 2x. The ether remaining in the aqueous phase after this operation was removed by placing the samples in a hot water bath for 30 min. The samples were shell frozen in an ethanol/solid CO$_2$ bath and lyophilised. The dried samples and standards were each dissolved in 0.2 ml and 1 ml of distilled water respectively and stored frozen until used.
The samples and standards (50 µl) were spotted on to Whatman No. 1 filter paper and the chromatographs run using a descending solvent system of ethyl acetate/pyridine/distilled water (10:4:3; v/v/v). After the solvent front had run approximately 150 mm from the spotting line, the chromatographs were removed from the tank containing the solvent and dried overnight in a fume cupboard. The dried chromatographs were cut into 1 cm² pieces and counted for tritium activity using a toluene based scintillant (section 2.2.10.).

The top graph shows the distribution of tritium activity from the chromatograph of the standards 2-deoxy[3H]-glucose (50 µl spotted from a solution of 1 µCi/ml; 20 Ci/mmol) and 2-deoxy[3H]-glucose-6-phosphate. The bottom graph shows the distribution of tritium counts of chromatographs of (i) combined medium samples (EFC₁₀) from 3 cultures of BHK cells (50 mm dishes) 5 h after removal of the labelling medium, (see figures 4 and 5), (ii) combined TCA extracts (section 2.2.9.) of 3 cultures of BHK cells (50 mm dishes) immediately after removal of the labelling medium (i.e. 0 h; see figures 4 and 5) and (iii) standard 2-deoxy[3H]-glucose-6-phosphate (0.2 µCi/ml) after 5 h incubation at 37°C in growth medium (EFC₁₀).
Figure 5 continued/

a/ Standards

2-deoxy-[\(^3\)H]-glucose-6-phosphate

2-deoxy-[\(^3\)H]-glucose

b/ Samples

2-deoxy-[\(^3\)H]-glucose-6-phosphate after 5 h incubation in EFC\(_{10}\)

TCA extracts

Labelled medium

Distance (cm) from spotting line →
Figure 6  **Half-life of the 2-deoxy-[^3]H]-glucose-6-phosphate Pool in Pure and Mixed Cultures**

Growing Kl, BHK and L cells (section 2.2.3.) were seeded on to separate 50-mm culture dishes (5 x 10^5 cells/dish in 3 ml EFC<sub>10</sub>) and grown at 37°C for 16 h. The medium from all the cultures was discarded and the cultures washed 3x with BSS (3 ml/wash/dish) pre-warmed to 37°C. pyrEFC<sub>10</sub> containing 2-deoxy[^3]H]-glucose (20 Ci/mmol; 1 μCi/ml; 3 ml medium/dish) was added to half of the BHK and L cell cultures (total of 36 cultures of each cell type per experiment) and to 4/5 of the Kl cultures (total of 90 cultures per experiment). (To ease the number of experimental manipulations carried out at one time the labelling of the cultures used in the pure culture experiment was carried out 30 min after the labelling of the other cultures used in the mixed culture experiment). To the remaining cultures (i.e. half of the BHK and L cell cultures and 1/5 of the Kl cultures) pyrEFC<sub>10</sub> was added (3 ml/dish). All the cultures were grown for a further 1 h after which the medium was removed and the cultures washed 3x with EFC<sub>10</sub> (3 ml/wash/dish). The unlabelled cultures were each suspended in 3 ml EFC<sub>10</sub> after trypsinization (section 2.2.2.) and then transferred to the labelled cultures of Kl cells; one 3 ml cell suspension being added to one labelled Kl culture. EFC<sub>10</sub> was added to the remaining labelled cultures of Kl, BHK and L cells (3 ml/dish) and all the cultures were re-incubated at 37°C. Sample cultures were removed at hourly intervals and the amount of tritium activity in the acid soluble cell fraction per
cultural dish was measured (section 2.2.9. and 2.2.10.).

The results are plotted as a percentage of the tritium counts originally present per culture at 0 h (i.e. average CPM/culture at 0 h). The error bars show the maximum variation between three separate experiments. Each experiment is the average of three replica cultures for each time point which varied by no more than 10% from their respective means. The insets show the log\textsubscript{10} plot of the percent tritium counts per culture described for the main figure.
Figure 6 continued/

a/ Separate culture

% CPM/dish of 0 h

Log_{10} %CPM/dish of 0 h

b/ Co-culture

% CPM/dish of 0 h

Log_{10} %CPM/dish of 0 h

h of culture after labelling

h of culture after labelling
Experimental details of the growth of donor BHK and L cells and recipient BHK cells, labelling of donor cultures and preparation and growth of co-cultures of donor and recipient cells are identical as those given in the legend to figure 2 (section 3.2.) except that EFC\textsubscript{10} was used throughout the experiment, the donor cultures were not washed before labelling and \( \text{[}^{3}\text{H}\text{]}\text{-glucosamine (10 Ci/mmol; 5 \( \mu \text{Ci/ml} \)) was used to label the donor cultures. The co-cultures were processed for autoradiography as described in sections 2.2.6. and 2.2.7. Counting of autoradiographic grains over donor and primary recipient cells (i.e. recipient cells directly in contact with donor cells) and estimation of the background cell density were carried out as described in section 2.2.8. (except for the L cell experiment where the grains over 50 cells of each type were counted). Autoradiographic grains were counted over 100 cells of each type on 4 or more coverslips for each part of the same experiment. Transfer of labelled materials from unchased donor BHK cells, but not from chased donor BHK cells and unchased donor L cells, was seen in another two experiments but was not quantitated.
Table 6 continued

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mean number of grains/cell (mean) ± S.E.</th>
<th>Number of days exposure (exp)</th>
<th>Background grain density (bkg)</th>
<th>Adjusted mean for 20 days of exposure, i.e. (mean - bkg) x 20/days of exp</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK primary recipient cells co-cultured with unchased BHK donors</td>
<td>9.9 ± 3.5</td>
<td>20</td>
<td>0.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Unchased BHK donors</td>
<td>41.7 ± 7.1</td>
<td>10</td>
<td>1.1</td>
<td>81.2</td>
</tr>
<tr>
<td>BHK primary recipient cells co-cultured with chased BHK donors</td>
<td>1.2 ± 0.9</td>
<td>20</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Chased BHK donors</td>
<td>28.7 ± 8.2</td>
<td>20</td>
<td>0.9</td>
<td>27.8</td>
</tr>
<tr>
<td>BHK primary recipient cells co-cultured with unchased L cell donors</td>
<td>1.1 ± 0.4</td>
<td>20</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Unchased L cell donors</td>
<td>52.7 ± 5.5</td>
<td>10</td>
<td>0.5</td>
<td>104.4</td>
</tr>
</tbody>
</table>
Table 7 Changes in the Activity in the Acid Soluble and Acid Insoluble Fractions of BHK Cells Over a 24 h Growth Period After Labelling with $[^3\text{H}]-\text{glucosamine or }[^3\text{H}]-\text{fucose}$

Growing BHK cells (section 2.2.3.) were seeded on to 50 mm culture dishes ($5 \times 10^5$ cells/dish and 3 ml EFC$_{10}$) and grown at 37°C for 16 h. The medium was discarded and fresh EFC$_{10}$ containing either $[^3\text{H}]-\text{glucosamine (10 Ci/mmol; 1 } \mu\text{Ci/ml)}$ or $[^3\text{H}]-\text{fucose (12 Ci/mmol; 1 } \mu\text{Ci/ml})$ added and the cultures grown for a further 3 h. The labelled medium was discarded and the cultures washed 3x with EFC$_{10}$ pre-warmed to 37°C (3 ml/wash/dish). Fresh medium was added back to the cultures (3 ml EFC$_{10}$) and the cultures re-incubated at 37°C. Sample cultures were removed as required and the tritium activity in the cellular acid soluble and the acid insoluble fraction of each culture measured as described in sections 2.2.9. and 2.2.10. The medium of the cultures used for the 2 h time point was changed at 4 and 20 h after labelling. Each data point is the average of 3 replica cultures from a single experiment. The replica cultures varied by no more than 5% from their respective means.
Table 7 continued/

<table>
<thead>
<tr>
<th>Time (h) of growth after labelling</th>
<th>Acid Soluble</th>
<th>Acid Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22,407</td>
<td>11,362</td>
</tr>
<tr>
<td>3</td>
<td>5,364</td>
<td>1,327</td>
</tr>
<tr>
<td>24</td>
<td>1,649</td>
<td>1,870</td>
</tr>
</tbody>
</table>
3.4. Study on the Transfer of Cellular Folates Between BHK Cells

3.4.1. Introduction

Cells in culture can be grown for prolonged periods in the absence of the vitamin folic acid, or in the presence of the folic acid analogue aminopterin, provided the growth medium is supplemented with glycine, thymidine and a purine (McBurney and Whitmore, 1974a and Littlefield, 1966a). Thus, loss of the metabolic function of the folic acid derived cofactor tetrahydrofolate (FH$_4$) can be overcome in cultured cells by appropriately supplementing the growth medium. As formate is dependant on this cofactor for its incorporation into cellular material (Bertino et al, 1962), cells depleted of folates* by growth in a folic acid deficient medium (or cells growing in the presence of aminopterin) will have a greatly reduced ability to incorporate formate. It should therefore be possible to detect intercellular transfer of cellular folates by culturing mixtures of folate depleted cells and undepleted cells in a folate deficient medium and measuring the level of formate incorporation in the depleted cells after co-culture. An increase in formate incorporation would indicate the transfer of cellular folates between the two types of cells during co-culture.

*Folates will be used to refer to folates and their derivatives, ex. tetrahydrofolate and dihydrofolate.
3.4.2. Preliminary Studies

The time required for the depletion of cellular folates by folate starvation and the suitability of $[^3\text{H}]$-formate incorporation as an indicator of intracellular levels of folates were first studied. BHK cells were grown in a medium having no added folate but which contained glycine, hypoxanthine and thymidine (fdEFC\textsubscript{10}; containing dialysed serum, see materials section 2.1.4.) and the levels of $[^3\text{H}]$-formate incorporation into acid insoluble material (expressed as CPM/mg protein) of these cells compared to that into BHK cells grown in normal medium (ghtEFC\textsubscript{10}; supplemented with glycine, hypoxanthine, thymidine and dialysed serum; section 2.1.4.) at regular intervals over a ten day period. The serum used in fdEFC\textsubscript{10} and ghtEFC\textsubscript{10} was dialysed against water to reduce its folate concentration to a minimum (section 2.1.4.). Also, in order to allow the cells to adapt to differences between their normal growth medium (EFC\textsubscript{10}) and the fdEFC\textsubscript{10}, the cells were grown for 2 days in ghtEFC\textsubscript{10} before the start of the experiment. The same medium (ghtEFC\textsubscript{10}) was used during the course of the experiment for the control, unstarved BHK cells.

BHK cells growing in the absence of added folate progressively lose their ability to incorporate $[^3\text{H}]$-formate into acid insoluble cellular material (figure 7). After 10 days in fdEFC\textsubscript{10} the incorporation is only 6.7% of that into control BHK cells growing in ghtEFC\textsubscript{10}. Thus the incorporation of $[^3\text{H}]$-formate appears to be
dependant on added folate. McBurney and Whitmore (1974a) found a similar time course for the depletion of cellular folates in CHO cells growing in a folate depleted medium. The residual incorporation after 10\(^6\) days growth in fdEFC\(^{10}\) is not reduced by further growth in fdEFC\(^{10}\) (6.8% after 20 days). This residual incorporation could be due to a low level of folate remaining in the serum after dialysis, a tetrahydrofolate independant incorporation of \([^3\text{H}]\)-formate, the incorporation of a \([^3\text{H}]\)-contaminant in the \([^3\text{H}]\)-formate preparation or a combination of these factors. However, this residual incorporation whatever its cause is so small that it does not interfere at all with the proposed experimental procedure.

The rate of fall of \([^3\text{H}]\)-formate incorporation into cells growing in fdEFC\(^{10}\) suggests that cellular folates are not lost by degradation or leakage from BHK cells. The fall is slow and is approximately exponential (after subtraction of the residual incorporation) which would be predicted if the fall in the intracellular concentration of folate was due only to cell division.

When 10 day folate starved BHK cells are placed back into a folate supplemented medium (ghtEFC\(^{10}\)) they rapidly regain the ability to incorporate \([^3\text{H}]\)-formate (figure 7). Within 4 h they are incorporating at the pre-starvation level. This result confirms the above observation that \([^3\text{H}]\)-formate incorporation into cellular material of BHK cells is dependant on added folate. Further, this
rapid recovery demonstrates that the effects of folate starvation are readily reversible, suggesting that the enzymes involved in formate incorporation are present at their normal levels either during starvation or shortly after transferring the cells back to a folate supplemented medium. McBurney and Whitmore (1974b) have presented experimental evidence suggesting that the enzymes of folate and tetrahydrofolate metabolism, are synthesised constitutively in CHO cells.

In conclusion, a growth period of 10 days in fdEFC\textsubscript{10} depletes BHK cells of most, if not all, of their folates and that measurement of incorporation of radioactivity derived from $[^3\text{H}]$-formate into cellular material may be used as an indicator of intracellular levels of folates in BHK cells.

During the course of the above experiment no differences could be seen in the growth rates of normal and folate starved BHK cells. To confirm this observation normal and starved BHK cells were seeded at low density ($2 \times 10^5$ cells/50 mm dish) in separate culture dishes in the appropriate growth medium and the cell number/dish measured daily until confluence had been attained (figure 8). Both cell types have similar growth curves with a maximum doubling time of 20 h between 1 and 3 days after seeding and reach confluence after 5 days with a maximum cell density of around $1.6 \times 10^6$ cells/dish ($2.09 \times 10^4$ cells/cm$^2$). The morphology of the cell sheets at confluence under both conditions is similar with the cells aligning themselves next to one another forming the pattern
characteristic of fibroblasts in culture. Thus, BHK cells starved of folate appear not to be adversely affected under the conditions used.

3.4.3. The Transfer of Cellular Folates Between BHK Cells

From the information provided by the above experiments, an experiment could now be carried out to discover if folates are transferred between BHK cells. Normal BHK cells and 10 day folate starved BHK cells were cultured together in a 1:1 ratio at an almost confluent density (1 x 10^6 cells/50 mm culture dish) in fdEFC\textsubscript{10} for 16 h. After this period the mixed cultures were suspended in fresh fdEFC\textsubscript{10} containing $[^3\text{H}]$-formate and re-seeded at a low density into new dishes (2 x 10^5 cells/dish) containing glass coverslips. The cultures were grown for a further 4 h period before processing for acid insoluble autoradiography. After a short exposure of the autoradiographs (6 days) activated photographic grains were counted over 800 single, isolated cells to measure the incorporation of $[^3\text{H}]$-formate/cell.

The incorporation of $[^3\text{H}]$-formate into separate cultures of normal and starved BHK cells was measured using the above procedure. Normal and starved BHK cells were cultured separately after seeding at an initial density of 1 x 10^6 cells/dish in fdEFC\textsubscript{10} over the same 16 h period as the co-culture. The cultures were similarly
re-seeded at low density \((2 \times 10^5 \text{ cells/dish})\) into separate dishes containing coverslips and labelled for 4 h in fdEFC\(_{10}\) with \(^3\text{H}\)-formate. The coverslips were processed for autoradiography as above and activated grains counted over 400 single, isolated cells. To show that labelled molecules derived from \(^3\text{H}\)-formate were not transferred through the medium during the labelling period, portions of the two cell suspensions of separately cultured normal and starved BHK cells obtained after the 16 h period, were mixed in a 1:1 ratio, re-seeded at low density \((2 \times 10^5 \text{ cells/dish})\) and labelled with \(^3\text{H}\)-formate in fdEFC\(_{10}\) for 4 h. Coverslips from these cultures were also processed for autoradiography. The grain counts from all the autoradiographs are plotted as histograms in figure 9.

In the experiment where the normal and starved BHK cells were cultured and labelled separately, the normal cells have an average grain count of 75.0/cell and the starved cells have an average of 13.7 grains/cell. After subtracting the photographic background of 5.2 grains/cell (see methods section 2.2.8.), the incorporation into starved cells is 12.3% of that into normal cells which compares favourably with the value of 7% obtained earlier by scintillation counting (figure 7) and confirms that the folate starved BHK cells have a much reduced ability to incorporate \(^3\text{H}\)-formate. In the co-culture experiment all the cells incorporate \(^3\text{H}\)-formate. This suggests that during the 16 h co-culture folate is transferred from the normal BHK cells to the starved BHK cells and thus eliminating the population of low \(^3\text{H}\)-formate incorporation.
The incorporation of radioactivity by the folate starved cells in the co-culture experiment could also be explained by the transfer through the medium from the normal to the starved BHK cell of $[^3\text{H}]$-compounds derived from $[^3\text{H}]$-formate. This possible explanation is excluded in the control experiment where the normal and starved cells were cultured separately but mixed together at low density during the 4 h $[^3\text{H}]$-formate labelling period. In this experiment two populations of cells are present which correspond exactly with the normal and folate starved cells in the experiment where they were cultured separately throughout the 16 h growth period and the labelling period. Thus, $[^3\text{H}]$-compounds derived from $[^3\text{H}]$-formate are not transferred through the medium in detectable amounts.

In this experiment extensive transfer of labelled material was seen from heavily labelled normal BHK cells to the starved cells in contact in a manner directly analogous to metabolic co-operation (section 1.6.3.) showing that the depletion of cellular folates has no effect on the formation of permeable junctions. Because of this extensive junctional transfer, it is necessary to count only single, isolated cells in the autoradiographs.

The transfer of folates between the normal and starved BHK cells in mixed culture could be by a direct junctional pathway, or through the medium, or both. The results obtained in the earlier experiment examining the time course of folate depletion (figure 7) suggest
that folates are not lost from BHK cells which argues against a medium mediated pathway of transfer. Another way to examine whether folates are transferred through the medium is to take the medium from cultures of normal BHK cells grown in fdEFC\textsubscript{10} and add it to cultures of folate starved BHK cells. This conditioned medium, if it contains released folates, should cause an increase of \textsuperscript{3}H\textsuperscript{-}formate incorporation in starved BHK cells. This was tested by transferring medium from cultures of normal BHK cells (10\textsuperscript{6} cells/50 mm dish) grown for 16 h in fdEFC\textsubscript{10} to cultures of starved BHK cells (10\textsuperscript{6} cells/50 mm dish) and growing these cultures in this conditioned medium for a further 16 h. These cultures were then re-seeded at a lower density into new dishes (5 x 10\textsuperscript{5} cells/dish) containing the conditioned medium and \textsuperscript{3}H\textsuperscript{-}formate and grown for 4 h. The incorporation of \textsuperscript{3}H\textsuperscript{-}formate into acid insoluble material (expressed as CPM/mg protein) was measured and compared with that into control cultures of normal and starved BHK cells. There is little or no increase in the amount of \textsuperscript{3}H\textsuperscript{-}formate incorporation by starved BHK cells in the presence of this conditioned fdEFC\textsubscript{10} (table 8) showing that this medium does not contain sufficient, utilizable folates to cause the elimination of the cell population with low \textsuperscript{3}H\textsuperscript{-}formate incorporation in the earlier co-culture experiment (figure 9).

Transfer of folates between BHK cells therefore appears to be by a direct route; for example intercellular junctions. The involvement of these junctions in transfer of folates can be tested
using L cells because these cells do not form permeable junctions (Gilula et al., 1972). Unstarved L cells were co-cultured with 10 day folate starved BHK cells in a 1:1 ratio at high density. After sub-culturing the mixture at low density $[\text{H}^3]\text{-formate}$ incorporation was analysed as described before. Grain counts over the starved BHK cells in these mixed BHK/L cultures could be measured separately as the two cell types have different morphologies. This experiment was carried out at the same time as the other experiments so that all the grain counts could be directly compared.

The average grain count over the starved BHK cells in co-culture with the normal L cells is very similar (14.0 grains/cell) to the average grain count over the control starved BHK cells cultured by themselves (13.7 grains/cell; figure 9) showing that there is no detectable transfer of folates from the normal L cells to the starved BHK cells. Transfer of folates between BHK cells would therefore appear to depend on the formation of permeable intercellular junctions. The lack of folate transfer from L cells to BHK cells is also a further evidence against a medium mediated transfer of folates between BHK cells because such a pathway of transfer would be expected to operate with both cell types.

The histograms of the grain counts from the experiment where folate starved BHK cells were co-cultured with normal BHK cells and then labelled in diluted culture (figure 9b) appears to be composed of two populations; a population of high $[\text{H}^3]\text{-formate}$
incorporation (estimated mean, 75 grains/cell) which is like that of the normal BHK cells cultured and labelled separately (figure 9a) and a second population of intermediate incorporation (estimated mean, 40 grains/cell) and which seem most likely to be derived from the starved BHK cells. This difference in $[^3H]$-formate incorporation between the two cell types could be due to either an unequal distribution of folates between the two cell types after co-culture or the starved BHK cells not having the full enzyme potential to incorporate $[^3H]$-formate even though the pool of folates has become equilibrated. However, it is known from the earlier work (figure 7) that the 10 day folate starved BHK cells have the full enzyme potential to incorporate $[^3H]$-formate 4 h after transferring back to normal folate supplemented medium, which suggests that the second possibility is incorrect. Furthermore, the normal BHK cells appear to have a similar level of $[^3H]$-formate incorporation after co-culture with the starved BHK cells to the control normal BHK cells showing that the normal BHK cells have not yet lost sufficient folates by transfer to cause a detectable decrease in their level of incorporation. This result would not be expected if the pool of folates had become equilibrated between the normal and starved BHK cells over the 16 h period because the dilution of intracellular folates by one cell division (i.e. a factor of 2) causes a detectable loss (20%) in $[^3H]$-formate incorporation ability (figure 7).

The apparent lack of folate equilibration over 16 h is at variance
with what has been found for metabolites such as purine nucleotides (Sheridan et al, 1975 and in preparation) and 2-deoxy-glucose-6-phosphate (section 3.3.) which rapidly equilibrate between BHK cells by junctional transfer. Before analysing the possible causes of this slow rate of folate transfer the rate and extent of folate equilibration was examined in further detail. The time of co-culture of folate starved and normal BHK cells was varied from 8 to 48 h and the $[^3H]$-formate incorporation into the subsequent sparse cultures measured by autoradiography as described above. For the 24 h and 48 h co-cultures the initial cell density was halved to $5 \times 10^5$ cells/dish so that confluent conditions would not be reached before re-seeding. At this lower density all the cells are still joined by intercellular junctions (Pitts, 1972). Control experiments to measure the $[^3H]$-formate incorporation into normal and starved BHK cells cultured separately were carried out simultaneously. The histograms of the grain counts from the autoradiographs are shown in figure 10.

As the time of the co-culture is increased, the formate incorporation of the starved BHK cells increases until after 24 h of co-culture the two populations representing the normal and starved cells are difficult to distinguish. By 48 h the normal and starved cells appear as a single population showing that eventually the pool of cellular folates becomes equally distributed between the starved and normal cells in the co-culture. The single population in the 48 h co-culture has a lower mean grain count/cell than that of the
normal BHK cells cultured separately (33.0 grains/cell as opposed to 54.9 grains/cell respectively; table 9) demonstrating that the normal BHK cells lose the ability to incorporate \(^3\text{H}\)-formate as would be expected if they transferred folates to the starved BHK cells.

The qualitative analysis of the data was extended by a statistical analysis. The histograms of grain counts in the control experiments where the normal and starved BHK cells were cultured and labelled separately (figures 9a and 10a/c/e) appear to be of Gaussian form. Therefore best-fit Gaussian curves were generated to the histograms of grain counts from the co-culture experiments by the method of least squares (Fraser and Suzuki, 1973 and section 2.2.12.) using a computer. For the 8, 16 and 24 h co-culture times two bands were predicted to occur on the assumption that there were two populations of cells present of unequal ability to incorporate \(^3\text{H}\)-formate, whereas for the 48 h co-culture time only one band was predicted. Estimates for the parameters of these bands were found by a visual inspection of the histograms; these estimates were needed to initiate the analysis.

For a comparison of the goodness of fit of these curves, best-fit Gaussian curves were similarly generated for the two histograms of grain counts over the normal and folate starved BHK cells cultured for 16 h and labelled separately in dilute culture (shown as combined histograms in figure 9a). The generated curves
and the residual deviation or error curves (see below) are shown in figure 11. A summary of the observed and generated data from these experiments is given in table 9.

That the generated curves represent the normal and folate starved BHK cells at the various co-culture times is evident from the following. (i) The unbiased estimates of the standard deviation for the various co-culture times and the pure cultures of normal and folate starved BHK cells are very similar (ranging from 1.049 to 2.585 for the co-cultures and 2.213 to 2.223 for the normal and folate starved BHK cells respectively). Therefore the generated curves for the histograms of the co-cultures are as good a fit as the curves generated to the separate histograms of normal and starved BHK cells in the control experiment. (ii) Because the initial ratio of normal to folate starved BHK cells was 1:1 and because both cell types have similar growth rate (figure 8) then the areas under the curves which represent the histograms of grain counts over these two cell types at every co-culture time will be in a 1:1 ratio. As is seen (table 9) the ratio of the areas under the generated curves at each co-culture time is approximately 1:1. (iii) The error curves for the co-culture histograms do not diverge to any greater extent from the abscissa than the error curves for the separate histograms of the control normal and starved BHK cells again showing that the generated curves for the co-cultures are as good a fit as the curves for the pure control cultures. An overall divergence from the abscissa indicates that the histograms are not of Gaussian form and a
divergence in a limited range on the negative side indicates that an extra band not included in the computer programme occurs in that range. The converse is true for a limited divergence on the positive side. (iv) The plot of the time of co-culture against the ratio of the means of the generated curves at each co-culture time crosses the ordinate (ratio axis) at 0.13 (figure 12). This value represents the basal level of $[^3H]$-formate incorporation by the folate starved BHK cells. However, it is already known (figure 7) the basal level of $[^3H]$-formate incorporation in 10 day folate starved BHK cells is 7% (i.e. 0.07) of the normal level of incorporation. These two values (0.13 and 0.07) are quite close considering the two very different approaches in obtaining these estimates and thus shows that the generated curves must closely represent the normal and starved BHK cells in the co-culture.

The statistical analysis confirms what was already obvious qualitatively, that with increasing co-culture times the folate starved BHK cells have an increasing ability to incorporate $[^3H]$-formate. This analysis further shows that there is a concomitant decrease after 16 h of co-culture of the ability of the normal BHK cells to incorporate $[^3H]$-formate as would be required if the normal cells are transferring folates to the starved cells. As a consequence of these changes, the two populations of normal and starved BHK cells in the co-culture merge together to produce by 48 h a single population of cells with respect to their ability to incorporate $[^3H]$-formate; that is the pool of folates present originally only in the normal cells has become equally distributed
between both normal and folate starved cells.

The significance of the linearity of the curve of the length of co-culture against the ratio of the means (figure 12) with respect to the rate of folate transfer cannot be interpreted as the formate incorporation is probably not a direct measure of intracellular concentration of folates as it only reflects the metabolic activity of folates. As mentioned earlier, the slow rate of transfer of folates through intercellular junctions is somewhat unusual. There are a number of possible causes of this phenomenon and these are discussed in the following section.

3.4.4. Study on the Possible Causes of the Slow Rate of Transfer of Folates

Intercellular junctions joining vertebrate cells appear to be impermeable to the dye Chicago sky blue (mol. wt. 1,000) but are freely permeable to procion yellow (mol. wt. 625; Imanaga, 1975) suggesting that the size limit for transfer through these junctions lies between the mol. wt. values of procion yellow and Chicago sky blue. Although the native folic acid has a mol. wt. of 425, inside the cell the most predominant form of folate is the pentaglutamate derivative of mol. wt. 960, formed by adding further glutamate molecules on to the pre-existing glutamate residue, (Houlihan and Scott, 1972). The junctions between BHK cells may therefore be
only slightly permeable to the pentaglutamate derivative and which could lead to a very slow rate of transfer of this folate derivative between cells. Indeed, these junctions may be impermeable to the pentaglutamate derivative and in such a case the added polyglutamate side chain would need to be wholly or partially removed before transfer of folate can take place. A slow rate of removal of the polyglutamate side chain would lead to a slow rate of production of transferable forms of folate and consequently a slow rate of transfer.

Again, if the junctions are freely permeable to the pentaglutamate derivative, then a slow rate of transfer of folates could also be accounted for by a poor equilibration between enzyme-bound and free folates. However, as the role of folates is to act as carriers of one-carbon groups it would be expected that folates would be freely mobile rather than tightly bound. The question "are junctions permeable to the pentaglutamate derivatives?", could possibly be answered by using a CHO mutant cell (AuxBl) isolated by McBurney and Whitmore (1974a). The AuxBl cells are auxotrophic for glycine, thymidine and a purine and this multiple auxotrophy appears to be due to a single lesion in folate metabolism.

The AuxBl cells have normal levels of activity for all the enzymes of folate metabolism so far examined. These include dihydrofolate reductase and the folate transport system. However, they have a greatly reduced folate pool and almost all of the folates are in the
monoglutamate form whereas in the wild-type CHO cells, the intracellular folates are mostly in the pentaglutamate form. As the activity of the enzyme responsible for the removal of the polyglutamate side chain appears to be at the normal level, the most likely lesion in the AuxB1 cells is a defect in the enzyme responsible for the addition of the glutamate side chain to folic acid (McBurney and Whitmore, 1974a and b).

If the explanation of McBurney and Whitmore for the multiple auxotrophy of the AuxB1 cells is correct these cells will incorporate little or no formate into cellular material. If intercellular junctions are permeable to the pentaglutamate derivative, then BHK cells will be able to supply this derivative to AuxB1 cells in mixed cultures in medium supplemented with folate. This will be seen as an increased ability of the AuxB1 cells to incorporate formate which can be measured autoradiographically. AuxB1 cells have been tested and shown to form permeable intercellular junctions with BHK cells (section 4.2.).

To confirm that AuxB1 cells are unable to incorporate formate into cellular material, separate cultures of AuxB1 cells and wild-type CHO cells were labelled for 4 h with $^{3}$H-formate in EFC10 supplemented with non-essential amino acids, hypoxanthine and thymidine (htEFC10NEAA: see materials). The incorporation of tritium into the acid soluble and acid insoluble fractions was measured for each culture dish. Duplicate, unlabelled cultures were
used to estimate the amount of protein/dish to allow a comparison to be made between the amount of incorporation into AuxB1 and into wild-type cells.

There is very little incorporation of $[^3\text{H}]$-formate into acid insoluble material of AuxB1 cells but, in contrast, there is a sizeable incorporation into acid soluble material (Table 10). Incorporation into acid soluble material can be explained as the AuxB1 cells have been shown (McBurney and Whitmore, 1974b) to have enzymic activity in the enzymes of one carbon metabolism and also a small pool of the lower glutamated forms of tetrahydrofolate. Incorporation into the acid soluble fraction is inconsequential in the co-culture experiment (to examine possible transfer of pentaglutamate folate) as this fraction is removed during processing for autoradiography.

To examine the permeability of junctions to the pentaglutamate form of folate, AuxB1 cells and BHK cells were grown in 1:1 mixtures for 48 h at high density in hTEFC$_{16}$NEAA and the ability to incorporate $[^3\text{H}]$-formate using autoradiography was measured as described above. The grain counts are plotted as histograms in Table 11.

After prolonged co-culture of AuxB1 with BHK cells, there is still only a low level of $[^3\text{H}]$-formate incorporation in the AuxB1 cells, indicating that there is little or no transfer of metabolically
active forms of folate. It would appear likely that intercellular
cjunctions are not permeable to the pentaglutamate form of folate.

In summary, folates can be transferred between BHK cells by a
direct pathway. The lack of transfer of folates between L cells
and BHK cells suggests that the movement of these molecules
between cells is through permeable intercellular junctions.
However, the rate of transfer of folates is unusually slow taking
as long as 2 days to reach equilibrium between BHK cells. This
is most likely due to the impermeability of intercellular junctions
to the pentaglutamate form of folate, which is the most
abundant form of folate inside mammalian cells (Houlihan and Scott,
1972), and a slow degradation of the pentaglutamate form to
lower glutamated and transferable forms of folate. A slow rate
of removal of the polyglutamate side chain is in keeping with
the suggestion of McBurney and Whitmore (1974b) that the role of
polyglutamation of folate is to aid its cellular retention.
Growing cultures of BHK cells in various stages of folate starvation (section 2.2.4.) and normal BHK cells (cultured in ghtEFC$_{10}$ for 48 h; section 2.2.3.) were suspended in fdEFC$_{10}$ and ghtEFC$_{10}$ respectively after trypsinization (section 2.2.2.). The cell suspensions were seeded in 50 mm dishes ($5 \times 10^5$ cells/dish) in medium (3 ml/dish); fdEFC$_{10}$ for the starved cells and ghtEFC$_{10}$ for the normal cells. $^{3}$H$_{2}$-formate (200 mCi/mmol; 5 μCi/ml) was added to half of the dishes of each cell type and the cultures were grown for a further 4 h. After this time the medium from all the dishes was removed and the incorporation of tritium into acid insoluble material in each $^{3}$H$_{2}$-formate labelled culture was measured (sections 2.2.9. and 2.2.10.). The unlabelled cultures were estimated for protein (section 2.2.11.). The results are expressed as the percentage of radioactivity (CPM/mg protein) incorporated by BHK cells at various stages of folate starvation compared to that incorporated by normal unstarved BHK cells (CPM/mg protein).

The recovery of $^{3}$H$_{2}$-formate incorporation by BHK cells in a folic acid supplemented medium was measured as follows. Cell suspensions of growing normal BHK cells (cultured in ghtEFC$_{10}$ in Roux flats; section 2.2.3.) and 9 day folate starved BHK cells (prepared as
described in section 2.2.4.) were suspended in \( \text{ghtEFC}_{10} \) and \( \text{fdEFC}_{10} \) respectively by trypsinization (section 2.2.2.). The cell suspensions were seeded into separate 50 mm dishes (5 \( \times 10^5 \) cells/dish) and the volume of medium adjusted to 3 ml/dish by adding \( \text{ghtEFC}_{10} \) to the normal cells and \( \text{fdEFC}_{10} \) to the starved cells. The cells were grown for 24 h at 37°C and the medium was then changed in all dishes to \( \text{ghtEFC}_{10} \) (this medium is supplemented with folate). \( [^3\text{H}] \)-formate (200 mCi/mmol; 10 \( \mu \)Ci/ml) was immediately added to a portion of the dishes of each cell type and these cultures were grown for a further 30 min before estimating the incorporation of radioactivity into acid insoluble material (sections 2.2.9. and 2.2.10.). A portion of the dishes of each cell type was also removed for an estimation of protein (section 2.2.11.). The incorporation of radioactivity (expressed as CPM/mg protein) was compared between the normal and previously starved BHK cells as a percentage of the normal BHK level of incorporation. The incorporation of \( [^3\text{H}] \)-formate/mg protein over a 30 min period of normal and starved BHK cells was likewise found and compared after 4, 16, 24 and 48 h growth in \( \text{ghtEFC}_{10} \).

Each data point shown is the average of 3 points in each of 2 separate experiments. The values of incorporation of the normal BHK cells over a 4 h period are 16,733 CPM/mg protein and 13,121 CPM/mg protein for the two experiments.
Figure 7 continued/

% of normal incorporation of $[^3H]$-formate

Medium changed to ghtEFC$^{10}$

Days of growth →
Growing cultures of 10 day folate starved BHK cells (section 2.2.4.) and normal BHK cells which had been grown for 48 h in ghtEFC\textsubscript{10}, were suspended after trypsinization (section 2.2.2.) in fdEFC\textsubscript{10} and ghtEFC\textsubscript{10} respectively. The suspensions were seeded in separate 50 mm culture dishes at a density of $2 \times 10^5$ cells/dish in 3 ml of the appropriate medium (i.e. either fdEFC\textsubscript{10} or ghtEFC\textsubscript{10}). The cultures were grown for 4 h at 37°C and then the medium from each dish discarded and appropriate fresh, pre-warmed medium added (3 ml/dish). Sample cultures were removed at this time and the number of cells/dish measured (section 2.2.2.) to give the initial cell density. The cultures were grown at 37°C and sample dishes removed every 24 h to allow an estimation of the number of cells/dish. The results are the average counts of 3 replica culture dishes for each data point.
Figure 8 continued/

Number of cells/50 mm dish \( \times 10^{-6} \)

Days of growth

- ghtEFC10
- ffEFC10
Figure 9  Histograms of Counts of Autoradiographic Grains over
\([^3H]\)-formate labelled Cells from Cultures of Normal BHK or L
Cells and Folate Starved BHK Cells Grown Either Separately or
in 1:1 Co-culture

The medium from cultures in Roux flats of normal BHK and L cells
(section 2.2.3.) was removed and the monolayers washed 4x with BSS
(20 ml/wash/Roux) pre-warmed to 37°C. These cultures had been
grown in ghtEFC\textsubscript{10} for 48 h prior to the start of the experiment.
The washed cultures of normal cells and 10 day folate starved BHK
cells (prepared as described in section 2.2.4.) were suspended
separately in fdEFC\textsubscript{10} after trypsinization (section 2.2.2.). For
the experiment to test possible transfer of folates between cells,
equal numbers of normal cells (i.e. BHK or L cells) and folate
starved BHK cells were seeded together in the same culture dish
(50 mm; 3 ml fdEFC\textsubscript{10}/dish) to give a final cell density of \(1 \times 10^6\)
cells/dish. For the control experiment to measure the incorporation
of \([^3H]\)-formate into normal and starved BHK cells, portions of the
respective suspensions were seeded into separate dishes (50 mm;
3 ml fdEFC\textsubscript{10}/dish) again to give a final cell density of \(1 \times 10^6\)
cells/dish. At this density all the cells in the culture dish
are joined by permeable intercellular junctions as seen by
nucleotide transfer (Pitts, 1972). All the cultures were grown at
37°C for 16 h.

For autoradiographic measurement of \([^3H]\)-formate incorporation/cell
after the 16 h period, the medium in each dish was removed and the cultures were suspended separately in fdEFC_{10} after trypsinization (section 2.2.2.). The cell suspensions were then re-seeded into separate dishes (50 mm) at low density (2 x 10^5 cells/dish). Portions of the two cell suspensions derived from the normal BHK cells and the starved BHK cells cultured separately, were mixed to give a 1:1 ratio of normal to starved BHK cells and also added to dishes (50 mm). All dishes contained glass coverslips. The volume of the medium in each dish was adjusted to 3 ml by adding fresh fdEFC_{10} and then [^{3}H]-formate was added to all the cultures (200 mCi/mmol; 5 µCi/ml). The cultures were grown at 37°C for 4 h and the coverslips then removed and processed for acid insoluble autoradiography (section 2.2.7.).

The autoradiographs were developed after 6 days exposure and stained with giemsa (section 2.2.7.). Autoradiographic grains over single, isolated cells were counted as described in section 2.2.8.; at least 6 coverslips were used for the quantitation of each part of the experiment. The background number of grains/cell for the experiment was estimated as described in section 2.2.7. and was found to be 5.2 grains/cell. The grain count data are grouped into classes; the size of each class is 4 grains/cell. Similar results were found in another experiment using an identical protocol.

a/ Combined histograms of autoradiographic grain counts over 400
isolated normal BHK cells and 400 isolated starved BHK cells cultured and labelled separately. Mean numbers of grains/cell are 75.0 for the normal BHK cells and 13.7 for starved BHK cells.

b/ Histograms of autoradiographic grain counts over 800 isolated cells from a 1:1 co-culture of normal BHK cells and folate starved BHK cells.

c/ Histograms of autoradiographic grain counts over 800 isolated cells from a 1:1 culture of normal and starved BHK cells cultured separately but labelled after mixing together.

d/ Histograms of autoradiographic grain counts over 400 isolated BHK cells from a 1:1 co-culture of normal L cells and the folate starved BHK cells. Mean number of grains over each BHK cell is 14.0. (For comparison the histogram of autoradiographic grain counts over the starved BHK cells cultured and labelled separately in a/ is also shown).
Figure 9 continued/

Number of cells

100
200

Number of grains/cell

100
50

16 h separate culture control

b/ 16 h co-culture

normal

starved
Figure 9 continued.

- e: starved PHK cells with normal I cells after 16 h co-culture
- f: control (from control PHK cells)
- g: 16 h separate culture control
- h: 48 h separate culture control
- i: 96 h separate culture control
- j: 168 h separate culture control
Table 8 The Incorporation of $[^3\text{H}]$-formate in the Presence of Conditioned Medium

Cultures of normal and 10 day folate starved BHK cells (section 2.2.4.) were trypsinized (section 2.2.2.) and seeded on separate 50 mm dishes ($5 \times 10^5$ cells/dish) in fdEFC$_{10}$ (final volume of 3 ml/dish). The normal cells had been grown in Roux flats for 48 h in ghtEFC$_{10}$ before the start of the experiment and the monolayers washed 4x with BSS (20 ml/wash/Roux) pre-warmed to $37^\circ$C prior to trypsinization. For a control of the normal level of $[^3\text{H}]$-formate incorporation in conditioned medium, normal BHK cells were similarly established in ghtEFC$_{10}$ in dishes ($5 \times 10^5$ cells/dish in 3 ml medium). All the cultures were grown at $37^\circ$C for 16 h. After this time, the three types of conditioned media (i.e. fdEFC$_{10}$ conditioned by normal BHK cells, fdEFC$_{10}$ conditioned by starved BHK cells and ghtEFC$_{10}$ conditioned by normal BHK cells) were collected aseptically. The conditioned media were spun at top speed for 5 min in an MSE bench centrifuge to pellet cells and cell debris. Portions of the conditioned media were warmed to $37^\circ$C ready for use and the remainder of the media were stored at $4^\circ$C for use approximately 16 h later.

Cultures of normal and 10 day folate starved BHK cells were now established in the conditioned media. The medium from cultures, in Roux flats, of folate starved and normal BHK cells (which had been grown for 48 h in ghtEFC$_{10}$) was discarded and the monolayers
washed 4x with BSS (20 ml/wash/Roux) pre-warmed to 37°C. The monolayers were trypsinized (section 2.2.2.) and suspended in the conditioned media in the following manner; one Roux culture of normal BHK cells was suspended in ghtEFC\textsubscript{10} conditioned by normal BHK cells (control of normal incorporation), one Roux of folate starved BHK cells was suspended in fdEFC\textsubscript{10} conditioned by folate starved BHK cells (control of starved incorporation), and one Roux of starved BHK cells was suspended in fdEFC\textsubscript{10} conditioned by normal BHK cells (experiment to test transfer through the medium). The 3 types of suspensions were seeded on to separate 50 mm culture dishes (5 x 10\textsuperscript{5} cells/dish) and the volume of medium adjusted to 3 ml/dish by adding the appropriate conditioned medium. The cultures were grown at 37°C for 16 h and after this time the medium was removed. The appropriate, fresh conditioned medium was added to the cultures (3 ml/dish and to half of the dishes \textsuperscript{3}H\textsuperscript{-}formate was added (200 mCi/mmol; 5 μCi/ml). The cultures were grown for 4 h and after this time the medium from all the cultures was removed. The incorporation of tritium into acid insoluble material of the \textsuperscript{3}H\textsuperscript{-}formate labelled cultures after extraction with TCA was measured as described in section 2.2.9. and 2.2.10. The unlabelled cultures were estimated for protein as described in section 2.2.11. The results are shown as CPM/mg protein. Each measurement of \textsuperscript{3}H\textsuperscript{-}formate incorporation and each estimation of protein is the average of 3 replica cultures.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incorporation of $^{3}\text{H}$-formate into acid insoluble material (CPM/mg protein)</th>
<th>% of Normal Control Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal BHK cells grown in ghtEFC10 conditioned by Normal BHK cells</td>
<td>10,466</td>
<td>100</td>
</tr>
<tr>
<td>Starved BHK cells grown in fdEFC10 conditioned by Starved BHK cells</td>
<td>773</td>
<td>7.25</td>
</tr>
<tr>
<td>Starved BHK cells grown in fdEFC10 conditioned by Normal BHK cells</td>
<td>1,008</td>
<td>9.65</td>
</tr>
</tbody>
</table>
To discover if the ability to incorporate $[^3\text{H}]$-formate could become the same for normal and folate starved BHK cells, the length of time of the high density co-culture was varied from 8 to 48 h. The 1:1 co-cultures of normal and folate starved BHK cells and also the control cultures of normal and starved BHK cells growing separately, were established as described in the legend to figure 9. For the 24 and 48 h time points, the initial cell density was halved to $5 \times 10^5$ cells/dish. After the required length of time of the high density co-culture (8, 24 and 48 h), the cultures were trypsinized (section 2.2.2.), seeded at low density in new dishes and labelled with $[^3\text{H}]$-formate (200 mCi/mmol; 5 pCi/ml) before processing for acid insoluble autoradiography (section 2.2.7.). After several days exposure the autoradiographs were developed and activated grains over single, isolated cells counted (section 2.2.8.). The background number of grains over each cell was estimated as described in section 2.2.8. Autoradiographic grains were counted over cells on at least 3 coverslips for each part of the experiment. The grain count data are grouped into classes; the size of each class is 4 grains/cell.

The statistical analysis of the co-culture experiment is given in
Also given in table 9 are the mean numbers of grains over each cell, adjusted for the background estimate, for the 8, 24 and 48 h control experiment.

a/ Combined histograms of autoradiographic grain counts over 200 isolated normal and 200 isolated folate starved BHK cells from 8 h control experiment where the normal and starved cells were cultured and labelled separately. Autoradiographs were developed after 4 days exposure and the background estimate is 4.2 grains/cell.

b/ Histograms of autoradiographic grains over 400 isolated cells from an experiment where normal and folate starved BHK cells were grown together in a 1:1 ratio for 8 h. Autoradiographs were developed after 4 days exposure and the background estimate is 4.2 grains/cell.

c/ Combined histograms of autoradiographic grain counts over 200 isolated folate starved BHK cells from the 24 h control experiment where the normal and starved cells were cultured and labelled separately. Autoradiographs were developed after 8 days exposure and the background estimate is 4.5 grains/cell.

d/ Counts of autoradiographic grains over 400 isolated cells from an experiment where normal and folate starved BHK cells were grown together in a 1:1 ratio for 16 h. Autoradiographs were developed after 8 days exposure and the background estimate is 4.5 grains/cell.
e/ Combined histograms of autoradiographic grain counts over 200 isolated normal and 200 isolated folate starved BHK cells from the 48 h control experiment where the normal and starved cells were cultured and labelled separately. Autoradiographs were developed after 10 days exposure and the background estimate is 8.2 grains/cell.

f/ Counts of autoradiographic grains over 400 isolated cells from an experiment where normal and folate starved BHK cells were grown together in a 1:1 ratio for 48 h. Autoradiographs were developed after 10 days exposure and the background estimate is 8.2 grains/cell.
Figure 10 continued/

Figure 10 continued/

Figure 10 continued/

Figure 10 continued/

Figure 10 continued/
Best-fit Gaussian curves were generated on a computer to the counts
of autoradiographic grains over the co-culture experiments by the
method of least squares described by Suzuki and Fraser (1973 and section
2.2.12.). The computer programme contained the raw data for each time
point (i.e. 8, 16, 24 and 48 h) and also estimates of the peak
parameters; i.e. maximum peak height, bandwidth at half peak height and
the mean. These estimates were found by a visual examination of the
histograms. For the 8, 16 and 24 h time points, two peaks were
assumed to occur whereas for the 48 h time point only one peak was
assumed. For a comparison of the goodness of fit of this type of
analysis to pure populations (as opposed to mixed populations of the
co-cultures), best-fit Gaussian curves were similarly generated for the
counts of autoradiographic grains of the normal and starved BHK cells
cultured and labelled separately from the 16 h time point control
(figure 11a). The curves are shown as dashed lines and the residual
deviation curves (i.e. observed value minus generated value) are shown
above. Because the raw data was given to the computer and not the
grouped data (class size being 4 integers along the abscissa) shown
in figures 11 and 12 the units along the ordinate are 1/4 the value of
the units shown in the aforementioned figures. The means of the
generated curves, the ratio of the areas under the curve for each time
point, and the unbiased estimates of the standard deviation are given
in table 9.
The generated curves are shown by the dashed lines and the residual deviation curves are shown by the solid lines. The number of grains/cell is given as a function of the number of cells. The results are for 16 h control (i.e., normal and starved cells) and are represented in Figure 11 continued.
Table 9  Summary of the Observed and Generated Data from the
Autoradiographic Analysis of the Transfer of Folates Between BHK Cells

The results of the autoradiographic analysis and the subsequent statistical analysis of these data (figures 11, 12 and 13) are given. The observed and the generated means have been corrected for the photographic background by subtracting the appropriate background estimate (given in figure 11 for the 16 h time point and figure 12 for the 8, 24 and 48 h time points) from the native value. The ratios of the generated means at each time point are plotted in figure 14.
<table>
<thead>
<tr>
<th>11 (III) Co-</th>
<th>48 h</th>
<th>24 h</th>
<th>16 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.4°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4°</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2.2°</td>
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<td></td>
</tr>
<tr>
<td>2.0°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.9°</td>
<td></td>
<td></td>
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<tr>
<td>1.8°</td>
<td></td>
<td></td>
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<tr>
<td>1.7°</td>
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<tr>
<td>1.6°</td>
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<td></td>
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<tr>
<td>1.5°</td>
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<td>1.4°</td>
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<td>1.3°</td>
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<td>1.2°</td>
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<tr>
<td>1.1°</td>
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<td></td>
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<tr>
<td>1.0°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9 continued/
The length of time of the high density co-culture of normal and starved BHK cells is plotted against the ratio of the generated means given in table 9. The ratio of the generated means at 0 h is 0.13 (cf. figure 7).
Table 10 The Incorporation of $[^3\text{H}]$-formate by Wild-type and AuxBl CHO Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Incorporation of $[^3\text{H}]$-formate</th>
<th>Acid Soluble</th>
<th>Acid Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPM/mg protein</td>
<td>% of wild-type</td>
</tr>
<tr>
<td>CHO/K1</td>
<td></td>
<td>7,516</td>
<td>100</td>
</tr>
<tr>
<td>CHO/K1-AuxBl</td>
<td></td>
<td>4,764</td>
<td>63.4</td>
</tr>
</tbody>
</table>

Wild-type CHO cells and AuxBl CHO cells growing in htEFC$_{10}$NEAA in Roux flats (section 2.2.3.) were trypsinized and suspended (section 2.2.2.) in htEFC$_{10}$NEAA. The cell suspensions were seeded into 50 mm dishes ($5 \times 10^5$ cells/dish) and the volume of the medium/dish adjusted to 3 ml by adding htEFC$_{10}$NEAA. The cultures were grown at 37°C for 16 h. The medium was removed after this time and fresh htEFC$_{10}$NEAA added. $[^3\text{H}]$-formate was added to half of the cultures (200 mCi/mmol; 5 μCi/ml) and all the cultures were grown for a further 4 h. The medium was removed from all the dishes and the cultures washed 2x with ice cold BSS (3 ml/wash/dish) and the tritium activity in the acid soluble and acid insoluble cell fractions of the labelled cultures measured (sections 2.2.9. and 2.2.10.). The unlabelled cultures were estimated for protein (section 2.2.11.). Each measurement of $[^3\text{H}]$-formate incorporation and each protein estimation is the average of 3 replica cultures.
Table 11  Counts of Autoradiographic Grains over BHK and AuxBl Cells after 48 h 1:1 Co-culture and Labelling with \(^{3}\text{H}\)-formate

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mean number of grains/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK</td>
<td>86.8 ± 9.7</td>
</tr>
<tr>
<td>AuxBl</td>
<td>2.9 ± 0.9</td>
</tr>
</tbody>
</table>

Growing cultures of AuxBl cells and BHK cells (section 2.2.3.) were trypsinized and suspended (section 2.2.2.) in htEFC\(_{10}\)NEAA. Mixed cultures were established in 50 mm dishes at a final cell density of 5 x 10\(^5\) cells/dish in 3 ml of htEFC\(_{10}\)NEAA/dish. The mixed cultures were grown at 37\(^\circ\)C for 48 h and then trypsinized (section 2.2.2.) and suspended in fresh htEFC\(_{10}\)NEAA before re-seeding into new 50 mm dishes at low density (2 x 10\(^5\) cells/dish in 3 ml htEFC\(_{10}\)NEAA). These dishes contained glass coverslips. The low density cultures were labelled for 4 h with \(^{3}\text{H}\)-formate (200 mCi/mmol; 5 μCi/ml) and processed for autoradiography (section 2.2.7.). The autoradiographs were developed after 10 days exposure and grains counted over single, isolated cells (section 2.2.8.). For BHK cells 50 cells were counted and for AuxBl cells 200 cells were counted on at least 5 separate coverslips. A preliminary experiment gave a similar result.
3.5. Study on the Transfer of Proline and its Precursors Between Cells in Culture

Johnson and Sheridan (1971) showed that the dansyl derivatives of the amino acids aspartate and glutamate could be freely transferred between electrically coupled Novikoff hepatoma cells. Whether unsubstituted amino acids are transferred through junctions between vertebrate cells is unknown. The pre-labeling technique of Pitts and Simms (1977) cannot be extended to examine intercellular transfer of amino acids because the pools of these metabolites are small, turnover rapidly and are easily washed out from cells (Pitts, unpublished results). However, mutant mammalian cells in culture have been isolated which are auxotrophic for certain amino acids and it should be possible using a "feeder" technique to use this auxotrophy to examine the transfer of these particular amino acids.

One well characterised amino acid auxotroph is the Kl mutant of CHO cells (Kao and Puck, 1968). Kl cells are auxotrophic for proline being unable to convert glutamate to glutamate-γ-semialdehyde. If proline and/or its precursors (glutamate-γ-semialdehyde and pyrrolin-5-carboxylate) can be transferred through intercellular junctions, then in a proline deficient medium, BHK cells will be able to feed Kl cells with their requirements and so allow growth of the Kl cells. However, because L cells are unable to form these junctions (Gilula et al, 1972), then in mixed cultures of L cells
and Kl cells in the same proline deficient medium, the Kl cells will not grow and will be lost from the culture by cell death.

The proline dependence of the Kl cells had not been tested for some years, thus, a growth analysis of Kl cells in the presence (11.5 µg/ml) and absence of proline was first carried out to check the proline dependence (figure 13). The serum used to supplement the growth medium was first dialysed to remove any proline and precursors (section 2.1.4.). The Kl cells grow quickly in the proline supplemented medium (psEFC_{10}) with a maximum doubling rate of 18 h, attaining a final cell density of 2.5 x 10^6 cells/50 mm dish 4 days after seeding at an initial density of 2 x 10^5 cells/50 mm dish. In the proline deficient medium (pdEFC_{10}) the Kl cells grow only poorly with a maximum doubling rate of 26 h. The cultures reach a maximum cell density of 1.1 x 10^6 cells/50 mm dish (just over 2 cell divisions) after 3 days, after which the cells begin to detach from the culture dish. Therefore these cells are still auxotrophic for proline.

To test whether proline (and/or its precursors) could be transferred through intercellular junctions the following experiment was carried out. Kl cells were seeded in 50 mm dishes at a 1:1 ratio with the HGPRT⁻ variants of BHK and L cells (TG2 and A9 cells respectively which are unable to incorporate hypoxanthine) at a final cell density of 5 x 10^5 cells/dish in psEFC_{10}. After 4 h growth in this medium the cultures were washed several times in BSS and half the cultures
grown in psEFC\textsubscript{10} and the other half grown in pdEFC\textsubscript{10}. Sample cultures were taken at this time (0 days) to estimate the ratio of K1 cells to TG2 and A9 cells by the following method. The cultures were trypsinized and suspended in psEFC\textsubscript{10} and seeded at low density in 50 mm dishes containing glass coverslips. The cultures were labelled for 16 h with \textsuperscript{3}H-hypoxanthine and the coverslips processed for autoradiography. An estimate of the ratio of K1 cells to TG2 and A9 cells was thus found by counting the labelled cells (K1 cells) versus the unlabelled cells (TG2 and A9 cells) in the exposed autoradiographs. The remaining cultures were grown for 3 days with daily medium changes before being suspended and re-seeded into new dishes at the same initial cell density in the appropriate medium. Portions of these suspensions were used to estimate the ratio of K1 cells to TG2 and A9 cells. The cultures were similarly sub-cultured after 6 and 9 days and the cell ratio measured. The results are shown in figure 14.

The ratio of K1 cells to TG2 cells remains approximately constant at 1:1 throughout the 9 day co-culture period both in the presence and absence of proline. Therefore TG2 cells are able to supply the K1 cells with sufficient proline (and/or its precursors) to allow a normal rate of growth of the K1 cells. However, the K1/A9 cell ratio only remains 1:1 over the 9 days in the presence of proline; in the absence of proline the ratio falls slowly at first followed by a more rapid fall and then tailing off by the end of the experiment. This result would be expected if the K1 cells were
being limited by the lack of proline. A9 cells are therefore unable to provide sufficient proline (and/or its precursors) for the K1 cells to support their growth. Hence, the likely pathway of transfer of proline (and/or its precursors) from TG2 cells to K1 cells is through intercellular junctions joining the two cell types.

However, this experiment does not wholly exclude the possibility of a medium mediated pathway of transfer. Indeed it could be argued that because TG2 cells are of fibroblastic origin (Stoker and MacPherson, 1963) they might overproduce proline; this being a major amino acid in collagen which is synthesised by fibroblasts. This could lead to a sufficient secretion of proline into the medium to support the growth of the K1 cells in the above experiment even though the medium was changed daily to overcome this problem and also remove dead cells. Such transfer can be easily examined by assaying the amount of growth of K1 cells in pdEFC\textsubscript{10} that has first been incubated (i.e. conditioned) with cultures of TG2 cells.

For this experiment the rate of growth of K1 cells in 50 mm culture dishes was followed over 6 days in the presence of pdEFC\textsubscript{10} which had been conditioned with growing cultures of TG2 cells for 24 h. To ensure that any secreted proline (or utilizable precursors) could be detected the ratio of the number of cells used to condition the medium was over twice that used in the previous experiment. Therefore, the concentration of any secreted proline in this medium
should be at least twice that in the medium in the previous experiment. Again, the medium in the culture dishes was changed daily. For controls, the growth of K1 cell in the presence of either psEFC\textsubscript{10} conditioned by cultures of K1 cells or pdEFC\textsubscript{10} conditioned by cultures of A9 cells was followed simultaneously. The results are shown in figure 15.

The results show that in the presence of the TG2 conditioned pdEFC\textsubscript{10} there is no more growth of the K1 cells than in the control experiment with A9 conditioned pdEFC\textsubscript{10}. This appears to be due to a limitation of proline as the K1 cells grow at the normal rate in psEFC\textsubscript{10} conditioned by K1 cells. Thus, TG2 cells do not release sufficient proline or utilizable precursors of proline into the medium to support the growth of K1 cells observed in the previous experiment and therefore the most likely pathway of transfer of these metabolites is through intercellular junctions.

This feeder technique could be readily extended to examine transfer of other metabolites. The drawback of the technique is the difficulty of obtaining auxotrophic mutants. Recently asparagine auxotrophs of Don Chinese hamster cells have been isolated (Goldfarb et al, 1977) which could readily be used in exactly the same way to investigate the possible transfer of this amino acid. The AuxBl mutant of CHO cells described earlier (section 3.4.4.; McBurney and Whitmore, 1974a) could likewise be used for investigating possible transfer of glycine.
Figure 13  Growth of Kl Cells in the Presence and Absence of Proline

Growing cultures of Kl cells in Roux flats (section 2.2.3.) were washed 4x with BSS pre-warmed to 37°C (20 ml/wash/Roux flat) and then trypsinized (section 2.2.2.). Half of the cultures were then suspended in a proline deficient medium (pdEFC\textsubscript{10} supplemented with 10% dialysed serum: section 2.1.4.) and the other half suspended in a proline supplemented medium (psEFC\textsubscript{10} supplemented with 10% dialysed serum and proline at a concentration of 11.5 ug/ml). The cells were seeded on to 50 mm culture dishes at a density of 2 x 10\textsuperscript{5} cells/dish and the volume of the medium in each dish adjusted to 3 ml by adding the appropriate medium. The cells were grown for 4 h at 37°C and the medium changed for the appropriate, fresh medium pre-warmed to 37°C. Samples of cultures were removed at this time and the numbers of cells/dish measured (section 2.2.2.) using a Coulter Counter to give the initial cell density. The remaining cultures were grown at 37°C and sample cultures removed at daily intervals for the first 4 days and then every second day to allow an estimation of the numbers of cells/dish. The medium in all the dishes was changed daily for the appropriate, fresh medium. Each data point is the average of 3 replica cultures.
Figure 13 continued/

Number of cells/ 50 mm dish \( \times 10^{-6} \)

Days of growth

- + proline
- - proline
Figure 14  Ratio Between Kl Cells and TG2 or A9 Cells in Mixed Cultures Growing in the Presence or Absence of Proline

Cultures of Kl, TG2 and A9 cells growing in psEFC<sub>10</sub> in Roux flats (section 2.2.3.) were trypsinized and suspended in fresh psEFC<sub>10</sub>. Equal numbers of Kl cells and TG2 cells or A9 cells were seeded on to the same culture dishes at a final cell density of 5 x 10<sup>5</sup> cells/dish. The volume of the culture medium was adjusted to 3 ml/dish by adding fresh psEFC<sub>10</sub> and the mixed cultures grown at 37°C for 4 h. The cultures were then washed 4x with BSS pre-warmed to 37°C (3 ml/wash/dish) and to half of the mixed cultures of each type (i.e. Kl/TG2 and Kl/A9) psEFC<sub>10</sub> was added and to the other half psEFC<sub>10</sub> was added. A sample culture from each type of mixed culture was taken at this time and trypsinized and re-seeded in psEFC<sub>10</sub> in new dishes (2 x 10<sup>5</sup> cells/dish) containing glass coverslips. [H<sup>3</sup>]-hypoxanthine (2 Ci/mmol; 2.5 μCi/ml) was added to these cultures and then they were grown at 37°C for 16 h before processing for autoradiography (sections 2.2.6. and 2.2.7.). After a 3 week exposure of the autoradiographs the ratio of Kl cells to TG2 or A9 cells was found by counting the number of labelled cells (Kl cells) and unlabelled cells (TG2 or A9 cells) using a microscope. A total of 400 cells were counted for each part of the experiment. The remaining cultures were grown for a further 9 days sub-culturing at 3 and 6 days on to new dishes at the same initial cell density (5 x 10<sup>5</sup> cells/dish) in the appropriate medium. Samples of the trypsinized suspensions after 3, 6 and 9
days were seeded into separate dishes (2 x 10^5 cells/dish) containing glass coverslips and psEFC_{10} and labelled with [^{3}H]-hypoxanthine to allow an estimation of the ratio of Kl cells to TG2 and A9 cells as described above. The medium in all the cultures was changed daily for the appropriate, fresh medium. Each data point is the average of 3 separate experiments and the error bars show the variation between individual experiments.

a) Ratio of Kl cells to TG2 cells in the presence or absence of proline

b) Ratio of Kl cells to A9 cells in the presence or absence of proline
Figure 14 continued/

**a/ Mixed cultures of Kl and TG2 cells**

- Ratio of Kl to TG2 cells
- Days of growth: 3, 6, 9
- Conditions: + proline, - proline

**b/ Mixed cultures of Kl and A9 cells**

- Ratio of Kl to A9 cells
- Days of growth: 3, 6, 9
- Conditions: + proline, - proline
Figure 15  Growth of Kl Cells in Medium Conditioned by Cultures of Kl, TG2, and A9 Cells

psEFC\textsuperscript{10} was conditioned by adding 100 ml of this medium to a roller culture (Winchester) of growing Kl cells and pdEFC\textsuperscript{10} was conditioned by adding 100 ml of this medium into each roller culture of TG2 and A9 cells. The cell density in each roller culture was $2 \times 10^7$ cells/Winchester at the start of the experiment and the medium was conditioned for 24 h. The conditioned media were spun in an MSE Mistral 2L at 2,000 RPM for 5 min to pellet cells and cell debris. The media was decanted off and stored at 4°C until used (no longer than 6 days).

The ability of these three types of conditioned medium to support the growth of Kl cells was measured as described in the legend to figure 14. Essentially, freshly trypsinized and growing Kl cells were seeded on to 50 mm culture dishes in psEFC\textsuperscript{10} and at a cell density of $2 \times 10^5$ cells/dish. After 4 h of growth at 37°C the medium was discarded and the cultures washed 4x with BSS pre-warmed to 37°C (3 ml/wash/dish). To 1/3 of the dishes Kl conditioned psEFC\textsuperscript{10} was added, to another 1/3 TG2 conditioned pdEFC\textsuperscript{10} was added and to the remaining 1/3 A9 conditioned pdEFC\textsuperscript{10} was added. Sample cultures were removed at this time and the cell numbers/dish measured using a Coulter Counter (section 2.2.2.). The remaining cultures were grown at 37°C and sample cultures removed daily to allow an estimation of the number of cells/dish.
The medium in all dishes was changed daily for the appropriate conditioned medium. Each data point is the average of 3 replica cultures.
Figure 15 continued/

Number of cells /50 mm dish x 10^-6

- psEFC_{10} conditioned by K1 cells
- pdEFC_{10} conditioned by A9 cells
- pdEFC_{10} conditioned by TG2 cells

Days of growth
Chapter 4  A STUDY ON THE ABILITY OF DIFFERENT
CELL TYPES TO FORM PERMEABLE JUNCTIONS WITH
BHK CELLS
4.1. Introduction

A number of different cell lines currently being studied in the Department of Biochemistry were examined for their ability to form permeable junctions. This survey was undertaken to discover if such junction formation is a common property of all different cell types and whether specificity of junction formation existed. The method chosen for detecting permeable junctions was pre-labelling of uridine nucleotide pools. This method has the advantage that junction formation can be readily examined between cells from the same cell line or from different cell lines and the extent of transfer (found by grain counting of recipients) is an approximate measure of the degree of junction formation (Simms, 1973). Also, the molecular basis of the method was fully understood at the start of this survey. The different cell lines examined and their origins are shown in section 2.1.3.

4.2. Junction Formation with BHK Cells (fibroblasts)

Dilute growing cultures of the various cell lines were established on coverslips in 50 mm dishes (1 x 10^5 cells/dish). The coverslip cultures were labelled for 3 h with [3H]-uridine and after washing off the labelling medium, fresh unlabelled BHK cells were seeded into the labelled cultures (5 x 10^5 cells/dish) and the mixed cultures grown together for a further 3 h. The co-cultures were then fixed
and processed for autoradiography. The autoradiographs were exposed either for short periods to allow grain counting of recipient BHK cells, or for long periods for photographing. Results of the grain counting are shown in table 12 and photographs are shown in figures 16 and 17.

Of the 6 cell lines which had not previously been studied for formation of permeable junctions, only the HTC cells (figure 16f) are totally incapable of forming these junctions with BHK cells. However, H35 cells (figure 16 g and h) can only form these junctions at very low levels with BHK cells. Indeed, the low level of transfer is not detectable by grain counting and can only be seen by long exposure of the autoradiographs. This correlates with the finding that these cells have been found to be weakly electrically coupled and joined by small numbers of small gap junctions (Sheridan, 1973).

The Chang liver cell (figure 16 c) and the 4629 P10 cell (figure 16b) both show similar degrees of junction formation with BHK cells (figure 16a; 83.5% and 109.0% respectively). The Kl cell line (figure 16d) shows intermediate interaction and the Int 407 cell line (figure 16c) has only poor interaction with BHK cells (42.5% and 10.6% respectively). Ledbetter and Lubin (1979) have recently found that CHO cells (Kl cells are a clonal subline) do not form permeable junctions as well as fibroblasts using a quite different approach.

The Chang, BSC-1, HeLa, Int 407, HTC and H35 cell lines are believed to be of epithelial origin (see materials). The data show that these
epithelial cell lines vary considerably in their ability to form permeable intercellular junctions with BHK cells. Taking into consideration other epithelial-like cells in culture (Fentiman et al., 1976; Pitts and Burk, 1976), the ability of epithelial cell lines to form junctions with BHK cells can be divided into 3 groups, (i) cell lines that form junctions readily with BHK cells (Chang, BSC-1, Hela and Int 407), (ii) cell lines that form junctions readily with themselves but not BHK cells (ex. BRL cells) and (iii) cell lines unable to form junctions (HTC and H35).

Whether such variation in the ability of epithelial cell types in vivo occurs is not known. It is possible that selection of epithelial cell types to grow in tissue culture may alter the properties of the plasma membrane.

The HTC and the H35 cell line are both unable to form permeable junctions to any degree and this is in agreement with earlier reports which showed a lack of electrical coupling between tumour cells of epithelial origin (Loewenstein and Kanno, 1966 and 1967; Jamokosmanovic and Loewenstein, 1967). However, the Hela cell is able to form permeable junctions and yet was also derived from an epithelial tumour (Gey et al., 1952). Whether the Hela cell line is representative of tumour epithelial cells with respect to this property is doubtful as Cox et al. (1974b) have found that different clones of Hela cells do not participate in metabolic co-operation. The possible role of lack of formation of permeable junctions in cancer was discussed in section 1.8.4.
Fibroblast cells in culture do not show such a variation (Pitts, 1971 and 1972). The K1 cells partially align up at confluence suggesting that they may have fibroblastic properties. The K1 cells are quite able to form junctions with BHK cells. The ability of fibroblasts to readily form junctions may reflect their physiological role in moving around tissues laying down a network of collagen fibrils.

The spleen 4629 P10 cell line was derived from a culture of murine splenic B-lymphocytes (Prekumar-Reddy et al., 1976). However, an immunological analysis carried out by Professor A.R. Williamson (personal communication) showed that these cells lacked most of the cell surface markers of B-lymphocytes. Thus, they may not reflect the communication properties of B-lymphocytes in the spleen.

4.3. A Simplified Method to Detect and Quantitate Permeable Intercellular Junctions

The method used above to detect and quantitate junctional permeability is slow and laborious requiring extensive grain counting. A simplified method was suggested from collaborative work carried out with Professor J.D. Sheridan (Sheridan et al., 1975 and manuscript in preparation). This method is based on the phenomenon of metabolic co-operation (section 1.6.3.) and measures metabolic interactions in mixed populations of TG2 cells (the HGPRT− variant of BHK cells and thus unable to incorporate hypoxanthine) and wild-type cells.
Sheridan and his colleagues found that there is more incorporation of $^3\text{H}$-hypoxanthine into mixed cultures of BHK and TG2 cells than would be expected from the amount of incorporation into the same numbers of BHK and TG2 cells grown in separate cultures. This increase in incorporation in mixed cultures was greatest at high exogenous hypoxanthine concentrations (50 uM). The incorporation of hypoxanthine increases as the exogenous hypoxanthine concentration is increased. The increase in incorporation in mixed cultures can be explained by an increased activity of HGPRTase in the wild-type BHK cells caused by the TG2 cells acting as a sink for the purine nucleotides synthesised from hypoxanthine. This is possible because BHK and TG2 cells form intercellular junctions permeable to these metabolites. L cells are unable to form these junctions and there is no such increase in incorporation of hypoxanthine in mixed cultures of wild-type L cells and TG2 cells (or wild-type BHK cells and A9 cells; these latter cells being the HGPRT$^-$ variant of L cells). Thus, the extra incorporation of hypoxanthine in mixed cultures of wild-type cells and TG2 cells over that calculated for the incorporation into separate cultures, should depend on the ability of the wild-type cells to form permeable junctions with TG2 cells.

Experiments to measure the junction forming ability of different cell types with TG2 cells by this method were carried out as follows. 1:1 co-cultures of wild-type and TG2 cells were established in 50 mm culture dishes at sub-confluent cell concentrations ($10^6$ cells/
The cultures were grown for 16 h and the medium then changed for fresh medium containing unlabelled hypoxanthine and $[^3H]$-hypoxanthine at a final total hypoxanthine concentration of 100 $\mu$M. After labelling the cultures for 2 h the amount of radioactivity incorporated into acid insoluble cellular material was measured. Results are expressed as the percentage increase in incorporation in the mixed cultures over the incorporation into separate wild-type cultures (the expected incorporation being half the incorporation into these latter cultures). The incorporation of hypoxanthine into the separate cultures of TG2 cells was always less than 0.1% of that into wild-type cultures. To compare different experiments, the percentage increases for the different cell types in mixed cultures with TG2 cells were related to the percentage increase for the BHK/TG2 cell in mixed culture in each experiment. The results are given in table 13.

The percentage increase in incorporation of $[^3H]$-hypoxanthine by the mixed cultures of BHK and TG2 cells varies in the three experiments (34.8% to 55.7%) and the average of these increases is approximately half of that seen in the earlier experiments carried out with Professor J.D. Sheridan although the same final concentration of exogenous hypoxanthine was used in both sets of experiments. However, in the earlier experiments the initial cell density was half that used in the present study ($5 \times 10^5$ cells/50 mm dish) and it has since been found (Sheridan, personal communication) that the incorporation in the mixed culture is affected by the cell
density being relatively lower (per cell) at near confluent cell
densities than at lower cell densities. It is possible that small
variations in the final cell densities in this latter set of
experiments may have caused the variations in incorporation in the
mixed cultures of BHK and TG2 cells.

Despite these moderate variations between different experiments, the
results compare favourably with other methods for measuring the ability
of different cell types to form permeable junctions with BHK cells;
n.i.e. uridine nucleotide transfer by grain counting (section 4.1.; Simms,
1973) and metabolic co-operation between wild-type and TG2 cells by
grain counting (Pitts, 1972). One interesting result though is the
high level of co-operation between BRL cells and TG2 cells. Uridine
nucleotide transfer experiments carried out by Pitts and Burk (1976)
have shown that BRL cells exhibit 'specificity of communication'. That
is, in a 3 h co-culture period only a very small percentage of BRL cells
form permeable junctions with BHK cells although in the homologous
systems all BRL cells form junctions and so do all BHK cells. However,
the infrequent junctions formed appear to be functionally similar to
those formed in homologous systems. Pitts (unpublished results) has
noted that with increasing times of co-culture there is an increasing
incidence of junction formation between BRL cells and BHK cells and thus
it might be expected that there would be a normal level of co-operation
between BRL cells and TG2 cells after a 16 h co-culture period.
Nevertheless, this result is still of interest as the BRL cells 'sort out'
from TG2 cells forming 'islands' which limits the number of BRL and TG2
cells which are in contact and thus able to form permeable junctions.
with one another. These junctions between the two cell types can only form at the borders between the BRL 'islands' and the surrounding BHK 'channels'. So the fact that there is a similar level of co-operation in BRL/TG2 and BHK/TG2 cultures even though there must be fewer permeable junctions between the two cell types in the former situation suggests, perhaps, that junctional permeability is normally in excess. Other factors such as the activity of HGPRTase in the wild-type cells, must also contribute to the amount of increase of incorporation in the mixed cultures of wild-type cells and TG2 cells. Therefore this method may only give a qualitative guide of the relative ability of different cell types to share their metabolites through permeable junctions.

Recently, a number of other methods have been published for estimating the ability of cells to form permeable junctions. Two of these methods (Corsaro and Migeon, 1975; Nicolas et al, 1978) also rely on using HGPRTmutants and thus may similarly only give a qualitative guide. Another method (Ledbetter and Lubin, 1979) relies on the differential sensitivity of human cells and rodent cells to oubain. Ledbetter and Lubin (1978) have recently found that at higher concentration of oubain (50 μM) normally used in their original experiments resulted in a reduction in the level of apparent co-operation between the cells. This might be due to the oubain acting directly or indirectly on the junctions (i.e. uncoupling; section 1.7.) or on the permeability of the non-junctional membranes of the human cells to $^{86}$Rb which they use as an analogue of K+. Thus, this method could give a false indication of the ability of cells to form junctions. Finally, it seems likely that all these methods lack the sensitivity of the autoradiographic techniques and thus might miss low levels of junction formation which could be functionally important.
Table 12  **Counts of Autoradiographic Grains Over Primary Recipient BHK Cells in Contact with Different Cell Types Pre-labelled with $[^3H]$-uridine**

Donor cultures of BHK, 4629 P10, Chang, K1, Int 407, HTC and H35 cells were prepared by plating suspensions of these cells harvested from growing cultures (section 2.2.3.) into separate 50 mm culture dishes (1 x $10^5$ cells/dish) containing EFC$_{10}$ (final volume 3 ml/dish) and glass coverslips (section 2.2.5.). The cultures were grown at 37°C for 16 h after which time the medium was changed for fresh EFC$_{10}$ containing $[^3H]$-uridine (26 Ci/mmol; 2.5 μCi/ml). The cultures were labelled for 3 h at 37°C and the radioactive medium was removed. The labelled donor cultures were washed 3x with EFC$_{10}$ (3 ml/wash/dish) pre-warmed to 37°C ready for the establishment of co-cultures with recipient BHK cells. The recipient BHK cells were prepared simultaneously with the donor cultures. Growing BHK cells were harvested (sections 2.2.2. and 2.2.3.) and seeded into 50 mm culture dishes (5 x $10^5$ cells/dish) containing EFC$_{10}$ (final volume of 3 ml/dish). The cultures were grown for 16 h at 37°C after which time the medium was changed for fresh EFC$_{10}$. The cultures were grown for a further 3 h at 37°C before being suspended by trypsinization (section 2.2.2.) in EFC$_{10}$ (3 ml final volume/culture). Co-cultures were established by transferring one suspension from one BHK recipient culture to one dish of labelled donor cells. The mixed cultures were grown for 3 h at 37°C before processing for autoradiography (sections 2.2.6. and 2.2.7.).
The autoradiographs were exposed for 8 days before developing. Autoradiographic grains over primary recipient BHK cells were counted as described in section 2.2.8. An estimate of the background grain density, carried out as described in section 2.2.8., was made and has been subtracted from the average number of grains/primary recipient BHK cell to give the figures shown in column 2. The results are from one experiment and the grains over the cells were counted over at least 3 separate coverslips for each part of the experiment.
Table 12 continued/

<table>
<thead>
<tr>
<th>Donor cell type</th>
<th>Average number of grains/primary recipient BHK cell</th>
<th>% of BHK value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK</td>
<td>117.1</td>
<td>100</td>
</tr>
<tr>
<td>4629 P10</td>
<td>86.4</td>
<td>109*</td>
</tr>
<tr>
<td>Chang</td>
<td>97.8</td>
<td>84</td>
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<tr>
<td>K1</td>
<td>52.9</td>
<td>45</td>
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<tr>
<td>Int 407</td>
<td>12.4</td>
<td>11</td>
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<tr>
<td>HTC</td>
<td>0</td>
<td>0</td>
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<tr>
<td>H35</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* A separate experiment was carried out for these cells and the average number of grains of primary recipient BHK cells with donor BHK cells in this experiment was 79.3
Figure 16  Long Exposure of Autoradiographs of Recipient BHK Cells Co-cultured with Different Cell Types Pre-labelled with $[^3H]uridine$

The experiments were carried out as described in the legend to table 12 except the autoradiographs were exposed for 4 weeks before developing. The photographs show typical microscopic fields of view. The experiment was carried out at least 3 times for each cell type and similar results were obtained on each occasion.

A/ Mixed cultures of donor BHK cells (heavily labelled) and recipient BHK cells (lightly labelled)- the magnification is approximately 500 times.

B/ Mixed cultures of donor 4629 cells (heavily labelled) and recipient BHK cells (lightly labelled)- the magnification is approximately 500 times.

C/ Mixed cultures of donor Chang cells (heavily labelled) and recipient BHK cells (lightly labelled)- the magnification is approximately 320 times.

D/ Mixed cultures of donor Kl cells (heavily labelled) and recipient BHK cells (lightly labelled)- the magnification is approximately 500 times.
E/ Mixed culture of donor Int 407 cells (heavily labelled) and recipient BHK cells (very lightly labelled) - the magnification is approximately 600 times.

F/ Mixed cultures of donor HTC cells (heavily labelled) and recipient BHK cells (unlabelled) - the magnification is approximately 650 times.

G/ Mixed cultures of donor H35 cells (heavily labelled) and recipient BHK cells (unlabelled) - the magnification is approximately 600 times.

H/ As in G/- the doubly arrowed recipient BHK cells can be seen to be labelled above background labelling of non-contacting recipient BHK cells. The magnification is approximately 900 times.
Figure 17  **Long Exposure of Autoradiographs of Recipient HTC and H35 Cells Co-cultured Respectively with Donor HTC and H35 Cells Pre-labelled with $^3$H-uridine**

The experiments were carried out as described in the legend to table 12 except the recipient cells used were HTC and H35 cells which were co-cultured respectively with donor HTC and H35 cells. Also the autoradiographs were exposed for 4 weeks before developing.

A/ Mixed cultures of donor HTC cells (heavily labelled) and recipient HTC cells (unlabelled) - the magnification is approximately 500 times.

B/ Mixed cultures of donor H35 cells (heavily labelled) and recipient H35 cells (unlabelled) - the magnification is approximately 800 times.
Table 13 The Incorporation of $[^3\text{H}]$-hypoxanthine in Mixed and Separate Cultures of Various Wild-type Cells and TG2 Cells

Growing cultures (section 2.2.3.) of BHK, Chang, BSC-1, HeLa, H35, HTC, L, K1, AuxB1, PyBHK, BRL, 3T3, 4629 P10 and TG2 cells were harvested by trypsinization (section 2.2.2.) and were seeded either into separate 50 mm culture dishes or in co-culture with TG2 cells (1 wild-type cell to 1 TG2 cell) at a final cell density in all cultures of 1 $\times$ $10^6$ cells/dish. The medium (EFC$^{10}$) was adjusted to a final volume of 3 ml/dish and the mixed and pure cultures grown for 16 h at 37°C. The medium in all the cultures was then changed for fresh EFC$^{10}$ containing hypoxanthine and $[^3\text{H}]$-hypoxanthine ($1 \text{ Ci} /\text{mmol}$) at a final hypoxanthine concentration of 100 $\mu$M and a final specific activity of 100 mCi/mmol for $[^3\text{H}]$-hypoxanthine. The cultures were labelled for 2 h at 37°C and the incorporation of tritium activity into acid insoluble material measured (sections 2.2.9. and 2.2.10). Each estimation is the average of 3 replica cultures from the same experiment. For convenience the experiment was carried out in 3 parts. The incorporation into mixed cultures (column 3) has been adjusted for the background incorporation by TG2 cells. These are Expt. 1 310 CPM, Expt. 2 425 CPM and Expt. 3 354 CPM/50 mm dish of TG2 cells seeded at the start of the experiment at $10^6$ cells/dish.
Table 13 continued/

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Incorporation into pure culture (A)</th>
<th>Incorporation into mixed culture with TG2 cells (B)</th>
<th>% Increase over expected i.e. $(B - A/2)100 \div B$</th>
<th>% of BHK/TG2 value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt (i)</strong></td>
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<tr>
<td>BHK</td>
<td>46,280</td>
<td>31,190</td>
<td>34.8</td>
<td>100</td>
</tr>
<tr>
<td>Chang</td>
<td>20,340</td>
<td>13,950</td>
<td>37.2</td>
<td>107</td>
</tr>
<tr>
<td>BSC-1</td>
<td>24,200</td>
<td>14,060</td>
<td>16.2</td>
<td>47</td>
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<td>Hela</td>
<td>67,420</td>
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<td>13</td>
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<td>H35</td>
<td>61,720</td>
<td>31,520</td>
<td>0</td>
<td>0</td>
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<td>HTC</td>
<td>39,600</td>
<td>19,400</td>
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<td>0</td>
</tr>
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<td>L</td>
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<tr>
<td>BHK</td>
<td>39,380</td>
<td>28,700</td>
<td>45.7</td>
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<tr>
<td>PyBHK</td>
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<td>33,690</td>
<td>38.3</td>
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<td>KL</td>
<td>43,920</td>
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<td>53</td>
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<td>AuxBl</td>
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<td><strong>Expt (iii)</strong></td>
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<td>23,820</td>
<td>55.7</td>
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<td>BRL</td>
<td>22,440</td>
<td>18,640</td>
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<td>4629 P10</td>
<td>29,890</td>
<td>14,940</td>
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<tr>
<td>3T3</td>
<td>21,820</td>
<td>18,660</td>
<td>65.0</td>
<td>117</td>
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</table>
Chapter 5 A DISCUSSION OF THE RESULTS ON THE
PERMEABILITY OF INTERCELLULAR JUNCTIONS FORMED
BETWEEN ANIMAL CELLS
5.1. The Permeability of Junctions Between Vertebrate Cells

Before the start of this study it was known that vertebrate cells which could form gap junctions could exchange a variety of substances with mol. wts. less than about 1,000. However, the only class of endogenous molecules known definitively to pass between such coupled cells was the nucleotides (Pitts and Simms, 1973). It was believed that the transfer occurred by passive diffusion through water filled pores located in the centre of the subunits, or connexons, of the gap junctions. From the existence of such pores it would be predicted that all small molecules move freely between cells joined by gap junctions. This present study was carried out to test this prediction. Rather than use fluorescently labelled, chemically synthesised probes to study permeability (cf. Simpson et al, 1976), it was decided to examine radioactive labelled endogenous molecules because these are more functionally significant indicators of junctional permeability than the former class of substances.

The results of this study show that several quite different metabolites are readily transferred between coupled cells. These metabolites include phosphoryl choline (and/or CDP-choline), lower glutamated forms of cellular folates, proline (and/or pyrrolin-5-carboxylate and glutamate-γ-semialdehyde), 2-deoxy-glucose-6-phosphate and possibly the phosphate and/or nucleotide phosphate derivatives of glucosamine. Together with the earlier studies examining junctional permeability (section 1.6.) it now seems reasonable to conclude that all small
molecules are freely transferred between coupled vertebrate cells.

The only apparent specificity found in this study for the intercellular transfer of substances, as has also been found in other studies on vertebrate systems (Imanaga, 1974; Pitts and Simms, 1977), is a limitation of size. The pentaglutamate forms of folate (mol. wt. around 960) are apparently not transferred between coupled cells whereas the lower glutamated forms are transferred (section 3.3.4.). This size exclusion limit is in close agreement with the results of Imanaga (1974) who found that the dye Chicago sky blue (mol. wt. around 1,000) is not passed between sheep myocardial cells whereas the dye procion yellow (mol. wt. 630) is transferred. Preliminary results of Flagg-Newton and Loewenstein (unpublished but see Loewenstein, 1978) show that fluorescently labelled oligopeptides of mol. wt. 901 are not transferred between cultured rat liver epithelial cells (RL cells).

However, as has been pointed out by Imanaga (1974), it is probably not possible to assign a precise cut-off point because the transfer of substances approaching this size limit will be influenced by shape and charge of the molecules. The size limit and the otherwise general permeability of the junctions between coupled cells are consistent with a mode of transfer by passive diffusion through water filled pores rather than by specific transport mechanisms such as permeases. Such pores are believed to occur at the gap junction (Makowski et al, 1977; Caspar et al, 1977; section 1.5.4.) and indeed the gap junction is the only type of intercellular junction so far found
between BHK cells (Revel et al., 1971). Thus this present study offers further support for the contention that gap junctions are the sites of intercellular communication through exchange of substances.

5.2. Lack of Direct Intercellular Movement of Phospholipids

In contrast to the finding of Peterson and Rubin (1970) phospholipids are not transferred between coupled cells in culture (they also used fibroblasts). The results of these earlier experiments appear to be due to artefacts of fixation (Peterson and Furshpan, unpublished results). Interference by such artefacts did not appear to occur in this present study (see final paragraphs of section 3.2.). Although phospholipids have a mol. wt. of around 750 and are thus appearing to be below the cut-off limit for junctional transfer (see above), they cannot be transferred between coupled cells through gap junctions because they are insoluble in water and hence not occurring free in the cytoplasm.

The lack of transfer of phospholipids provides indirect evidence that the two plasma membranes at the gap junctions are not fused. It can be argued though that the area occupied by gap junctions between BHK cells is so small (0.05% of the total surface area; Revel et al., 1971) that even if the membranes are fused at gap junctions there may be such a low level of phospholipid exchange between the contacting BHK cells that it would not be detected by the methods employed in
this present study. Although it is now well established that lipids can rapidly move in the plane of the plasma membrane (Edidin, 1974), it is possible that the gap junction might impede the movement of lipids as indicated by their resistance to detergents (Benedetti and Emmelot, 1967). Thus it cannot be readily argued that despite the very limited area occupied by gap junctions between BHK cells, phospholipid transfer due to membrane fusion at such points of contact could still be easily detected in the experiments used in this present study. From a morphological standpoint it appears that the outer leaflets of two interacting plasma membranes at gap junctions are not fused (Revel and Karnovsky, 1967). This can be seen by the very definite gap of around 2 nm between the two membranes at these cell surface specializations.

5.3. The Extent of Junctional Transfer Between Cells in Culture and Cells in vivo

A pertinent question to ask in deciding what roles gap junctions may have in tissue is 'how fast are ions and metabolites transferred between cells through these junctions?'. Are substances just slowly 'leaked' between cells or is there such rapid transfer that the ionic and metabolite contents of a population of coupled cells in a tissue can be considered as a common pool? One way of approaching this question is to understand how fast ions and metabolites are exchanged between BHK cells as there is information available on the communication
capacities of these cells in comparison to the capacities of some cell types in vivo (Sheridan, 1973).

For purine nucleotides it has been found that in a coupled population of BHK cells there is sufficient junctional flux of these metabolites that the nucleic acids are synthesised from a common pool of purine nucleotides (Pitts, 1971; Pitts, 1976; Sheridan et al, manuscript in preparation). Moreover, it has been shown that such extensive exchange results in the integration between the cells of their relative activities of de novo and salvage pathways of purine nucleotide synthesis (Sheridan et al, manuscript in preparation). As the mechanism of intercellular junctional exchange is most likely to be by passive diffusion then it would be expected that other metabolites of similar size are exchanged between coupled BHK cells as extensively as the purine nucleotides. It is not possible to calculate the junctional flux of the metabolites shown in this present study to be transferred between BHK cells as has been done for the purine nucleotides (Pitts, 1976). However, the results are consistent with a high rate of intercellular transfer of substances through junctions.

For example, the pool of 2-deoxy-[\(^3\)H]-glucose-6-phosphate appears to be shared equally between Kl and BHK cells possibly within 15 min after establishing mixed cultures. This includes the time for the BHK cells to settle, make contact and ultimately form gap junctions with Kl cells. The results on the ability of Kl cells to form functional junctions with BHK cells (section 4.1. and 4.2.) show that Kl cells do
not efficiently form such junctions with BHK cells compared to BHK cells forming junctions with themselves. Ledbetter and Lubin (1979) using a very different approach have similarly found that CHO cells (of which K1 is a subline) are inefficient junction formers in comparison to fibroblasts. Thus it would be expected that in similar experiments, BHK cells would share pools of 2-deoxy-[\(^{3}\text{H}\)]-glucose-6-phosphate between themselves considerably faster than mixed cultures of K1 and BHK cells.

Similarly for proline and its precursors, there is sufficient transfer of these metabolites from BHK cells to support the normal growth rate of K1 cells. For phosphoryl choline (and CDP-choline) the proportion of labelled material found in the primary recipient BHK cells is close to that found in the very similar experiments of Pitts and Simms (1977) examining uridine nucleotide transfer between BHK cells.

In conclusion, it is reasonable to believe that coupled BHK cells rapidly share their metabolites between themselves which in at least one aspect results in the integration of their metabolic activities. However, it would be expected that there are limitations on transfer of metabolites and ions which are sequestered to cellular compartments other than the cytoplasm. Interestingly Pitts (1971) has shown that even for one of these metabolites, (the thymidine nucleotides which are located primarily in the nucleus; Adams, 1969), there is sufficient transfer between BHK cells that in a population where only half the cells can synthesise thymidine nucleotides, all the cells are still
capable of a normal growth rate.

It might be thought that the junctional communication capacity of BHK cells is unusually high. However, it appears that the converse is true. Sheridan (1973) has calculated that in comparison to rat liver hepatocytes and mouse brown fat cells, the communication capacity of BHK cells is 30 to 40 times lower. Sheridan has not only taken into account in his calculations the area of gap junctions joining the different cell types, but also their size which is an important parameter of communication capacities. The many freeze-fracture studies on vertebrate tissues suggest that most epithelial cells are as extensively joined by gap junctions as hepatocytes and fat cells. Indeed, many excitable cells are joined quite extensively by gap junctions such as mammalian myocardial cells (Revel and Karnovsky, 1967) and intestinal smooth muscle (Friend and Gilula, 1972) as well as some neurons (Sotelo, 1976).

Of course there are cell types which do not form gap junctions, for example skeletal muscle cells, circulatory cells and many neurons (Bennett, 1973). However gap junctions between these cell types might severly impair their physiological function. Also some cell types only poorly form gap junctions. Perhaps the case in point here is early embryonic cells which appear to form few gap junctions in relation to their large size resulting in the difficulty of observing transfer of injected tracer dyes (Sheridan, 1976; section 1.6.9.). Malignant cells also appear to be poorly joined by gap junctions.
(Weinstein et al, 1976; section 1.8.4.).

In summary, it appears that most vertebrate cell types joined by gap junctions will share their inorganic ions and metabolites to a similar, or in many cases, to a greater extent than BHK cells. Before going on to discuss the consequences of this sharing, the junctional communication capacities of invertebrate cells will be assessed to discover the generality of such consequences.

5.4. Permeability of Invertebrate Junctions; Extent of Intercellular Exchange

As is discussed in detail in Chapter 1 (section 1.6.) the permeability properties of invertebrate gap junctions appears to be very similar as those for vertebrate gap junctions. That is, they allow for the free intercellular exchange of all substances below mol. wt. 1,000. Perhaps the only difference may be in the cut-off limit. Thus arthropod gap junctions may allow transfer of molecules up to mol. wt. 1,500 (Simpson et al, 1976). Again the rate of transfer of substances appears to be quite rapid with for example, the connexons of the annelid gap junctions being several orders of magnitude more permeable to a variety of fluorescein anions than the non-junctional membrane (Brink and Barr, 1978). Thus it seems reasonable to believe that invertebrate cells joined by gap junctions do have the same potential to share their ionic and metabolic contents as coupled
vertebrate cells.

As mentioned above this potential is dependent upon the relationship of the cell volume to the area of gap junctions joining the cells. With this in view Sheridan (1973) has similarly demonstrated that the communication capacities of the cells of the hepatopancreas of the arthropod *Limulus* are similar to vertebrate counterparts such as the mammalian hepatocyte (which as mentioned above is at least 30 more efficient at sharing ions and metabolites than BHK cells). As yet there is little information on the communication capacities of other invertebrate cells. However, there are a number of morphological studies which suggest that many epithelial-like cells from different invertebrate phyla are also extensively joined by gap junctions. For example the epidermis of *Hydra* (Hand and Globel, 1972), the gastrodermis of *Planaria* (Quick and Johnson, 1977), the columnar epithelia from the diverticula of the mollusc *Aplysia* (Finbow, unpublished observations), the hemocyte capsule of cockroach (Baerwald, 1975) and the salivary gland of the blowfly (Skaer et al, 1975) are all rich in gap junctions. Thus the consequences of rapid junctional exchange of substances are probably widespread occurring in perhaps the majority of non-excitable tissues and a few excitable tissues (ex. myocardial and smooth muscle cells).

5.5. Consequences of Junctional Permeability; Homeostasis and Evolution
This present study offers support for the homeostatic roles proposed for cell coupling (see section 1.7.2. and 1.7.3.). The sharing of ions and metabolites introduces a level of internal stability not possible if the cells remain as single units. Coupling will act as an efficient way of transporting nutrients and end products of metabolism through tissues. The activities of coupled cells will become integrated and thus a population of cells will in many ways act as a functional unit (cf. a syncytium with respect to ions and metabolites). These roles may explain the prevalence of gap junctions in non-excitable tissues and of course, these roles may be of equal importance in excitable tissues even though it has been established that they can act as electrotonic synapses.

The finding of low resistance junctions (Loewenstein, 196/a) in the most primitive multicelled animal, the sponge, suggests that junctional communication is important for the maintenance of the multicelled state. This suggestion is supported by the finding that even multicelled algae as well as higher plants are joined by the plant counterpart of the gap junction, the plasmodesmata (Spanswick and Casterton, 1967; Spanswick, 1972). So far, on a morphological basis, only one multicelled organism has been found to lack the type of intercellular communication afforded by gap junctions (or plasmodesmata). This is the slug stage of the slime mould Dictostelium discoideum (Johnson et al, 1977). However, under favourable growth conditions this species is unicellular and it is only in times of starvation that the cells aggregate to form a transient, multicelled form.
where most of the cells are committed to death in order to propagate the species. The slime mould is therefore interesting in that it can differentiate and have spatial orientation (pattern formation) in the apparent absence of junctional mediated intercellular communication. Thus, if morphogenesis at a simple level does not require intercellular junctions, then it raises the possibility that gap junctions (and may be plasmodesmata) were evolved for homeostatic purposes and not for their ability to transmit developmental signals. But it could be that later on in evolution gap junctions assumed this transmission function as well as other transmission roles (cf. electrotonic synapses). In other words a society of cells may not be able to evolve into a composite whole, that is a multicelled organism, unless they find some way of achieving a means to produce a sameness in their contents to allow for integration of their activities. Gap junctions would provide this means for the animal kingdom and likewise plasmodesmata would provide the means for the plant kingdom.

An interesting feature of intercellular communication through gap junctions is its size specificity. Large molecules do not pass between cells whereas small molecules and ions are freely transferred. This could be important as it allows for intercellular transfer of substances between different cell types with no adverse effects on their states of differentiation as the molecules which define these states, mRNA and proteins, are held within the cells (or daughter cells) in which they were synthesised unless they are destined for export. Cellular molecules conveniently fall into two classes; metabolites,
cofactors and small control molecules which are generally below mol. wt. 1,000 and macromolecules which range from mol. wts. of several thousand to several million. Thus, it would be relatively easy to select for a pore size which would safely exclude transfer of macromolecules but allow for exchange of most small cellular molecules. Also, it is likely that it would be easier to control the permeability of a small pore than a large pore. As is discussed earlier (section 1.7.), there is some evidence that the permeability of gap junctions can be modulated possibly by a change in the effective diameter of the pores in the centre of each connexon (Rose et al, 1976). A third advantage of a small diameter pore as opposed to one of a large diameter is that it is inherently a less complex structure making it thus easy to assemble and disassemble and, moreover, less structural components are needed and so it will be less open to lesions arising from mutations.

The ability for gap junctions to allow cells of varying differentiated states to directly communicate with each other could greatly increase the evolutionary capacity of multicelled animals. It would permit a level of versatility in tissue function and organization not possible if the cells remained as separate entities. This evolutionary aspect can be best illustrated by describing a number of systems which may exemplify such versatility.

The work of Orci and his colleagues (Orci, 1974; Orci et al, 1973) has shown that the pancreatic islet alpha and beta cells are
joined by gap junctions both with themselves and with each other. These two cell types respectively secrete the antagonistic hormones glucagon and insulin. Thus by their ability to share low molecular weight intracellular second messengers they may be able to integrate their production and secretion of these two hormones. Such communication could introduce a level of control and responsiveness that would not be possible if the cells were separate entities. This example shows how two cell types together in the same tissue and which have antagonistic roles may complement each other for the benefit of the organism.

The epidermis of the amphibian skin perhaps demonstrates how communication through junctions can increase the functional capacities of a tissue. The epidermis has a protective function against physical trauma and dehydration but in the amphibian it also has a role in salt balance by actively transporting sodium from the outside of the animal to the inside (Ussing and Zerahn, 1951). The outermost living layer, the stratum granulosa, is destined to become the dead cornified layer and as a consequence of cornification it contains little or no active sodium pump sites and yet is joined by tight junctions and thus restricting paracellular flow of these ions (Mills et al., 1977; Mills and DiBonna, 1978). However, because this layer is joined by gap junctions to the inner layers where the sodium pump sites are located, this tissue can act very efficiently in sodium transport (Mills et al., 1977; Farquhar and Palade, 1966).
Communication through gap junctions may also allow some versatility in metabolism. The mammalian oocyte uses pyruvate, but not glucose, as an energy source (Biggers et al., 1967; Eppig, 1976) and this appears to be supplied by the surrounding granulosa cells (Donahue and Stern, 1968). The functional significance of this metabolic restriction is as yet unclear but the very early stages of the mammalian embryo appear to have similar restrictions (Biggers et al., 1967). The oocyte, at least in the antral stages, is joined by gap junctions to the surrounding granulosa cells (cumulus) and these cells are in turn joined extensively by gap junctions with other granulosa cells (Gilula, et al., 1978; Albertini et al., 1975). It is possible that this metabolic adaptation may not have occurred if the oocyte and follicular cells were not joined by gap junctions which could act in this system as an efficient pathway for metabolite movement. This possibility is supported by the finding that the pre-antral oocyte appears to be dependent for growth upon having intimate contact with surrounding granulosa cells (Eppig, 1977).

Thus the diversity of form and function in multicelled animals, at least at the tissue level, may in part be due to the increased evolutionary capacity afforded by communication through gap junctions.

5.6. **Uncoupling and Cell Death**

The finding of this present study offers support for the suggestion
of others (Bennett, 1973; Rose and Loewenstein, 1976) that coupled cells may need to have some mechanism to break junctional communication at times of cell death to prevent loss from healthy cells to dead or dying cells of metabolites which may in turn cause their own cell death. There is now an increasing amount of experimental evidence that cells can rapidly and reversibly break junctional communication (see section 1.7. for references). As yet the mechanism for uncoupling is not understood but it has been proposed that it could occur by a structural change of the gap junction (Perrachia and Dulhunty, 1976) or by a breakdown of the gap junction either by internalisation to form 'annular' gap junctions or a dispersal of the connexons in the plane of the membrane (Yancey, Easter and Revel, manuscript in preparation).

5.7. Predictions on the Control of Discrete Cell Functions

The fact that gap junctions tend to cause the interiors of cells within a population to become similar with respect to their metabolite and ion content leads to some predictions on how cells control their own discrete processes.

There is now an increasing amount of evidence that factors which initiate DNA synthesis are located in the cytoplasm. For example the transplantation experiments of Gurdon (1960) showed that a nucleus transplanted from an intestinal epithelial cell to the frog oocyte will de-differentiate and begin DNA synthesis. Also, the nuclei of
heterokaryons formed by cell fusion become synchronous. This synchronisation appears to be achieved at S-phase or mitosis (Rao and Johnson, 1974). These factors must be large molecules if they are not to be lost to surrounding cells. There is now evidence that at least some of these factors are proteins which is therefore consistent with the prediction (Ley, 1975; Benbow et al, 1976).

5.8. Conclusions

In summary, this present study shows that gap junctions are permeable to a variety of quite different metabolites and taken together with other studies, suggests that these junctions are freely permeable to all small substances. Thus their prime function in non-excitatable tissues may be to integrate the metabolic activities of the cells. For excitatable tissues rich in gap junctions, such integration may be as equally as important as their role as synapses. The ability of gap junctions to integrate cellular activities and act as synapses may explain their ubiquity in multicelled animals. Integration of cellular activities by gap junctions may have evolutionary significance as it may allow for considerable versatility in tissue function and organization.
Chapter 6  A CHARACTERISATION OF THE THYMIDINE INCORPORATION OF BHK CELLS AND TWO OF ITS TK- DERIVATIVES
6.1. Introduction and Results

Collaborative experiments carried out with Professor J. D. Sheridan showed that in mixed cultures of HGPRT- BHK cells and wild-type BHK cells, the mutant cells responded as wild-type cells to high concentrations of exogenous hypoxanthine due to the presence of permeable junctions between the cells (Sheridan et al., 1975 and manuscript in preparation). A similar situation may occur between TK- mutants and wild-type BHK cells.

Tissue culture cells can be blocked in S-phase by the presence of high concentrations of thymidine in the growth medium (Xeros, 1962). This is due to an expansion of the thymidine nucleotide pool inside the cell which feeds back on the synthesis of deoxycytidine triphosphate resulting in a shortage of the latter nucleotide for DNA synthesis (Gentry et al., 1965). The cells become arrested in S-phase and so are unable to proceed into G2 and ultimately cell division. TK- mutant cells should not be affected by high levels of thymidine as they are unable to synthesise thymidine nucleotides from thymidine. However, in co-culture with wild-type cells, it would be predicted that the TK- cells will likewise be blocked in S-phase due to the wild-type cells sharing their expanded thymidine nucleotide pool with the TK- mutants through permeable junctions.

To test this prediction BHK and TK- BHK cells (B1) were seeded into 50 mm dishes either separately or together in a 1:1 ratio at a cell
density of $5 \times 10^5$ cells/dish. The cultures were grown either in the presence (10 mM) or absence of thymidine added to the medium and the cell density measured after 24 and 48 h of growth (figure 18).

In the presence of thymidine the growth of the BHK cells and the Bl cells in mixed culture with BHK cells is inhibited to the same extent. Quite surprisingly the growth of Bl cells in pure culture is similarly inhibited by the added thymidine suggesting that they can incorporate thymidine into thymidine nucleotides as efficiently as the BHK cells. However, Pitts (1971) found by autoradiography that Bl cells do not detectably incorporate thymidine into cellular material. These two different results perhaps can be explained by the difference in the exogenous concentrations of thymidine used. For Pitts' experiments the concentration of thymidine in the culture medium was less than $10^{-6}$ M whereas for these experiments the concentration was 10 mM (i.e. $10^4$ fold higher). Nevertheless, the inhibition of the growth of Bl cells by thymidine was not expected. Therefore a number of experiments were carried out to characterize this aspect of the phenotypes of Bl cells and BHK cells. Also, another TK$^-$ mutant (2601; a subline of PyBHK cells) was used because it was reported that these cells were resistant to high concentration of 5-bromodeoxyuridine* (3 mM; Dr. C. Slack, personal communication). These cells thus should serve as a useful comparison.

* This nucleoside is an analogue of thymidine and is often used to select for TK$^-$ mutants.
As the mechanism of inhibition of DNA synthesis by high concentrations of exogenous thymidine is believed to be by an expanded intracellular thymidine nucleotide pool, the incorporation of $\text{[^3H]}$-thymidine into the acid soluble fraction of BHK, Bl and 2601 cells was first investigated. Cultures of these cells were established in separate 50 mm dishes (5 x $10^5$ cells/dish) and after 16 h growth the old medium was discarded and fresh medium containing various concentrations of thymidine plus $\text{[^3H]}$-thymidine was added. The cells were grown for a further 1 h before measuring the incorporation of radioactivity into the acid soluble fraction. The results are shown in figure 19.

It can be seen that with increasing concentrations of thymidine there is a proportionate increase in the amount of tritium counts in the acid soluble fraction for all three cell types. The amount of incorporation of $\text{[^3H]}$-thymidine over the range of the exogenous concentration (0.1 mM to 10 mM) of this nucleoside is similar for both wild-type BHK cells and the two TK$^{-}$ cells. Thus it appears that these two TK$^{-}$ cells can incorporate thymidine as efficiently as the wild-type BHK cells at high exogenous concentrations of thymidine resulting in a large increase in the size of the thymidine nucleotide pool derived from exogenous sources. This explains why the growth of the Bl cells was inhibited in the earlier experiment.

A second set of experiments was now carried out to study the incorporation of both thymidine and its analogue 5-bromodeoxyuridine into the acid insoluble fraction (i.e. DNA) of these cells. A wider
range (0.001 mM to 10 mM) of concentrations of these two nucleosides was used than in the above experiment so as to cover the possible concentrations of thymidine where it is known that the B1 cells incorporate thymidine much less efficiently than the wild-type BHK cells (Pitts, 1971). Growth and labelling the cells was carried out as described above. The results are shown in figure 20.

Again, with increasing concentrations of these two nucleosides there is an increasing amount of incorporation of tritium activity into the acid insoluble fraction of all three cell types. However, for the wild-type BHK cells the increase in incorporation is not initially directly proportional to the increase in exogenous concentration of the two nucleosides and it is only above 1 mM concentrations where there is direct proportionality between the two. This distinguishes the wild-type cells from the two mutant cells as the incorporation of either nucleoside by the wild-type cells is much higher at the lower exogenous concentrations of the two nucleosides.

It is also seen that the incorporation of 5-bromodeoxyuridine by 2601 cells is nearly as high (65%) as that of the wild-type BHK cells at a concentration of this analogue (3 mM) at which these former cells can survive (Dr. C. Slack, personal communication).

There is still a sizeable incorporation of $[^3H]$-thymidine into acid insoluble material (DNA) of all three cell types at concentrations of the nucleoside known to block S-phase. This suggests that there is a sufficient amount of deoxycytidine nucleotides present inside
these cells to allow near normal levels of DNA synthesis for at least 1 h. Of course, it is possible that some of the deoxycytidine nucleotides could be provided by intercellular transfer from these cells not in S-phase which may possess residual constitutive pools of these nucleotides.

To further confirm that high levels of exogenous thymidine can inhibit DNA synthesis in TK" cells, the incorporation of another DNA precursor was measured. Growing BHK, B1 and 2601 cells were seeded into 50 mm dishes (5 x 10^5 cells/dish) and after 4 h growth the medium was changed for fresh medium containing various concentrations of added thymidine. The cultures were grown for a further 16 h before pulsing with [3H]-deoxyguanosine and measuring the incorporation of tritium counts into the acid insoluble fraction. The results are shown in figure 21.

It can be seen that in all three cell types there is a progressive fall, possibly exponential at higher concentration (about 0.1 mM), in the incorporation of [3H]-deoxyguanosine into cellular material. This is consistent with increasing concentrations of exogenous thymidine progressively inhibiting DNA synthesis. Thus, it seems reasonable to believe that high levels of exogenous thymidine can inhibit DNA synthesis in both wild-type BHK cells and in these two types of TK" cells.
6.2. Discussion

The data show that at high exogenous concentrations of thymidine (i.e. above 0.01 mM) or its analogue 5-bromodeoxyuridine, B1 and 2601 cells incorporate both nucleosides into cellular material to the same extent as wild-type BHK cells resulting in the same metabolic consequences. That is, a thymidine nucleotide pool expansion, reduced incorporation of deoxyguanosine into cellular material and, for at least the BHK and B1 cells, the loss of growth. These consequences are all consistent with the belief that DNA synthesis can be inhibited in all three cell types at high concentrations of thymidine in the growth medium. However, the phenotypes of the B1 and 2601 cells manifest themselves at the lower concentrations of thymidine and 5-bromodeoxyuridine (less than 0.1 mM) by having a much reduced incorporation of these two nucleosides than the wild-type BHK cells.

It might be thought that some of these results could be brought about by some toxic contaminant in the thymidine reagent. However, such a contaminant could not explain the level of incorporation of thymidine and 5-bromodeoxyuridine by the two types of TK" cells reaching the wild-type BHK level.

Thymidine is not phosphorylated by other pyrimidine kinase (Sköld, 1959; Durham and Ives, 1970) and therefore its incorporation into cellular material of B1 and 2601 cells must be through thymidine kinase. Thus, the mutation in both these cells could lie either
in the thymidine kinase or in the transport of thymidine into the cell. Mutants defective in either of these two genetic loci have been isolated (Kit et al, 1963; Breslow and Goldsby, 1969). The most likely explanation of the above data is that the mutant thymidine kinase or thymidine transport system of Bl and 2601 cells has an altered affinity for thymidine, i.e. a Km mutant. It is not known if other so called TK^- cells are Km mutants.

The result of 2601 cells incorporating 5-bromodeoxyuridine at an exogenous concentration of 3 mM into cellular material at near the wild-type rate and yet still able to survive at this level of the analogue (Dr. C. Slack, personal communication) could be explained in two ways. Davidson and Bick (1974) have isolated a mutant cell dependent on the analogue for growth, which has thymidine kinase activity, and it would seem likely that a mutation elsewhere, such as in the DNA polymerase and transcription system, would allow for 5-bromodeoxyuridine incorporation into DNA with no adverse effects. An alternative explanation is that the DNA repair mechanism of these cells is very efficient. This second explanation can be tested as it predicts that after pulsing with [^3H] 5-bromodeoxyuridine the activity in the acid insoluble fraction will fall over the next generation period.

Thymidine kinase rises sharply during S-phase (Littlefield, 1966b) and is also encoded on the Herpes virus genome (Keir, 1968) which suggest it is an important enzyme in DNA synthesis (Howard et al, 1974). Total loss of the enzyme may therefore be lethal. The results
with 2601 and B1 cells support this as they still appear to have enzymic activity. Also, this would explain why the mutant cells isolated by Davidson and Bick (1974) and possibly 2601 cells, have developed elaborate systems to survive in the presence of 5-bromo-deoxyuridine whilst retaining thymidine kinase activity.
Growing BHK and B1 cells (section 2.2.3.) were seeded on to separate 50 mm culture dishes or on to the same 50 mm culture dishes in a 1:1 ratio (final cell density in each case was $5 \times 10^5$ cells/dish). The medium ($EFC_{10}$) in each dish was adjusted to a final volume of 3 ml and the cultures grown for 4 h at $37^\circ$C. The medium in half the cultures was then changed for fresh $EFC_{10}$ containing 10 mM thymidine. Sample cultures were removed at this time to allow an estimation of the initial cell density using a Coulter Counter (section 2.2.2.). The cultures were at $37^\circ$C and sample cultures were removed at daily intervals for an estimation of cell density/dish. Each data point is the average of 3 replica cultures.
Figure 18 continued/

Number of cells/dish $\times 10^{-6}$

- BHK (-Tdr)
- Bl (-Tdr)
- BHK/Bl (-Tdr)
- BHK (+Tdr)
- Bl (+Tdr)
- BHK/Bl (+Tdr)

h of growth after addition of Thymidine (Tdr)
Growing BHK, B1 and 2601 cells (section 2.2.3.) were seeded on to separate 50 mm culture dishes (5 x 10^5 cells/dish) in EPC_10 (3ml/dish) and grown at 37°C for 16 h. The medium in all the cultures was removed and fresh medium containing various concentrations of thymidine and [\textsuperscript{3}H]-thymidine (sp. act. at each thymidine concentration was 0.65 mCi/mmol) was added to half of the cultures and to the remaining half of the cultures fresh medium containing various concentrations of thymidine was added. The cultures were grown for 1 h at 37°C after which the labelled cultures were used to estimate the incorporation of thymidine into acid soluble cellular material (sections 2.2.9. and 2.2.10.) and the unlabelled cultures were estimated for protein after TCA extraction (sections 2.2.9. and 2.2.11.) For each estimation of thymidine incorporation and each estimation of protein 3 replica cultures were used.
Figure 19 continued/

Exogenous thymidine concentration (mM) →

CPM/mg protein

10^5

10^4

10^3

0.1 1.0 10.0

B1

BHK

2601
Figure 20 The Increase in Incorporation of $[^{3}\text{H}]$-thymidine and 5-bromo-2'-deoxy$[^{3}\text{H}]$-uridine into Acid Insoluble Material of BHK, B1 and 2601 Cells with Increasing Thymidine and 5-bromodeoxyuridine Concentrations in the Growth Medium

The experiment was carried out as described in the legend to figure 19 except incorporation into acid insoluble cellular material was measured (sections 2.2.9. and 2.2.10.). Also the incorporation of 5-bromodeoxyuridine was measured and a wider range of concentrations of both pyrimidine nucleosides in the growth medium was used. The results are corrected a final specific activity of $[^{3}\text{H}]$-thymidine and 5-bromo-2'-deoxy$[^{3}\text{H}]$-uridine of 0.65 mCi/mmol. For the two lowest concentrations, 0.001 mM and 0.01 mM, the specific activity used was 6.5 mCi/mmol. Each estimation of incorporation and each protein estimation is the average of 3 replica cultures.
Figure 20 continued/

Incorporation of thymidine

<table>
<thead>
<tr>
<th>CPM/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5</td>
</tr>
<tr>
<td>10^4</td>
</tr>
<tr>
<td>10^3</td>
</tr>
<tr>
<td>10^2</td>
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<tr>
<td>10^1</td>
</tr>
</tbody>
</table>

Exogenous thymidine concentration (mM)

BHK, 2601, B1
Figure 20 continued/

b/ Incorporation of 5-bromodeoxyuridine

CPM/mg protein

Exogenous 5-bromodeoxyuridine (mM)
Growing BHK, B1 and 2601 cells (section 2.2.3.) were seeded on to separate 50 mm culture dishes (5 x 10^5 cells/dish) in EFC\textsubscript{10} (3 ml/dish) and grown for 4 h at 37°C. The growth medium from all the dishes was discarded and fresh EFC\textsubscript{10} containing various concentrations of thymidine was added. The cultures were grown for a further 16 h at 37°C and the medium again changed for fresh EFC\textsubscript{10} containing $[^3\text{H}]$-deoxyguanosine (4 Ci/mmol; 1 µCi/ml). and the appropriate concentration of thymidine in half the cultures and in the other half of the cultures fresh EFC\textsubscript{10} containing just the appropriate concentration of thymidine was added. After 1 h growth at 37°C the incorporation into acid insoluble cellular material was measured in the labelled cultures and the protein was measured in the unlabelled cultures (sections 2.2.9., 2.2.10. and 2.2.11.). Each estimation of incorporation of $[^3\text{H}]$-deoxyguanosine and each protein estimation is the average of 3 replica cultures.
Figure 21 continued

Exogenous thymidine concentration (mM) →

CPM/ mg protein
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