Summary

Interactions between mammalian cells in tissue culture

by James W. Simms, B.Sc.

Summary of a thesis presented for the degree of Doctor of
Philosophy at the University of Glasgow, November, 1973.

In any animal the individual cells must interact to
co-ordinate and control their combined development, growth
and functions. These interactions are both direct, between
adjacent cells, and indirect by means of humoral factors.
Humoral or hormonal responses have been extensively studied,
but direct interactions are only poorly understood.

One type of direct cell-cell interaction recently observed,
both in vivo and in tissue culture, in many animal cells from a
wide variety of species has been termed metabolic co-operation.
Variant cells, lacking an enzyme function such as inosinic
pyrophosphorylase (IPP) or thymidine kinase (TK) and unable to
incorporate a nucleotide precursor, do incorporate the precursor
when cultured in contact with wild-type cells. However, the
change of the variant phenotype is not permanent, and reversion
is observed after separation of the two cell types.

The possible explanations for this phenotypic modification
of variant cells, growing in contact with wild-type cells, fall
into two classes:-

       1) the transfer of the enzyme itself from
wild-type to variant cells or transfer of information (DNA, mRNA)
to allow synthesis of the appropriate enzyme in the variant cells.

       2) the transfer of nucleotides, beyond the
enzyme block, or of nucleic acid from wild-type to variant cells.

In the first class, the ability to incorporate the nucleotide
precursor is transferred, in the second it is not.

However, earlier work showed that, after separation of the

ProQuest Number: 10647549

ProQuest 10647549

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

variant cells from wild-type cells, no enzyme activity could be detected in variant cells. This eliminates explanation 1). It was therefore suggested, as DNA was not thought to be transferred between cells, that the most probable basis of metabolic co-operation was nucleotide transfer. However, a recent report suggested that RNA, but not nucleotides, is transferred between cells, so the clarification of the basis of metabolic co-operation (nucleotide transfer or nucleic acid transfer) required further investigation. In this present study the nature of the molecules which can be transferred from cell to cell in tissue culture has been examined in detail. Radioactive tracers have been used to prelabel specific classes of molecules in donor cells, and the transfer of these labelled molecules to unlabelled recipient cells during co-culture has been investigated by autoradiography.

When $[^3H]$ uridine is used to label the uridine nucleotides and the RNA of donor cells, and such donor cells are co-cultured with unlabelled cells, label is transferred to the recipient cells. However, if the labelled uridine nucleotide pool is chased into RNA, prior to addition of recipient cells, the extent of label transferred to recipient cells is reduced by 97%. The label still transferred (3%) was shown to be due to transfer of residual labelled nucleotide pool in the donor cells, produced by RNA turnover. This strongly suggests that RNA is not transferred from cell to cell, but that RNA precursors are. These precursors must be nucleotides, and not nucleosides, to explain the observations on metabolic co-operation.

A similar conclusion can be drawn from quantitative observations of the effect of actinomycin D on the transfer of label from $[^3H]$ uridine labelled donor cells to unlabelled recipient cells. Actinomycin D prevents the appearance of labelled RNA in recipient cells by blocking the synthesis of RNA from the

transferred nucleotides.

Similar observations on the transfer of label from $[^3H]$ thymidine labelled donor cells to unlabelled recipient cells, before and after a chase to reduce the label in the DNA precursor pools, showed that thymine nucleotides, but not DNA, are transferred between cells.

When donor cells were prelabelled with glucose-2-H^3, labelled acid soluble pools, but not labelled acid insoluble material, were transferred to recipient cells. This suggests that phosphorylated sugars are also transferred between cells.

Using donor cells prelabelled with $[^3H]$ amino acids, it was also shown that protein, like RNA, is not transferred in detectable amounts from donor to recipient cells.

To summarise, nucleotides, but not RNA, DNA or protein, appear therefore to be transferred between cells. However, nucleotides cannot cross the cytoplasmic membrane and it is necessary to propose that intercellular junctions are formed with modified permeability properties.

Using RNA and protein inhibitors (actinomycin D and cycloheximide respectively) to study the mechanism required for metabolic co-operation, it was shown that neither prior synthesis nor continuous synthesis of either RNA or protein is required for nucleotide transfer to occur. This suggests that a stable determinant(s) in or on the cell membrane is involved in junction formation.

It has been shown that it is possible to locate the chromosomal locus (loci) for the protein(s) required for metabolic co-operation by the analysis of mouse-human hybrid cells.

Intercellular junctions could play an important role in co-ordination in multicellular systems by allowing free passage of small control molecules such as cyclic nucleotides. Junctions

with properties such as these have already been invoked as a necessary route of intercellular communication during embryonic development. Further work is required to define more clearly the nature and properties of these junctions and to establish their functions.

Interactions between mammalian cells in tissue culture
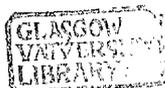
by

James W. Simms, B.Sc.

Thesis presented for the degree of

Doctor of Philosophy,

The University of Glasgow

Department of Biochemistry,
November, 1973.

TO MY PARENTS

Acknowledgements

Abbreviations

These are laid down in the Biochemical Journal Instructions to Authors (revised, 1973) with the following additions:-

APP   Adenine pyrophosphorylase.

BSS   Balanced Salt Solution.

Con A   Concanavalin A.

G-6-PD   Glucose-6-phosphate dehydrogenase.

IPP   Inosinic pyrophosphorylase.

Pancreatic RNase   Pancreatic ribonuclease.

PHA   Phytohaemagglutinin.

PHMB   p-Hydroxymercuribenzoate.

SDS   Sodium dodecyl sulphate.

TCA   Trichloroacetic acid.

TK   Thymidine kinase.

Cells (for details see Materials & Methods).

BHK   Baby hamster kidney cells.

PyBHK   Polyoma virus transformed BHK cells.

3T3   a mouse cell line.

SV3T3 SV_{40} transformed 3T3 cell line.

L   L929; mouse fibroblast cell line.

HeLa   Human epithelial cell line.

CONTENTS

1. INTRODUCTION

1. General Introduction.

Interactions between cells are required for the normal development, and for the continued normal functioning of multicellular organisms.

Such interactions between cells can be classified as either direct or indirect. A direct interaction requires cell-to-cell contact, and may be mediated by membrane-membrane interactions or by transfer of molecules from cell to cell. An indirect interaction is mediated by a molecule which is released from a cell into the extracellular fluid, and this either diffuses to, or is transported to, a target cell. Although many of the interactions between cells in a multicellular organism are lost when cells are grown in tissue culture, direct and indirect interactions between these cells are still observed (Stoker, 1967b).

Many of the molecules (e.g. hormones) which mediate indirect interactions between cells have been isolated and characterised, but the nature of the molecules which mediate direct interactions between cells is unknown. However, studies on two examples of direct interaction, the formation and function of low resistance junctions between cells and metabolic co-operation between cells, are helping to elucidate the types of molecules which can be transferred directly between cells.

1.1. Low resistance junctions between cells.

The flow of electrical current directly between the cytoplasms of contiguous cells has been detected by electrophysiological methods. It is mediated by a pathway (junction) between the cells which has a much lower resistance to ion flow than the cytoplasmic membrane (Loewenstein & Kanno, 1964).

Low resistance junctions between cells, which were first described between excitable cells such as nerve cells (Furshpan &

Potter, 1959), smooth muscle cells and heart muscle cells (see Bennett et al., 1967a), can provide rapid co-ordination of electrical activity in these tissues.

Low resistance junctions are different from chemical synapses, which also transmit electrical impulses between excitable cells. Unlike excitation via low resistance junctions, no direct transfer of electric current across the chemical synapse can be detected (Castillo & Katz, 1954; Hagiwara & Tasaki, 1958), and a special chemical transmitter, which is not transferred directly between the cells but diffuses across the synaptic cleft (Katz, 1966) from the excited cell to the adjacent cell, has been shown to mediate the impulse.

Low resistance junctions have also been detected between non-excitable cells, both in embryonic and adult tissues and in tissue culture.

1.1.1. Low resistance junctions between non-excitable cells.
1.1.1.1. Low resistance junctions in embryonic and adult tissues.

Studies with embryonic tissues (Potter et al., 1966; Ito & Hori, 1966; Sheridan, 1966; Slack & Palmer, 1969; Tupper et al., 1970) show that low resistance junctions are formed extensively throughout the early embryo, although at later stages of growth many of the tissues previously coupled may no longer be coupled (Potter et al., 1966).

Studies with adult tissue have also shown that low resistance junctions are commom features in many tissues from widely different origins (Table 1).

Table 1.

Interaction between cells in adult tissues.

Cell type	Low resistance junctions	Dye transfer	Gap junctions
Glial cells of leech	+[a]		
Drosophia salivary glands	+[b]	+[b]	
Toad urinary bladder	+[c]		
Mammalian liver (mouse)	+[d]		+[j]
Amphibian skin	+[e]		
Mouse brown fat cells	+[f]	+[g]	+[f,k]
Newt white fat cells	+[g]	+[g]	
Chironomus salivary glands	+[c]	+[h]	+[h]

a: Kuffler & Potter (1964); b: Loewenstein & Kanno (1964);

c: Loewenstein et al. (1965); d: Penn (1966); e: Loewenstein &

Penn (1967); f: Revel & Sheridan (1968); g: Sheridan (1971a);

h: Rose (1971); j: Revel & Karnovsky (1967); k: Revel et al. (1971).

1.1.1.2. Low resistance junctions between cells in tissue culture.

Many cell types growing in tissue culture have been shown to be electrically coupled. These include primary cells of spleen, kidney and heart from new born mice, diploid human lung fibroblasts, diploid minnow fibroblasts (Furshpan & Potter, 1968), and others shown in Table 2. O'Lague et al. (1970), using chick embryo fibroblasts, have shown that mitotic cells, in contact with interphase cells, are electrically coupled, suggesting that coupling persists throughout mitosis.

Interaction between cells of different strains and species has also been studied in tissue culture. Furshpan & Potter (1968) showed electrical coupling between BHK cells (a baby hamster kidney cell line isolated by Macpherson & Stoker, 1962) and PyBHK cells (polyoma virus transformed BHK cells); between 3T3 cells (a mouse fibroblast cell line isolated by Todaro & Green, 1963) and PyBHK cells; and between BHK cells and polyoma virus transformed 3T3 cell. Michalke & Loewenstein (1971) showed electrical coupling between rabbit lens epithelial cells (lens) and rat liver epithelial cells (liver), between lens cells and BHK cells, between lens cells and SV3T3 cells, and between liver cells and BHK cells. Therefore, both intraspecific and interspecific mixtures of cells in tissue culture have been shown to be electrically coupled.

1.1.1.3. Low resistance junctions between normal cells and

tumour cells.

Loewenstein & Kanno (1966, 1967) reported that although low resistance junctions were formed in vivo between rat liver cells, they were not detected in primary or transplantable tumours of rat liver. Also, it was reported that cells in tumours of thyroid epithelium (Jamakosmanovic & Loewenstein, 1968a,b), and in tumours

Table 2.

Interaction between cells in tissue culture.

Cell type	Low resistance junctions	Fluorescein transfer	Gap junctions	Metabolic co-operation
BHK/BHK	+[a]	+[g]	+[h]	+[k]
PyBHK/PyBHK	+[a]	+[g]		+[k]
3T3/3T3	+[a]			+[l]
SV3T3/SV3T3	+[a]			+[l]
Chinese hamster cells	+[b]		+[b]	+[b]
L/L	-[b]		-[b]	-[b,k]
Chick embryo	+[c]		+[j]	+[k]
RSV-chick embryo	+[j]		+[j]	+[k]
Liver epithelial cells/PyBHK	+[d]	+[d]		+[d]
Liver tumour epithelial cells/PyBHK	-[d]	-[d]		-[d]
Novikoff hepatoma cells	+[e]	+[e]	+[e]	
Reaggregated cells from Xenopus laevis embryo	+[f]	+[f]		

a: Potter et al. (1966); b: Gilula et al. (1972); c: O'Lague et al. (1970); d: Azarnia et al. (1972); e: Johnson & Sheridan (1971); f: Sheridan (1971b); g: Furshpan & Potter (1968); h: Revel et al. (1971); j: Pinto da Silva & Gilula (1972); k: Pitts (1972); l: Pitts (Personal communication).

For abbreviations see Page iii; also, RSV-chick embryo = Rous sarcoma virus transformed chick embryo cells.

of stomach epithelium (Kanno & Matusi, 1968), lack low resistance junctions although they are present in normal thyroid and normal stomach epithelium.

Borek et al. (1969), in a more detailed study of normal and tumour cells in tissue culture, assayed normal fibroblasts, normal epithelial cells, tumour fibroblasts and tumour epithelial cells for low resistance junctions. They found that both normal fibroblasts (3T3, hamster embryo and rat) and tumour fibroblasts [Novikoff (rat), hamster embryo (x-ray induced), cells derived from SV40 induced hamster tumours and SV3T3 cells] were electrically coupled, in agreement with Potter et al. (1966). However, although normal epithelial cells (rat liver and hamster embryo) were electrically coupled, tumour epithelial cells [Morris (rat liver), H$_4$11EC (rat liver) and hamster embryo (x-ray induced)] were not, in agreement with the in vivo results of Loewenstein & Kanno (1966, 1967).

Therefore, it appears from studies, in vivo and in vitro, on cells from many different species, that, in general, normal fibroblasts, tumour fibroblasts and normal epithelial cells do form low resistance junctions, whereas tumour epithelial cells do not form low resistance junctions.

1.1.2. Transfer of dyes between cells.

The transfer of molecules, larger than the ions involved in current flow, between cells has been studied. The method used involves injection of a tracer molecule into a cell and studying its transfer to surrounding cells. Fluorescein (mol.wt.332) has been widely used as such a tracer, since it is detectable by fluorescence on excitation with ultra-violet light at concentrations of 10^{-8} molar (Loewenstein & Kanno, 1964; Slack & Palmer, 1969). Transfer of fluorescein has been observed between

cells in vivo in Drosophila salivary glands (Loewenstein & Kanno, 1964), in crayfish (Pappas & Bennett, 1966), in lobsters (Furshpan & Potter, 1968), in Chironomus salivary glands (Rose, 1971), and between cells in tissue culture (Table 2). Electrical coupling, when tested for, was present in the cell populations studied above. Slack & Palmer (1969), however, reported that, although cells in the early embryos of Xenopus laevis were electrically coupled, transfer of fluorescein between the cells was not observed.

Another tracer widely used is Procion Yellow (mol.wt.500). Transfer of Porcion Yellow was observed in vivo between cells of crayfish septate axon (Payton et al., 1969), between cells of dogfish retina (Kaneko, 1971), between cells of Chironomus salivary gland (Rose, 1971), and in vitro between cells of Novikoff hepatoma (Johnson & Sheridan, 1971). Electrical coupling between all the cells studied above was also demonstrated.

When cells are immersed in either fluorescein (Johnson & Sheridan, 1971; Rose, 1971) or Procion Yellow (Johnson & Sheridan, 1971; Payton et al., 1969), no detectable dye penetrates into the cells, although Procion Yellow strongly stains the plasma membrane (Payton et al., 1969). This shows that the cytoplasmic membrane has a very limited permeability to these tracers. The requirement for cell-cell contact for the transfer of fluorescein between cells has been emphasised by Furshpan & Potter (1968). They observed that the transfer of fluorescein between BHK cells was never seen in the absence of cell contact, but that effective spread was seen between cells with only small areas of contact.

Kanno & Loewenstein (1966) studied the transfer of a range of tracers, with different molecular weights, between Drosophia salivary gland cells which were electrically coupled. When fluorescein (mol.wt.332), orange G (mol.wt.452), Solantine

turquoise (mol.wt.700), trypan blue (mol.wt.960) or Evans blue (mol.wt.961) was injected into a cell, the tracer moved to surrounding cells within minutes. Also, they reported that serum albumin (mol.wt.69,000), conjugated with fluorescein, spread slowly between cells, although polylysine (mol.wt.127,000) was not transferred. However, Bürk has suggested that it was the transfer of fluorescein, and not the conjugated complex between cells, which was detected (Pitts, 1971).

Reese et al. (1971) reported that although fluorescein (mol.wt.332) and microperoxidase (mol.wt.1800) could cross the septum of crayfish (electrically coupled), horseradish peroxidase (mol.wt.40,000) could not.

It appears, therefore, that fluorescein and possibly larger molecules can be transferred between cells, and that there may be a correlation between dye transfer and the presence of low resistance junctions between cells.

1.1.3. Nature of the junctions involved in ion transfer and dye transfer between cells.

Although various types of contacts between cells have been observed in thin sections, using the electron microscope (Furshpan & Potter, 1968), examination of excitable tissues, known to be electrically coupled, has shown that tight junctions were the only forms of specific contact between cells seen invariably in all the coupled tissues studied (Bennett et al., 1967a).

Revel & Karnovsky (1967), using alkaline lanthanum nitrate as a marker for extracellular space, could distinguish between two types of tight junctions:-

a) true tight junctions, where no interstitial space is present, and where the external layers of the two unit membranes appear to be fused, and

b) gap junctions where the two unit membranes are separated by a $20A^o$ gap, the gap being detected by the presence of a thin layer of lanthanum. The gap is also characterised by the presence of a polygonal array of units, which are seen in the occasional tangentially cut gap junction.

The lanthanum tracer technique has been used to investigate the nature of the contacts between cells, which can form low resistance junctions and exchange dyes. Payton et al. (1969) showed that Procion Yellow is transferred between septate axons of the crayfish, previously shown to be electrically coupled (Watanabe & Grundfest, 1961; Bennett et al., 1967b). Since there are only small areas where the axons are in contact and not separated by the septal connective tissue, this suggested that both ions and tracer molecules were transferred between axons at these sites. Analysis of electron micrographs of lanthanum treated sections through septa, for junctions between the axons, showed that only gap junctions could be detected. Pappas, Asada & Bennett (1971) reported that a change of coupling resistance between axons could be correlated with either the presence of many gap junctions, only a few, or the complete absence of them.

Johnson & Sheridan (1971) reported that Novikoff hepatoma cells, growing in clumps, or small chains, in suspension culture, were electrically coupled and transferred dye tracers (mol.wt. 332-500). They found that intermediate junctions (zonula adhaerens: unit membranes separated by $200A^o$, with dense, amorphous material on the cytoplasmic surfaces) and gap junctions were the only junctions frequently observed between these cells. They suggested that intermediate junctions were probably partly responsible for the adherence of the cells, and that gap junctions were the sites for ion and tracer transfer.

These reports therefore suggest that gap junctions are the

sites of transfer for both ions and dye molecules between cells.

Although tight junctions and gap junctions may form the main junctional elements of vertebrate epithelial cells (Revel & Karnovsky, 1967; Brightman & Reese, 1969), septate junctions (septate desmosome: unit membranes separated by 150-200A$^{\circ}$ gap but cross-linked by "septa", 60-80A$^{\circ}$ wide with a periodicity of roughly 200A$^{\circ}$) are the main junctional element of many invertebrate epithelial cells (Satir & Gilula, 1970). The septate junction has been suggested as a possible mediator of electrical coupling between mussel gill cells (Satir & Gilula, 1970) and between Chironomus salivary gland cells (Bullivant & Loewenstein, 1968) where gap junctions were not observed. However, after several reports of the presence of gap junctions between other invertebrate cells (see Gilula & Satir, 1971), Gilula & Satir (1971), using a freeze-cleaving technique, which splits the plasma membrane in the plane of the membrane and shows the fracture surfaces in relief by heavy metal shadowing, showed clearly that gap junctions are present between mussel gill cells. Rose (1971), using the lanthanum tracer technique, also reported that gap junctions were present between Chironomus salivary glands, but suggested, however, that gap junctions alone may not be able to account quantitatively for the electrical coupling observed between these cells.

However, Revel et al. (1971), using the freeze-cleaving technique to study junctions between BHK cells and between brown fat cells, both of which are electrically coupled, found that only gap junctions are present. This strongly suggests that gap junctions are the sites of electrical coupling between cells.

Gilula et al. (1972) have emphasised the correlation between the presence of gap junctions and electrical coupling between cells. Gilula et al. (1972) reported that Chinese hamster cells were

electrically coupled, and that several types of interactions
(gap junctions, possible focal tight junctions, close associations
of the unit membranes ($<130A^{o}$), microvillar interactions and
pinocytosis) were present between these cells. L cells, on the
other hand, were not electrically coupled with each other or with
Chinese hamster cells and lacked gap junctions, whereas all other
forms of interaction were still observed.

In summary, it would appear that gap junctions are probably
the sites for ion and dye transfer between cells.

Staehelin (1972) has further shown, using the freeze-cleaving
technique, that there are three types of gap junctions between rat
intestinal epithelial cells. He suggests that these junctions,
which differ in the size of the particles (units) which form the
gap junctions, may control the transfer of different types of
molecules between cells. This observation could therefore explain
the report of Slack & Palmer (1969), who found that, although
early Xenopus laevis embryo cells were electrically coupled, they
did not allow fluorescein transfer.

1.2. Metabolic co-operation between cells.

Metabolic co-operation between cells in tissue culture was first described by Subak-Sharpe et al. (1966). A culture (1:300) of wild-type PyBHK cells and variant PyBHK cells (PyBHK-IPP⁻), lacking the enzyme inosinic pyrophosphorylase (IMP, GMP: pyrophosphate pyrophosphoribosyl transferase; E.C.2.4.2.8.) and therefore unable to incorporate hypoxanthine, was grown in the presence of $[^3H]$hypoxanthine. It was shown by autoradiography that the wild-type cells (heavily labelled) could be easily identified from the variant cells (unlabelled). It was also noted that an intermediate number of grains was present in cells in contact with the heavily labelled wild-type cells. These intermediately labelled cells could either be variant cells, which had become labelled due to interaction with wild-type cells, or wild-type cells whose incorporation of $[^3H]$hypoxanthine had been considerably reduced by interaction with variant cells.

Subak-Sharpe et al. (1969) reported that the number of intermediately labelled cells increased faster than could be accounted for by cell divison of wild-type cells, which suggested that they were variant cells.

Bürk et al. (1968) grew a confluent 1:1 mixture of wild-type cells and variant cells in the presence of $[^3H]$hypoxanthine. Autoradiographs showed that all the cells were labelled. The disappearance of the variant IPP⁻ phenotype in such a mixed culture confirms that IPP⁻ cells become labelled when in contact with wild-type cells. The possibility that a selective death of the variant cells had occurred, leaving only labelled wild-type cells, was ruled out by Pitts (1971). A confluent 1:1 mixture of BHK cells and BHK-IPP⁻ cells growing together were subcultured to form either a confluent culture (99% of the cells), or a sparse culture (1% of the cells), where most of the cells were not in contact. The

cultures were incubated in the presence of $\left[^3H\right]$hypoxanthine.
Autoradiographs showed that although all the cells in the confluent
culture incorporated label, only 50% of the cells in the sparse
culture incorporated label, showing that a selective death of
BHK-IPP⁻ cells had not occurred.

Stoker (1967a) grew a mixed population of PyBHK-IPP⁻ variant
cells, marked with carbon granules (Stoker, 1964), and mouse embryo
cells (wild-type), in the presence of $\left[^3H\right]$hypoxanthine.
Autoradiographs showed that label was present in variant cells in
contact with the heavily labelled wild-type cells, but was absent
from variant cells not in contact with wild-type cells.

It appears, therefore, that the phenotype of certain IPP⁻
variant cells, growing in contact with certain wild-type cells, is
modified. This phenomenon is termed metabolic co-operation.

Metabolic co-operation between IPP⁻ variant cells and
wild-type cells has been reported between normal cells, between
transformed cells, between normal and transformed cells, and between
cells of heterologus species (Tables 3 and 4).

Table 3 also shows that, although in many instances the mutant
phenotype was completely suppressed in 1:1 mixtures of wild-type
and variant cells (100% interaction), examples of both intermediate
interaction (e.g. BSC-1/BHK-IPP⁻) and no interaction (e.g. L/L-IPP⁻)
between cells were also observed. Therefore, metabolic co-operation
between cells ranges from complete interaction (100%) to zero
interaction (0%).

Although most IPP⁻ variant cells used in the above studies
have been isolated by resistance to 6-thioguanine (Subak-Sharpe,
1965; Marin and Littlefield, 1968), skin fibroblasts isolated from
patients with the Lesch-Nyhan syndrome have been shown to have a
IPP⁻ phenotype. A mosaic of IPP⁺ and IPP⁻ cells is present in
cultures of skin fibroblasts taken from heterozygote mothers of

Table 3.

Interaction properties of different cell types.

Wild-type cell.	Variant IPP⁻ cell.	Extent of interaction (%)*
BHK	BHK	100
BHK	3T3	100
RSV-BHK	BHK	100
3T3	BHK	100
SV3T3	BHK	100
Mouse embryo	BHK	100
Human embryonic lung	BHK	100
Hel 2000	BHK	100
Chick embryo	Chick embryo	100
RSV-chick embryo	RSV-check embryo	100
BHK-L hybrid	BHK	100
PyBHK	PyBHK	Intermediate
BSC-1	BHK	55
HeLa	BHK	20
L	L	0
BHK	L	0
L	BHK	0
Mast cell	Mast cell	0

For abbreviations see Page iii; also, RSV-BHK = Rous sarcoma virus
transformed BHK; Hel 2000 = a human fibroblast line; RSV-chick
embryo = Rous sarcoma virus transformed chick embryo cells; BHK-L
hybrid = a hybrid cell line formed by fusion with Sendai virus of
BHK and L-929 cells; BSC-1 = an African green monkey cell line
(Hopps et al., 1963).

    * In a 1:1 mixture, ratio of mean number of grains/variant
cell : mean number grains/wild-type cell.

Data taken from Pitts (1972).

Table 4.

Metabolic co-operation in intra and interspecific mixtures.

Wild-type	Lesch-Nyhan (IPP⁻)	BHK-IPP⁻	L-IPP⁻
Human skin fibroblast	+	+	-
BHK	+	+	-
3T3	+	+	-
Py3T3	+		
Clone 1D	-		
HeLa$_{65}$	-		
HeLa$_{71}$	+		

For abbreviation see P(ii); also, Lesch-Nyhan = Human skin

fibroblast; clone 1D = a variant L-929 cell, lacking thymidine

kinase activity; Hela$_{65}$ and Hela $_{71}$ = two clonal derivatives

of HeLa cells, having modal numbers of 65 and 71 chromosomes

respectively.

Data taken from Cox et al., (1972).

patients with this syndrome (Rosenbloom et al., 1967), although virtually all cells in a mixed confluent culture incorporate $[^3H]$hypoxanthine (Dancis et al., 1969). Since the IPP⁻ gene is X-linked, a random inactivation of one of the X-chromosomes in female somatic cells in early embryonic development (Lyon, 1961) may explain this mosaic pattern.

Variant IPP⁻ cells have been isolated from the mothers of Lesch-Nyhan patients by cloning (Migeon et al., 1968), or by selection with thioguanine (Fujimoto et al., 1971). Metabolic co-operation has been reported between Lesch-Nyhan (IPP⁻) skin fibroblasts and various wild-typecells (Friedmann et al., 1968; Table 4). Also, metabolic co-operation has been reported between the mixed (IPP⁺ and IPP⁻) amniotic cells from a female foetus heterozygous for the Lesch-Nyhan disease (Fujimoto et al., 1968).

Therefore, metabolic co-operation has been shown between wild-type cells and IPP⁻ cells, selected by drugs, and between wild-type cells and naturally occurring IPP⁻ cells.

Pitts (1972) has reported that tumours formed from BHK-IPP⁻ cells in hamsters can incorporate $[^3H]$hypoxanthine, demonstrating metabolic co-operation between cells in vivo.

Therefore, metabolic co-operation between IPP⁻ cells and wild-type cells appears to be a common phenomenon between many different cell lines in tissue culture, and can also be demonstrated in vivo.

1.2.1. Metabolic co-operation between variant cells, lacking adenylic pyrophosphorylase or thymidine kinase activity, and wild-type cells.

Metabolic co-operation between cells is not limited to interaction between IPP⁻ variant cells and wild-type cells.

When a 1:1 mixture of wild-type PyBHK cells and variant PyBHK cells (PyBHK-APP⁻), lacking adenylic pyrophosphorylase activity (AMP: pyrophosphate phosphoribosyl transferase; E.C. 2.4.2.7.) and therefore unable to incorporate adenine, is grown in the presence of $[^3H]$ adenine, all the cells are labelled. This shows that the variant phenotype of APP⁻ cells is modified when growing in contact with wild-type cells (Burk et al., 1968). Subak-Sharpe (1969) confirmed this by growing a (1:300) mixed population of PyBHK wild-type cells and PyBHK-APP⁻ cells in the presence of $[^3H]$ adenine. He found that variant cells in contact with wild-type cells contained label. Cox et al. (1972) have reported metabolic co-operation between PyBHK-APP⁻ cells and human skin fibroblasts, 3T3 cells and BHK cells.

Pitts (1971) has also reported metabolic co-operation between BHK wild-type cells and BHK variant cells (BHK-TK⁻), which lack thymidine kinase activity (E.C. 2.7.1.21.) and cannot incorporate thymidine. When a 1:1 mixed population of wild-type BHK cells and BHK-TK⁻ cells are grown together in the presence of $[^3H]$ thymidine, all cells are labelled, except a small proportion which presumably have not synthesised DNA during the experiment (not passed through the S phase), showing that the phenotype of the variant BHK-TK⁻ cells has been modified.

Therefore, metabolic co-operation between cells has been shown using three different variant cell types. This suggests that metabolic co-operation may be a general phenomenon, at least for variant cells deficient in nucleotide synthesis from base or nucleoside precursors.

1.2.2. Requirement for direct cell-to-cell contact for metabolic

co-operation between cells.

Stoker (1967a) investigated the requirement for direct
cell-to-cell contact between cells participating in metabolic
co-operation. When PyBHK-IPP⁻ cells had attached to a confluent
layer of mouse embryo cells and adjoining areas of glass, which
had been scraped free of mouse embryo cells, the cells were
cultured in the presence of $\left[^3H\right]$hypoxanthine and then processed
for autoradiography. IPP⁻ cells on the bare areas, including those
adjoining but not touching the edge of the confluent mouse embryo
cells, showed only a few grains above background. However, IPP⁻
cells touching the edge or lying on top of the confluent mouse
embryo cells contained a considerable number of grains.

Published autoradiographs of studies on metabolic co-operation
between cells consistently show that variant cells in direct contact
with wild-type cells, or labelled interacting variant cells, are
labelled, but that variant cells, lying close to but not in contact
with labelled cells, are not labelled (Friedmann et al., 1968;
Subak-Sharpe et al., 1969; Subak-Sharpe, 1969).

If direct contact between two interacting cell populations is
prevented, metabolic co-operation between these cells is not
observed. Cox et al. (1970) showed that, although most cells of a
confluent 1:1 mixed monolayer culture of wild-type human skin
fibroblasts and IPP⁻ human skin fibroblasts were labelled in the
presence of $\left[^3H\right]$hypoxanthine, only approximately 50% of the cells
in a similar mixed population were labelled, when grown in
suspension in the presence of $\left[^3H\right]$hypoxanthine.

Van Zeeland et al. (1972) showed that, when a fibrin layer
which is thought to be permeable to molecules, but not to cell
processes, was used to separate human skin fibroblasts from mouse
cells (IPP⁻), metabolic co-operation did not occur between these cells.

Therefore, direct contact between interacting cells appears to be an essential requirement for metabolic co-operation.

1.2.3. Reciprocal exchange between cells participating in metabolic co-operation.

The modification of the phenotype of variant cells by wild-type cells requires transfer of a molecule(s) from wild-type cells to variant cells (see Section 1.2.6.). Metabolic co-operation is therefore based on a donor to recipient relationship. The possibility that a variant cell (recipient) could also function as a wild-type cell (donor) has been investigated.

A 1:1 mixed population of PyBHK-IPP⁻ and PyBHK-APP⁻ cells, growing at a cell density where most cells were in contact, were labelled with either $[^3H]$hypoxanthine or $[^3H]$adenine and processed for autoradiography (Bürk et al., 1968). Irrespective of which labelled purine was used, metabolic co-operation was observed when cells were in contact, indicating that both PyBHK-IPP⁻ and PyBHK-APP⁻ cells can function as donors or recipients, showing a reciprocal exchange between both cell types during metabolic co-operation.

1.2.4. Failure of L cells to participate in metabolic co-operation.

Although most cell types studied to date participate in metabolic co-operation, it is consistently found that L cells do not (Tables 3 & 4).

Metabolic co-operation between L-IPP⁻ variant cells and L-IPP⁺ cells does not occur (Pitts, 1971; Cox et al., 1972; Widmer-Favre, 1972). Pitts (1972) showed that mixed populations of wild-type BHK cells and L-IPP⁻ cells, or wild-type L cells and BHK-IPP⁻ cells, did not show metabolic co-operation; that is, L cells, whether wild-type or variant, are dominant in mixed culture.

This has been confirmed by Cox et al. (1972) for the BHK/L combinations of the above cells. Cox et al. (1972) also showed that mixed populations of wild-type L cells and human skin fibroblast-IPP⁻, or wild-type human skin fibroblast-IPP⁺ and L-IPP⁻ cells, did not interact.

This dominance of L cells is not peculiar to variant IPP^- cells alone. Cox et al. (1972) showed that mixed population of $L-APP^+/L-APP^-$ cells, human skin fibroblast-$APP^+/L-APP^-$ cells, $3T3-APP^+/L-APP^-$ cells and $L-APP^+/PyBHK-APP^-$ cells did not show metabolic co-operation. Also, metabolic co-operation between $L-TK^+/L-TK^-$ cells (Pitts, 1972; Widmer-Favre, 1972), or between human skin fibroblasts-$TK^+/L-TK^-$ (Cox et al., 1972), does not occur.

Therefore, for metabolic co-operation to occur, both the wild-type cells and the variant cells must have the ability to interact. Since mouse embryo fibroblasts interact (Stoker, 1967a; Pitts, 1972), but L cells (mouse fibroblasts) fail to interact with themselves or with other interacting cells, it has been suggested that L cells may fail to form some essential membrane component required for interaction (Pitts, 1971; Cox et al., 1972). McCargow and Pitts (1971) investigated this point using a BHK-L cell hybrid, isolated from a mixture of BHK-TK⁻ and L-IPP⁻ cells fused with inactivated sendai virus. They showed that the hybrid cells interacted with BHK cells but not with L cells, and by using immunological methods, they demonstrated that the hybrid contained both L cell and BHK cell cytoplasmic membrane determinants. This is consistent with the idea that L cells lack some membrane determinant, essential for metabolic co-operation.

1.2.5. Possible involvement of gap junctions in metabolic co-operation between cells.

Evidence presented above (section 1.2.2.) shows that direct cell-to-cell contact is required for metabolic co-operation between cells. Since direct transfer of ions and tracers between cells has been shown to be mediated by gap junctions between cells (Section 1.1.3.), the possibility that metabolic co-operation between cells may use a similar pathway has been investigated.

BHK cells and PyBHK cells which show metabolic co-operation (Subak-Sharpe et al., 1966, 1969; Pitts, 1971, 1972) have both been shown to be electrically coupled and to exchange fluorescein (Furshpan and Potter, 1968). Azarnia et al. (1972) showed that a mixture of liver epithelial/PyBHK cells were electrically coupled, exchanged fluorescein and showed metabolic co-operation. However, a mixture of tumour epithelial/PyBHK cells failed to form low resistance junctions, did not exchange fluorescein and did not show metabolic co-operation. This therefore suggests that a similar pathway may mediate the transfer of all three types of cell-cell interactions.

Gilula et al. (1972) reported a combined study of metabolic co-operation, low resistance junctions and gap junctions between cells (see also Section 1.1.3.). They found that Chinese hamster cells were electrically coupled, showed metabolic co-operation and a number of different types of interaction including gap junctions; but that L cells were not electrically coupled, did not participate in metabolic co-operation and lacked gap junctions, though all the other types of interaction were still observed. This suggests that gap junctions mediate ion transfer, dye transfer and also metabolic co-operation between cells.

1.2.6. The nature of the molecules transferred between cells in

metabolic co-operation.

Explanations (Subak-Sharpe et al., 1966, 1969; Subak-Sharpe, 1969) for the modification of the phenotype of variant cells, growing in contact with wild-type cells, are all based on transfer of a molecule(s) from wild-type cells to variant cells. The transfer of any one of the following classes of molecules could explain metabolic co-operation.

1) transfer of $\left[^3H\right]$ labelled nucleotides, synthesised in wild-type cells, to variant cells and their incorporation into variant cell nucleic acid.

2) transfer of $\left[^3H\right]$ labelled polynucleotides (RNA and/or DNA), synthesised in wild-type cells, to variant cells.

3) transfer of the wild-type enzyme from wild-type cells to variant cells.

4) transfer of informational molecules (mRNA or gene(DNA) coding for the enzyme) from wild-type cells to variant cells.

5) transfer of a "regulator" molecule, required in the variant cell for normal synthesis of the enzyme, from wild-type to variant cells (the site of the molecular lesion resulting in the IPP⁻ phenotype is unknown).

Possibilities 1 & 2 would require that, after separation of interacting wild-type and variant cells, there should be a prompt reversion of the variant cells to the variant phenotype.

Possibilities 3, 4 & 5 would require that, since a functional enzyme would be present in variant cells growing in contact with wild-type cells, wild-type enzyme activity should be present in the variant cells after separation.

Cox et al. (1970) have assayed variant cells after separation

from interacting wild-type cells for enzyme activity. Equal numbers of human Lesch-Nyhan fibroblasts ($\underline{IPP}^-$) and wild-type human fibroblasts were grown together in confluent culture, in unlabelled medium, to allow exchange to take place. A single cell suspension of these cells was formed and incubated with $[^3H]$ hypoxanthine. Autoradiographs showed that only 50% of the cells were labelled, suggesting that either a prompt reversion to the variant phenotype had occured, or that the enzyme activity must be very unstable. To obtain an estimate of the stability of the $\underline{IPP}^+$ enzyme, $\underline{IPP}^+$ cultures were treated with cycloheximide for a period of 12h. It was found that the $\underline{IPP}^+$ activity did not decrease during this time. However, since cycloheximide may inhibit factors that normally degrade the enzyme (Kenney, 1967), cell suspensions of mixed cultures which had undergone metabolic co-operation were incubated in the presence of $[^3H]$ hypoxanthine and cycloheximide (conditions where $\underline{IPP}^+$ activity is stable for at least 12h). A prompt reversion of the variant phenotype was still observed, showing that detectable quantities of the enzyme are not transferred to the variant cells.

Pitts (1971) confirmed this result using BHK cells. A mixed population of BHK-IPP$^-$ and BHK wild-type cells, which had undergone metabolic co-operation, were rapidly subcultured to form a sparse culture (1% of the cells), where most cells were not in contact. Incubation was continued in the presence of $[^3H]$ hypoxanthine. Autoradiographs showed that a prompt reversion of the variant phenotype occurred. Reconstruction experiments, using prolonged autoradiographic exposures, showed that if the BHK-IPP$^-$ cells had incorporated label at the wild-type rate for only 10 minutes, this could have been detected.

Cox et al. (1972) also showed that a prompt reversion of the

variant phenotype occured after separation of a heterospecific

mixture of interacting PyBHK-APP⁻ cells and wild-type human skin

fibroblasts.

Cox et al. (1972) extended the study to examine the

possibility of enzyme transfer using glucose-6-phosphate

dehydrogenase deficient cells (G-6-PD⁻). They analysed a mixed

population (1:100) of wild-type human skin fibroblasts and

G-6-PD⁻ human skin fibroblasts for G-6-PD⁺ activity. A

cytochemical method was used to assay the cells for enzyme activity

and this did not require separation of the cells. The assay

produced large granules of precipitated formazan in G-6-PD⁺ cells,

but not in G-6-PD⁻ cells. G-6-PD⁺ enzyme was not detected in the

variant cells.

Therefore, the available data suggest that a functional

enzyme is not present in variant cells during co-culture with

wild-type cells, suggesting that 3, 4, 5 above are unlikely.

This therefore suggests that 1 & 2 above are the most probable

explanation of metabolic co-operation: the transfer of radioactive

nucleotides and/or radioactive polynucleotides from wild-type cells

to variant cells.

1.2.7. Transfer of nucleotides and/or polynucleotides between cells.

From general considerations of the primary role of DNA in the

cell, it is unlikely that DNA would be transferred to the

significant extent required to explain the level of label present

in BHK-TK⁻ cells, growing in contact with BHK-TK⁺ cells in the

presence of [³H]thymidine (Pitts, 1971). Therefore, this suggests

that transfer of radioactive nucleotides from wild-type cells

to variant cells is the explanation of metabolic co-operation.

Since dyes up to 1000 molecular weight have been shown to be

transferred directly between cells (section 1.1.2.), it is not impossible, on the basis of molecular weight, that nucleotides (mol.wt.300 - 500) could be transferred between cells.

Pitts (1971) showed that, although BHK-IPP$^-$ and BHK-TK$^-$ cells fail to grow when cultured separately in HAT medium (medium containing aminopterin, hypoxanthine and thymidine) because they are unable to synthesise IMP and dTMP respectively, a near-confluent (1:1) mixed population grew at the wild-type rate. This suggests efficient reciprocal exchange and utilisation of nucleotides. Although nucleotides could be transferred from donor cells and incorporated immediately into RNA and DNA in the recipient cells, polynucleotides would have to be first synthesised in donor cells, transferred to recipient cells, degraded to nucleotides, and finally reincorporated into RNA and DNA in the recipient cells. This scheme seems less attractive than the simple exchange of nucleotides.

However, in direct conflict to the suggestion of nucleotide transfer, Kolodny (1971, 1972) reported that RNA, but not nucleotides, was transferred between 3T3 cells and between SV3T3 cells. Kolodny concluded that all major cytoplasmic RNA species, prelabelled in a donor cell with either $\left[^3H\right]$uridine or $\left[^3H\text{-methyl}\right]$ methionine, could be transferred to recipient cells, but that the transfer of nucleotide pools from donor to recipient cells did not occur.

Therefore, although the transfer of radioactive molecules from wild-type cells to variant cells is the most probable basis of metabolic co-operation, the question of the nature of the actual molecules transferred (nucleotides and/or polynucleotides) has not been resolved.

Aim of present work.

This study considers three aspects of direct interaction between cells: 1) the nature of the molecules which are transferred directly from cell to cell in tissue culture, 2) the mechanism of the intercellular transfer of these molecules, and 3) a preliminary genetic analysis of this interaction.

2. MATERIALS AND METHODS

2.1. Materials.

2.1.1. Chemicals.

Actinomycin D and cycloheximide (actidione) were obtained from Calbiochem, London.

Aminopterin, mitomycin C, RNase-A (bovine pancreatic, protease-free: ribonucleate nucleotido-2'-transferase (cyclizing); E.C. 2.7.7.16.), p-hydroxymercuribenzoate (PHMB) and 5-bromodeoxyuridine were obtained from Sigma, St.Louis, Missouri, U.S.A.

Concanavalin A (Con A) was obtained from Miles-Yeda Ltd., Kankakee, Illinois, U.S.A.

Dioxan, sodium dodecyl sulphate (SDS) and 2,5-diphenyloxazole (PPO) were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England.

Gelatine was obtained from Hopkin & Williams Ltd.

Hyamine hydroxide (1M-solution in methanol) and naphthalene were obtained from Nuclear Enterprises Ltd., Edinburgh.

Hydroxyurea was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Metol and Photo-Flo were obtained from Kodak Ltd., London.

Nupercaine was obtained from Ciba Laboratories, Bucks., England.

Phytohaemaggutinin (PHA) was obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

Other chemicals were obtained from either British Drug Houses Ltd., London or Sigma, St.Louis, Missouri, U.S.A.

2.1.2. Radiochemicals.

All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., England, and included D-$[2\text{-}H^3]$ glucose, 500 mCi/mmole; $[^3H]$hypoxanthine (G), 1 Ci/mmole; L-$[4,5\text{-}H^3]$ leucine, 36 Ci/mmole; L-$[methyl\text{-}H^3]$ methionine, 5 Ci/mmole; $[methyl\text{-}H^3]$ thymidine, 22.4 Ci/mmole or 27.7 Ci/mmole; $[5\text{-}H^3]$ uridine, 30 Ci/mmole.

2.1.3. Biological materials.

2.1.3.1. Tissue culture cells.

Two variant cell lines (Marin & Littlefield, 1968) of the BHK-21/C13 cell line (baby hamster kidney fibroblasts; Macpherson & Stoker, 1962) were used:-

1) BHK-IPP⁻ cells (TG$_2$), BHK cells which lack inosinic pyrophosphorylase (IMP, GMP: pyrophosphoribosyl transferase; E.C. 2.4.2.8.) and

2) BHK-TK⁻ cells (B$_1$), BHK cells which lack thymidine kinase (ATP: thymidine 5'-phosphotransferase; E.C. 2.7.1.21.).

A variant cell line (Littlefield, 1966) of the L929 cell line (a mouse fibroblast; Sanford et al., 1948), L-TK⁻ (B82), which lacks thymidine kinase, was also used.

These variant cell lines were kindly provided by Dr. J.W. Littlefield, Massachusetts General Hospital, Boston, Mass., U.S.A.

HeLa cells, a human epithelial cell line (cervical carcinoma; Gey et al., 1952), 3T3 cells (a mouse fibroblast line; Todaro & Green, 1963) and L-929 cells were supplied by the Wellcome Tissue Culture Unit, Department of Biochemistry, University of Glasgow.

H116 pass 15, a human embryo lung fibroblast line, and clones 3 & 201, isolated from the inactivated Sendai virus induced

fusion of Hl16.15 and L-IPP⁻ cells (A9; Littlefield, 1966) were
kindly provided by Dr. Gormley, MRC Clinical & Population
Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh.

2.1.3.2. Cell culture.

Amino acids, vitamins, calf serum, foetal calf serum and
minimum essential amino acids lacking methionine or leucine, were
obtained from Bio-cult Laboratories, Glasgow.

Penicillin and streptomycin were obtained from Glaxo
Laboratories Ltd., Greenford, Middlesex, England.

Trypsin (E.C. 3.4.4.4.) was obtained from Difco Laboratories,
Surrey.

2.1.4. Solutions.

Amfix
solution contained 20% (v/v) conc.
Amfix (May & Baker, Dagenham,
England) in water.

Balanced Salt Solution (BSS)
had the composition shown in Table 5.

Dioxan based scintillation fluid
contained 10% naphthalene + 0.7%
2,5-diphenyloxazole (PPO) in
1,4-dioxan (A.R.).

D19b
developer had the composition
shown in Table 6.

Eagle's medium
had the composition shown in Table 7.

Formol saline
solution contained 0.08M-NaCl,
0.1M-Na_2SO_4 in 4% (v/v) formaldehyde.

Gelatine-chrome alum
solution had the composition shown
in Table 8.

Gimesa
stain contained 0.75% Gimesa
(Gurr, London) in glycerol-methanol
(1:1, v/v).

<u>Toluene based scintillation fluid</u>   contained 0.5% 2,5-diphenyloxazole

(PPO) in A.R. toluene.

<u>Trypsin</u>   solution had the composition

shown in Table 9.

2.1.5. Cell culture media.

EC 10 contained Eagle's medium + calf serum (9:1).

EFC 10 contained Eagle's medium + foetal calf serum (9:1).

0%, 1% & 5% normal glucose medium contained Eagle's medium

with either 0%, 1% or 5% normal glucose conc. respectively.

1%, 10% & x10 normal leucine medium contained Eagle's medium

with either 1%, 10% or x10 normal leucine conc. respectively.

1% & x10 normal methionine medium contained Eagle's medium

with either 1% or x10 normal methionine conc. respectively.

Table 5

Balanced Salt Solution(BSS)

NaCl	6.8g
KCl	0.4g
$CaCl_2.6H_2O$	0.393g
$MgSO_4.7H_2O$.	0.2g
$NaH_2PO_4.2H_2O$	0.14g
$NaHCO_3$	2.2g
Phenol red	0.015g
Total volume	1 litre

Table 6

D19b

$Na_2SO_3.7H_2O$	144g
Na_2CO_3	48g
KBr	4g
Hydroquinone	8.8g
Metol	2.2g
Total volume	1 litre

Table 7

Eagle's medium

NaCl	6.8g
KCl	0.4g
$CaCl_2.6H_2O$	0.393g
$MgSO_4.7H_2O$	0.2g
$NaH_2PO_4.2H_2O$	0.14g
$NaHCO_3$	2.2g
Glucose	4.5g
L-arginine.HCl	0.0421g
L-cystine	0.0240g
L-glutamine	0.2920g
L-histidine.HCl	0.0192g
L-isoleucine	0.0525g
L-leucine	0.0525g
L-lysine.HCl	0.0731g
L-methionine	0.0149g
L-phenylalanine	0.0330g
L-threonine	0.0476g
L-tryptophan	0.0082g
L-tyrosine	0.0362g
L-valine	0.0469g
D-calcium pantothenate	0.002g
Choline chloride	0.002g
Folic acid	0.002g
i-inositol	0.004g
Nicotinamide	0.002g
Pyridoxal.HCl	0.002g
Riboflavin	0.0002g
Thiamine.HCl	0.002g
Penicillin	100,000 units
Streptomycin	0.1g
Phenol red	0.015g
Total volume	1 litre

Table 8

Gelatine-chrome alum

Gelatine	5g
Chrome alum ($CrK(SO_4)_2 \cdot 12H_2O$)	0.5g
Formaldehyde (40% (v/v) solution)	5ml
Photo-Flo	1ml
Total volume	1 litre

Table 9

Trypsin solution

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

2.2.1. Cell cultures.

All cultures were maintained at $37^{o}C$ in an atmosphere

containing 5% CO_2 in air. Cultures in plastic Petri dishes

(Nunclon) were maintained in a humidified incubator, continually

flushed with air containing 5% CO_2. Cultures propagated in 8 oz.

bottles were flushed with CO_2 immediately after subculture and

then incubated at $37^{o}C$.

BHK-TK⁻ cells, BHK-IPP⁻ cells and L-TK⁻ cells were grown

in 8 oz. bottles containing 15 ml EFC 10 medium. These cell lines

were propagated continuously by trysinising the monolayers of

cells every 3-4 days (BHK cells), or 5-6 days (L cells), and

subculturing one-twelfth of the cell suspension.

HeLa cells, 3T3 cells and L cells were propagated in EC 10

by trypsinising cell monolayers every 3-4 days and subculturing

one-tenth of the cell suspension.

H116 cells and clones 3 & 201 were propagated in EFC 10

in 8 oz. bottles by trypsinising the monolayer of cells every

5-6 days and subculturing one-twelfth of the cell suspension.

All cell lines were regularly checked for bacterial, fungal

and PPLO contamination.

Cells were counted in an "improved Neubauer" haemocytometer.

2.2.2. Preparation of a co-culture containing a 1:1 ratio of

donor cells to recipient cells.

Separate cultures of donor and recipient cells were plated

out in either 35mm dishes (2 ml EFC 10) or 50mm dishes (3 ml

EFC 10) and grown until they were confluent. The old medium in

both the donor and recipient cultures was then replaced with fresh

medium. Donor cells were labelled with a radioactive tracer
(usually for 4h). At the end of the labelling time, the donor
and recipient cells were washed x3 and x1 respectively with BSS
at 37°C. Donor and recipient cells were treated briefly with
trypsin, and the trypsin discarded. The cell sheets were dispersed
in fresh medium (1 ml/35mm dish; 1.5 ml/50mm dish) using a pasteur
pipette. One donor culture was combined with one recipient culture
in a fresh dish (35mm or 50mm), containing clean, sterile 13mm
diameter glass coverslips (Section 2.2.4.), and co-cultured together
for a time (usually 3h). The coverslips were then processed for
autoradiography (Sections 2.2.5. & 2.2.6.).

2.2.3. Preparation of a co-culture containing few donor cells
to many recipient cells.

Recipient cells were plated out in either 35mm dishes (2 ml
EFC 10) or 50mm dishes (3 ml EFC 10), and grown until they formed a
confluent culture. Sparse cultures of donor cells were plated out
in dishes (35mm or 50mm), containing clean, sterile 13mm diameter
glass coverslips (Section 2.2.4.), and cultured for a minimum of
8h before being used for an experiment.

At the same time as radioactive tracer was added to the donor
cultures, the old medium in the recipient cultures was replaced
with fresh medium. At the end of the labelling time (usually 4h),
the donor and recipient cultures were washed x3 and x1 respectively,
with BSS at 37°C. The recipient cultures were then treated briefly
with trypsin, and the trypsin discarded. The cells were dispersed
in fresh medium (1 ml/35mm dish; 1.5 ml/50mm dish), using a
pasteur pipette. Each recipient culture was transferred to a donor
culture (containing 1 ml medium/35mm dish or 1.5 ml/50mm dish),
and co-cultured together for a time (usually 3h). The coverslips
were then processed for autoradiography (Sections 2.2.5. & 2.2.6.).

2.2.4. Preparation of coverslips for cell culture.

Glass coverslips (Chance No.1; Macfarlane Robson Ltd.,
Glasgow), on which cells were to be grown, were immersed in
boiling 0.1M-NaOH for 10 min. The NaOH was then discarded, and the
coverslips were washed exhaustively under running tap water, with
occasional stirring, for 4-5h to remove all traces of NaOH. The
coverslips were then thoroughly washed several times with distilled
water, followed by ethanol, and sterilised at $160^{o}C$ for 1h.

Transfer of coverslips to plastic Petri dishes was carried out
under asceptic conditions. 35mm dishes can only accommodate 3
coverslips, whereas 50mm dishes usually contained 3 or more coverslips.

2.2.5. Preparation of cells, cultured on coverslips, for
autoradiography.

2.2.5.1. Acid insoluble autoradiography.

The method used was a modification of that of Subak-Sharpe
et al. (1969).

The medium was removed from culture dishes containing
coverslips, and the cells were washed x1 with formol saline (5
ml/35mm dish; 12.5 ml/50mm dish) for a minimum of 30 min to fix
the cells. The coverslips were then placed in procelain coverslip
racks (A.H. Thomas Company, Philadelphia, U.S.A.). The racks were
immersed x3 in 10% (w/v)TCA ($+4^{o}C$) for 10 min, x2 in distilled
water ($+4^{o}C$) for 5 min and dipped in ethanol. The coverslips were
then left in the rack to dry at either room temperature or $37^{o}C$.

2.2.5.2. Acid soluble autoradiography.

The medium was removed from culture dishes containing
coverslips, and the cells were washed x5 with ice-cold BSS.
The coverslips were then removed from the dishes, and after
draining off excess BSS, they were dried flat at $37^{o}C$.

2.2.6. Preparation of autoradiographs.

The dry coverslips, cells uppermost, were mounted with DePeX (Gurr, London) on microscope slides (0.8-1mm glass microscope slides (Macfarlane Robson Ltd., Glasgow), which had been degreased in ethanol and coated with a film of gelatine-chrome alum), and left overnight at room temperature. The slides were covered with AR.10 stripping film (Kodak Ltd., London), using a Kodak safelight (filter: Wratten Series 1), and placed in light tight boxes, which contained activated silica gel. The gel was replaced with fresh activated silica gel 24h later. After an exposure at room temperature, the autoradiographs were developed at $20^{o}C$ in D19b for 5 min, fixed with Amfix (no hardener) for 4 min, and rinsed in water. The autoradiographs were then stained with Gimesa (Gurr, London), freshly diluted 1:20 (v/v) in water, for 45s.

Photographs were taken with a Zeiss photomicroscope.

2.2.7. Preparation of autoradiographs for grain counting.

Autoradiographs for grain counting must contain only small numbers of grains per cell. This is achieved by using less radioactive precursor and/or a shorter exposure time, prior to development. However, at early exposure times (1-2 days), water present in the film quenches the tritium radiation, giving unpredictable grain concentrations. The water was therefore removed by blowing cold air over the slides for 30 min, immediately after covering with film.

2.2.8. Measurement of [^{3}H] thymidine incorporation into acid insoluble and soluble material.

Incorporation of label into the cells was terminated by removing the medium from the dishes and washing the cells x5 with ice-cold BSS. The cells were left overnight in 2 ml 5%

(w/v) TCA at +4°C, then scraped into the TCA, and the acid

insoluble material was collected by filtration on glass fibre

filters (GF/B Whatman; W.R. Balston Ltd., England). The dishes

were further washed x2 with 2 ml and x1 with 4 ml 5% ice-cold

TCA and the washings added to the filter. The acid soluble

effluent was also collected. The filters were dried in

scintillation vials in an oven at 55-60°C for 12h. 0.5 ml hyamine

hydroxide was added to each filter and incubation continued at

55-60°C for a further 20 min. 10 ml toluene based scintillation

fluid was added to each vial. 10 ml dioxan based scintillation

fluid was added to 1 ml of acid soluble effluent. The

radioactivity in each sample was then determined with a Philips

liquid scintillation spectrometer.

2.2.9. Measurement of [^{3}H]uridine incorporation into acid insoluble
and acid soluble material.

This method was based on that of Weber & Rubin (1971).

Incorporation of label into the cells was terminated by

removing the medium from the dishes, and washing the cells x5

with ice-cold BSS. Acid soluble pools were extracted by adding

x2 2 ml 5% (w/v) TCA (0°C) to each dish for 5 min. The acid

insoluble material was washed x2 with 5% TCA (0°C). These washings

were discarded. 2 ml of 10% (w/v) TCA (preheated) was added to

each dish and incubated at 70°C for 1h. The 10% TCA, containing

the RNA, was combined with a 2 ml 10% TCA wash of the dish. 1 ml

of solubilised RNA or 1 ml of acid soluble pool was combined with

10 ml of dioxan based scintillation fluid. The radioactivity in

each sample was then determined with a Philips liquid

scintillation spectrometer.

3. RESULTS

3. Results.

Although mechanisms may be envisaged for the direct transfer
of all types of molecules from one cell to another, the evidence
available by 1970 (see introduction) suggested that macromolecules
were not commonly transferred, but that small molecules and ions
were, and it was thought that transfer of radioactive nucleotides
was the basis of metabolic co-operation between cells.

However, Kolodny (1971, 1972) then presented evidence that RNA,
but not nucleotides, could be transferred between mammalian cells
in tissue culture. Since these results were completely contrary
to the earlier suggestions of nucleotide pool transfer, a study of
the types of molecules which can be transferred between cells
was undertaken.

The method used by Kolodny to study transfer of radioactivity
from prelabelled donor cells to unlabelled recipient cells
involved the separation of donor and recipient cells after
co-cultivation. This separation was achieved by allowing donor
cells to ingest tantalum particles, prior to co-culture, to make
them dense. After co-culture of the dense donor cells with
untreated recipient cells, the culture was trypsinised and the
recipient cells were separated from the dense donor cells on a
Ficoll gradient. The cells were then analysed for the presence
of label.

Another quite different method which can be used to analyse
the transfer of $[^3H]$ compounds from cell to cell is autoradiography.
This method has several advantages over that used by Kolodny.
After co-culture of prelabelled donor cells with unlabelled
recipient cells, a physical separation of the donor cells from
recipient cells is not required and, therefore, ingestion of

tantalum particles by donor cells is unnecessary. Moreover, both
the geometry of the interaction between donor and recipient cells,
and the sites of incorporation of label within the cells, can be
observed. Also, the extent of label transferred from donor to
recipient cells can be quantitated by grain counting. For these
reasons, the technique of autoradiography was chosen to study
transfer of labelled molecules between cells.

3.1. Transfer of molecules labelled with $[^3H]$uridine between cells.

Initially, a co-culture procedure, similar to that reported
by Kolodny (1971), was used to establish that a transfer phenomenon
could be detected by autoradiography.

A culture of donor cells, prelabelled with $[^3H]$uridine for
4h, was trypsinised and co-cultured for 5h, in the presence of
excess unlabelled uridine, with unlabelled recipient cells. The
cells were then processed for autoradiography. Fig. 1 shows that
all cells have incorporated label. The donor cells can be easily
identified from recipient cells, as the donor cells are heavily
labelled and the recipient cells are lightly labelled.

Grain counting on donor and recipient cells, from a similar
co-culture, showed that there was 7% transfer of donor cell
label to recipient cells in 3h. Kolodny (1971) found in a similar
co-culture, using 3T3 cells, that there was 10% transfer of donor
cell label to recipient cells in 4h. It appears, therefore, that
the transfer phenomenon described by Kolodny can be detected
to a quantitatively similar extent using autoradiography.

Fig. 1

Transfer of $[^3H]$uridine labelled molecules from donor cells to recipient cells

1×10^5 TG$_2$ cells (donors) and 1×10^5 B$_1$ cells (recipients) were plated out separately in 35mm dishes and the cultures were grown until they were confluent. A 1:1 co-culture of donor and recipient cells was formed as in Section 2.2.2. Donor cells were labelled for 4h with $[^3H]$uridine (5μCi/ml). Donor and recipient cells were co-cultured together for 5h in the presence of 0.04mg/ml unlabelled uridine. Cells were processed for autoradiography (Section 2.2.5.1.). Autoradiographs were developed after 34 days. Magnification x 700.
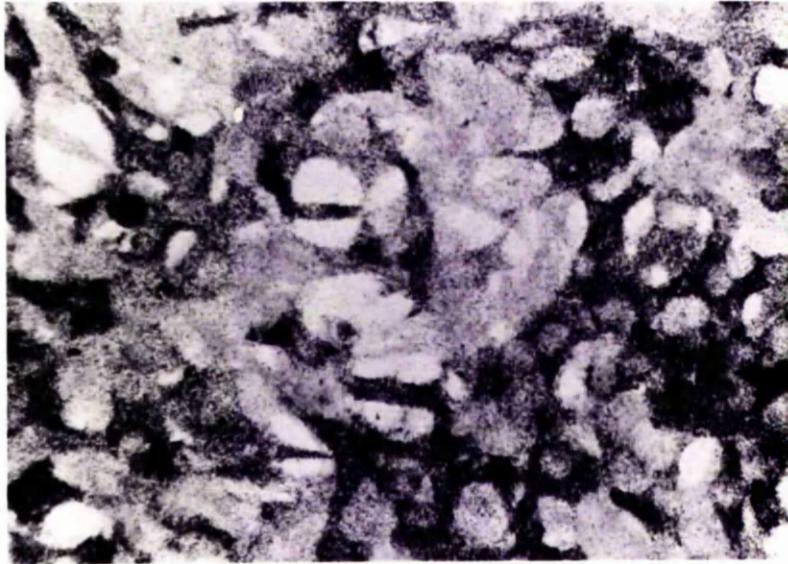
3.1.1. Effect of excess unlabelled uridine on the transfer of

donor label to recipient cells.

Kolodny (1971, 1972) added 1000-fold excess unlabelled
uridine during co-culture in an attempt to prevent the $\left[^3H\right]$ uridine
labelled acid soluble pool being incorporated into RNA, so that
he could conclude that any label present in recipient cells
could only be due to RNA transfer. However, Kolodny did not
report the effect of unlabelled uridine on the incorporation of
the pre-existing labelled donor cell acid soluble pool, so this
point was examined.

Confluent cultures of donor cells, prelabelled with $\left[^3H\right]$ uridine
for 4h, were co-cultured for 10h, in the presence of either 0 or
0.04 mg/ml unlabelled uridine, with confluent cultures of
unlabelled recipient cells. Autoradiographs show that the quantity
of label present in recipient cells is similar, whether excess
unlabelled uridine (Fig. 2a) or no uridine (Fig 2b) is present
during co-culture. Using a few donor cells to many recipient cells
(see Sections 3.1.3.1. & 3.1.3.2.), the quantity of label present
in donor and recipient cells, co-cultured together in the
absence or presence of excess unlabelled uridine (Fig. 6a,b,d),
was quantitated by grain counting (Fig. 3). The mean number of
grains/donor cell is similar in both cultures (103.4 for no
uridine; 100.0 for excess uridine). The quantity of label present
in recipient cells (in direct contact with donor cells),
co-cultured in the presence of excess unlabelled uridine, is
still 97% of that present in similar recipient cells, co-cultured
in the absence of excess unlabelled uridine (mean number of
grains/recipient cell is 14.6 and 15.1 in the presence and
absence of unlabelled uridine respectively). This data shows that
excess unlabelled uridine has little effect on the extent of
transfer of label from donor cells to recipient cells. Although

Fig. 2

Effect of excess unlabelled uridine on transfer of [3H]uridine

labelled molecules between cells.

(a)
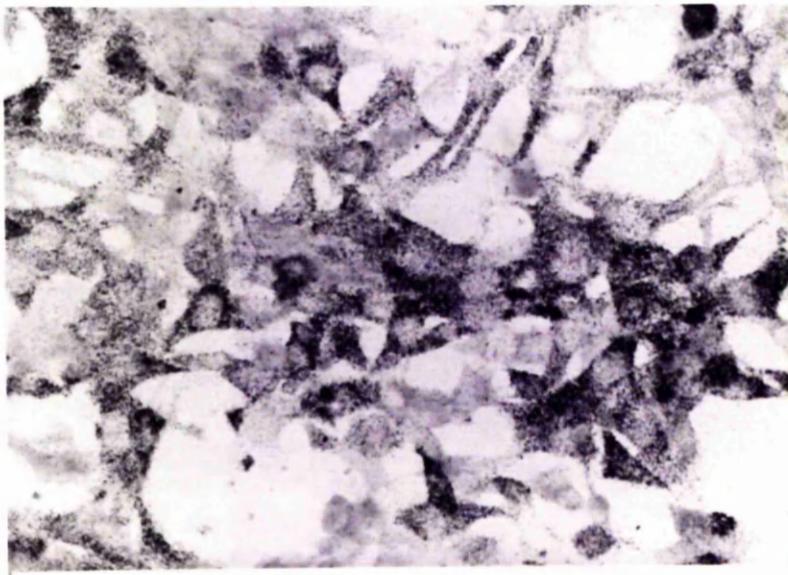
(b)

For details, see Legend to Fig. 1. Co-culture was 10h in the
presence of either a) 0.04mg/ml uridine or b) no uridine.
Autoradiographs were developed after 28 days. Magnification x 300.

Fig. 3

Quantitation of the effect of excess unlabelled uridine on the transfer of $[^3H]$ uridine labelled molecules between cells

5×10^5 B_1 cells (recipients) were plated out in 50mm dishes. 24h later, 3×10^4 TG_2 cells (donors) were plated out in 50mm dishes and grown overnight. Co-cultures (few donor cells to many recipients) were formed as in Section 2.2.3. Donor cells were labelled for 4h with $[^3H]$ uridine (0.17μCi/ml). Donor and recipient cells were co-cultured for 3h in the absence (a,b) or presence (c,d) of 0.04mg/ml unlabelled uridine. Cells were processed for grain counting.

Autoradiographs were developed after 22h for grain counting on donor cells (a,c) and after 232h for grain counting on recipient cells (b,d).

Only recipient cells in contact with donor cells were counted. 200 donor cells were counted; >350 recipient cells were counted.

 a) donor: no uridine; b) recipient: no uridine;

 c) donor: + uridine; d) recipient: + uridine.

Mean number of grains/cell a)103.4 b)15.1 c)100.0 d)14.6

Donor cells

Recipient cells

(a)

(b)

(c)

(d)

+ Urd.

+ Urd.

Cells

Cells

grains / cell
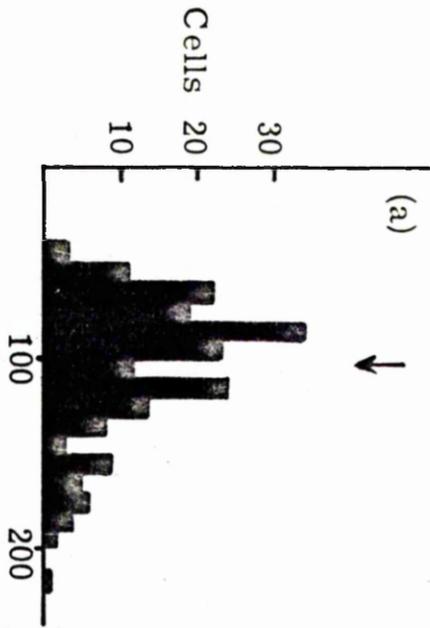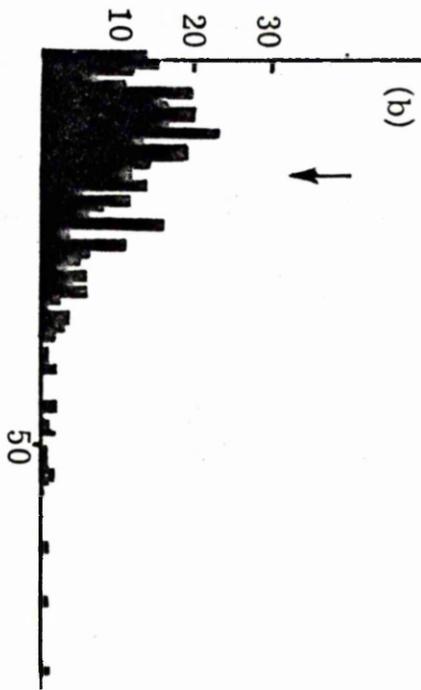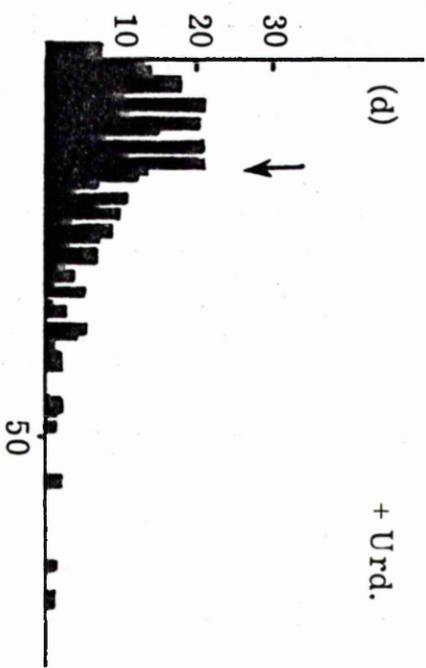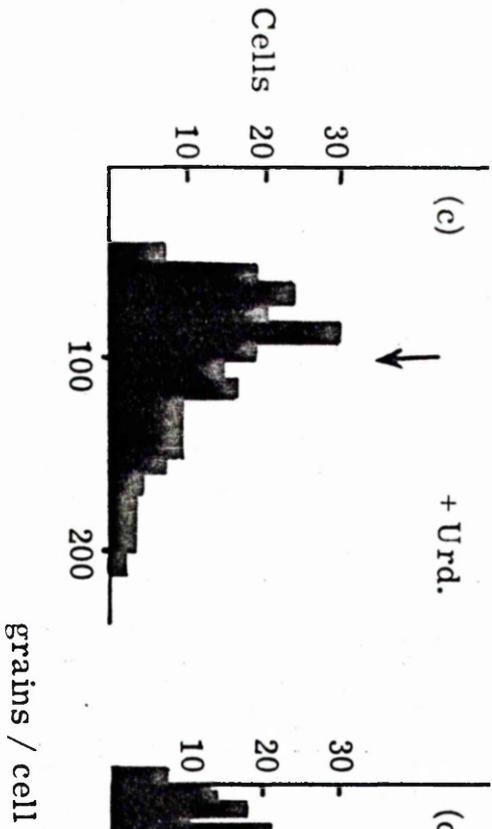
this data is apparently consistent with Kolodny's suggestion
that the acid soluble pool is not transferred between cells,
interpretation of the data requires caution, because several
reports (Sisken & Kinosita, 1961; Perry, 1963; Warner et al.,
1966; and see below) have shown that excess unlabelled
nucleosides are unsatisfactory in preventing the incorporation
of appropriately prelabelled nucleotides into nucleic acid.

3.1.2. Effect of excess unlabelled uridine on the incorporation of $[^3H]$ uridine labelled acid soluble pool into RNA.

It is particularly important to ascertain whether the labelled
acid soluble pool, present in cells after a pulse with $[^3H]$ uridine,
is incorporated into RNA during a chase in the presence of excess
unlabelled uridine. If the pool is incorporated, then its transfer
from donor to recipient cells, with its subsequent incorporation
into recipient RNA, could explain the appearance of labelled RNA
species in the recipient cells. However, if the pool is not
incorporated, this would strongly suggest that only labelled RNA
is transferred.

Cultures, prelabelled with $[^3H]$ uridine for 4h, were incubated
in the absence or presence of excess unlabelled uridine for 3h.
It was found that total counts in the acid insoluble material
(RNA) increased considerably during the chase, even in the
presence of excess unlabelled uridine, indicating that the labelled
acid soluble pool was being used for RNA synthesis, in preference
to the exogenous unlabelled uridine (Table 10). The presence of
excess unlabelled uridine reduces the incorporation of labelled
acid soluble pool by only 10%. The labelled acid soluble pools at
the end of the 3h chase were similar in both cultures, suggesting
that the decay of the labelled acid soluble pool was not affected
by excess unlabelled uridine. However, not all the labelled acid
soluble pool is incorporated into acid insoluble material.

Table 10

Effect of unlabelled uridine on the incorporation of a $[^3H]$ uridine

prelabelled acid soluble pool

Uridine conc. in chase (mg/ml)	Length of chase (h)	Acid insoluble material (c.p.m.)	Acid soluble material (c.p.m.)
—	0	40,804	46,016
0	3 ·	62,934	12,418
0.04	3	60,710	12,820

1×10^5 TG_2 cells were plated out in 35mm dishes and grown for 18h. Cultures were labelled for 4h with $[^3H]$ uridine (2.5μCi/ml). The radioactive medium was discarded and the cultures were washed x3 with BSS (37^oC). Three cultures were harvested at this time (zero time). To another three cultures, 2ml EFC 10 was added and to a further three cultures, 2ml EFC 10 containing 0.04mg/ml unlabelled uridine was added. These cultures were incubated for 3h. Incorporation of label into acid insoluble and acid soluble material was determined as in Section 2.2.9.

RNA, extracted by the cold phenol method at various times during a chase in the presence of excess unlabelled uridine, was analysed on sucrose gradients (Fig. 4). This shows that the stable RNA species, rRNA and tRNA, in the cell have all increased in activity.

These results show that added excess unlabelled uridine fails to inhibit the incorporation of a $\left[^{3}\text{H}\right]$uridine labelled acid soluble pool into RNA during a chase. This means that the direct transfer of the labelled acid soluble pool from labelled donor cells to recipient cells, with its subsequent incorporation into RNA in the recipient cells, may account for at least part of the label present in recipient cells. Therefore, experimental techniques which distinguish between labelled RNA precursor transfer and labelled RNA transfer must be used.

3.1.3. Effect of actinomycin D on transfer between cells.

Actinomycin D, present during co-culture at a high concentration to suppress total RNA synthesis, will distinguish between acid soluble pool transfer and RNA transfer between donor and recipient cells. If label is located in RNA of recipient cells after co-culture under such conditions, this must be due to labelled RNA transfer from donor cells, and not to transfer and incorporation of labelled acid soluble pool from donor cells. Kolodny (1971) reported that actinomycin D, at a final concentration of 12.5 µg/ml, still allowed 12% transfer of donor label to recipient cells during a 5h co-culture, similar to that found in a 5h untreated co-culture. This is strong evidence that RNA, and not the acid soluble pool is transferred, and this result was a major foundation for his conclusions.

This experiment was repeated using autoradiographic analysis to follow transfer, but the level of actinomycin D required to

Fig. 4

Effect of excess unlabelled uridine on incorporation of
$[^3H]$uridine prelabelled acid soluble pool into RNA

0.75×10^6 TG$_2$ cells were plated out in 90mm dishes in 10ml
EFC 10 and grown for 20h. $[^3H]$uridine (0.5μCi/ml) was added for
4h. Each dish was then washed x 3 with 5ml BSS (37°C). One dish
was harvested at this time (zero time). The remaining cultures
were chased in 10ml EFC 10 containing 0.04mg/ml uridine for 3h
and 24h. These cultures were washed x 5 with BSS (5ml; 0°C).
Cultures were harvested by adding 2ml 0.1%(w/v)SDS/0.05M-NH$_4$Ac
pH 5.1 to each dish for 5min. The SDS lysate was scraped into
centrifuge tubes. 2ml 80% phenol/0.05M-NH$_4$Ac pH 5.1 was added
to each tube and the mixture vortexed for 2 min. The resulting
emulsion was separated by centrifuging at 10,000g for 10 min.
The aqueous phase was re-extracted with a further equal volume
of 80% phenol/0.05M-NH$_4$Ac pH 5.1. 2 vol absolute ethanol was
then added to the aqueous phase and the mixture left at
-20°C for 18h. The RNA was collected by centrifugation at
10,000g for 30 min and was resuspended in 1ml 0.1%SDS/0.05M-NH$_4$Ac
pH 5.1. 2ml ethanol was added and the mixture left at -20°C for
18h. The RNA was pelleted by centrifugation at 10,000g for 30
min. The pellet was resuspended in 1ml 0.1%SDS/0.05M-NH$_4$Ac pH 5.1.
RNA (0.2ml) was separated through 5-20%(w/v) sucrose gradients,
containing 0.01%SDS/0.05M-NH$_4$Ac pH 5.1, at 100,000g for $3\frac{1}{2}$h at 4°C.
Gradients were harvested by collecting 2 drop fractions on 3mm
Whatman discs. Discs were dried at room temperature, washed x 3
in 5% TCA (0°C) for 10min, rinsed in ethanol and finally ether.
Each sample was counted in 10ml toluene based scintillation
fluid with a Philips liquid scintillation spectrometer. Sterile
solutions and glassware were used throughout to eliminate
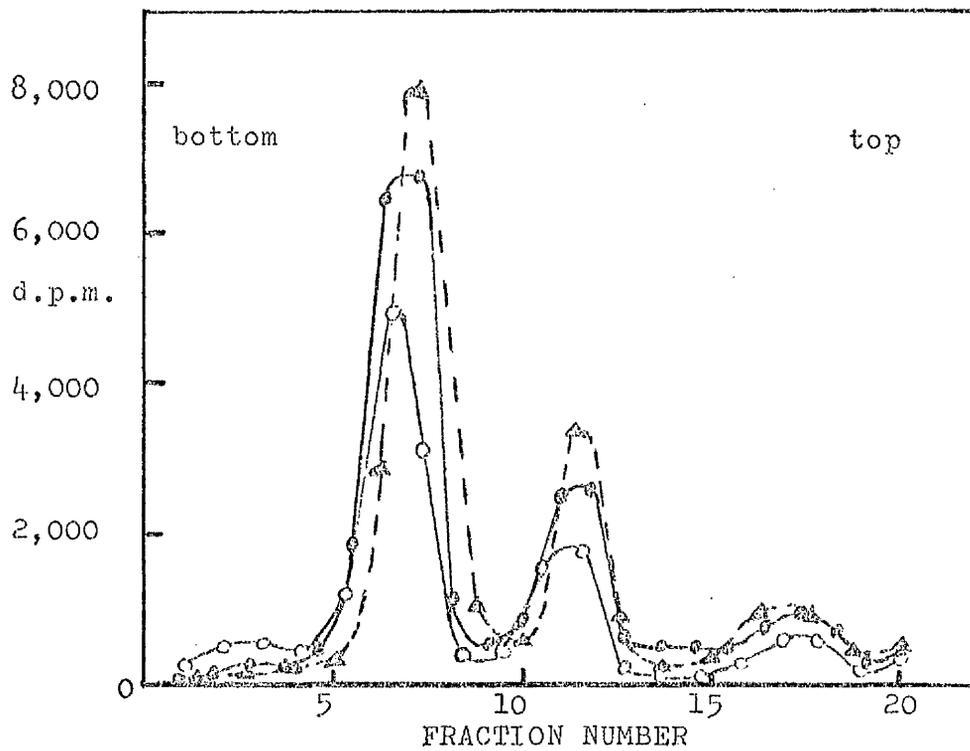
degradation by RNase.

———O——— : 4h label; no chase. — ——△—— — : 4h label; 3h chase.

———⊕——— : 4h label; 24h chase.

inhibit RNA synthesis in BHK cells was determined first, 5 µg/ml
final concentration actinomycin D inhibits $[^3H]$ uridine incorporation
into RNA by 99% of the control value, obtained in the absence of
actinomycin D. However, this concentration inhibits normal
spreading of cells, although the cells do attach (Table 11).
3 µg/ml actinomycin D allows spreading, although the process is
slower than the control without actinomycin D. 1 µg/ml and 2 µg/ml
actinomycin D allow normal spreading of the cells, and still give
95% and 97% inhibition respectively.

3.1.3.1. Effect of actinomycin D on a co-culture of a 1:1 mixture of donor and recipient cells.

Donor cells, prelabelled with $[^3H]$ uridine for 4h, were
co-cultured for 5h, in the presence of actinomycin D (0, 1, or
2 µg/ml) and excess unlabelled uridine, with recipient cells,
pretreated for 30 min with actinomycin D (0, 1, or 2 µg/ml).
Autoradiographs show that both concentrations of actinomycin D
give similar results (Fig. 5b,c), and that, compared to the
untreated control (Fig. 5a), there is a considerable reduction
in the transfer of label to recipient cells. This suggests,
contrary to Kolodny's data, that labelled RNA precursors, and not
labelled RNA itself, are transferred to recipient cells.

A reduction in the proportion of donor cells used for
co-culture allows an easier and more informative analysis of
autoradiographs. Gradients of label are formed extending from
donor cells to surrounding recipient cells in contact. A
qualitative assessment of the effect of any factor(s) which
reduces the transfer of label to recipient cells from prelabelled
donor cells, as well as an analysis of the geometry of interaction
between the donor and recipient cells, can be made. Also, compared

Table 11

Effect of actinomycin D on the spreading of cells

Actinomycin D conc. (μg/ml)	Appearance of cells after plating			
	1½h		3h	
	Attachment	Spread	Attachment	Spread
0	++++	++++	++++	++++
1	++++	++++	++++	++++
2	+++	++++	+++	++++
3	+++	—	+++	++++
4	++	—	++	+++
5	++	—	++	—

3.5×10^5 TG$_2$ cells (donors and recipients) were plated out in 35mm dishes and grown for 17h. Co-cultures were formed as in Section 2.2.2. The medium was renewed in each culture. 3½h later, actinomycin D (0,1,2,3,4,5μg/ml) was added to each recipient culture and incubation of all the cultures was continued for a further 30min. The cells (donors and recipients) were trypsinised and co-cultured together in the presence of actinomycin D (0,1,2,3,4 or 5μg/ml). Cultures were examined after 1½ and 3h.

Fig. 5

Effect of actinomycin D on a 1:1 co-culture of donor and
recipient cells.

1×10^5 TG$_2$ cells (donors) and 1×10^5 B$_1$ cells (recipients)
were plated out separately in 35mm dishes and the cultures
were grown until they were confluent. 1:1 co-cultures of donor
and recipient cells were formed as in Section 2.2.2. Donor cells
were labelled for 4h with [^{3}H]uridine (5µCi/ml). Recipient cells
were incubated with actinomycin D (0,1 or 2µg/ml) for 30 min
before the trysinisation and co-culture step. Donor and
recipient cells were co-cultured together for 5h in the
presence of 0.04mg/ml uridine and actinomycin D (0,1 or 2µg/ml).
Cells were processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 34 days. Magnification x **300**.

   a) 0µg/ml actinomycin D;   b) 1µg/ml actinomycin D;
   c) 2µg/ml actinomycin D.
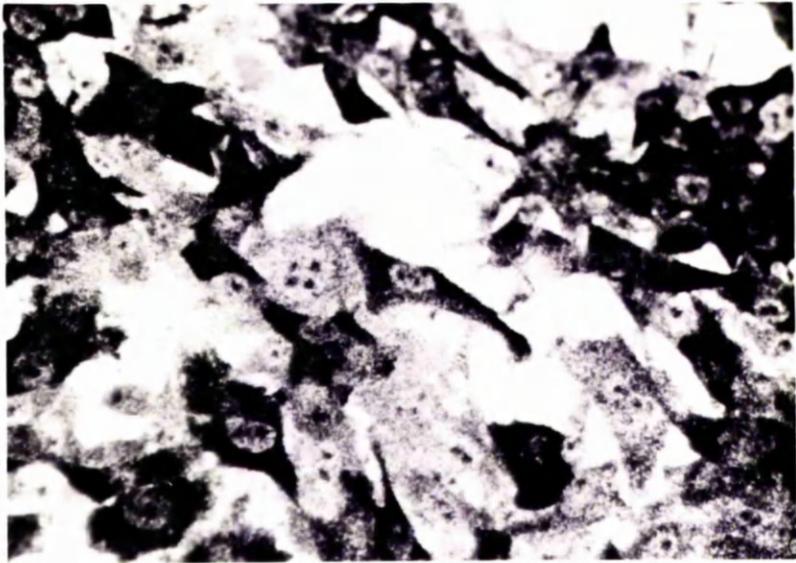
(a)
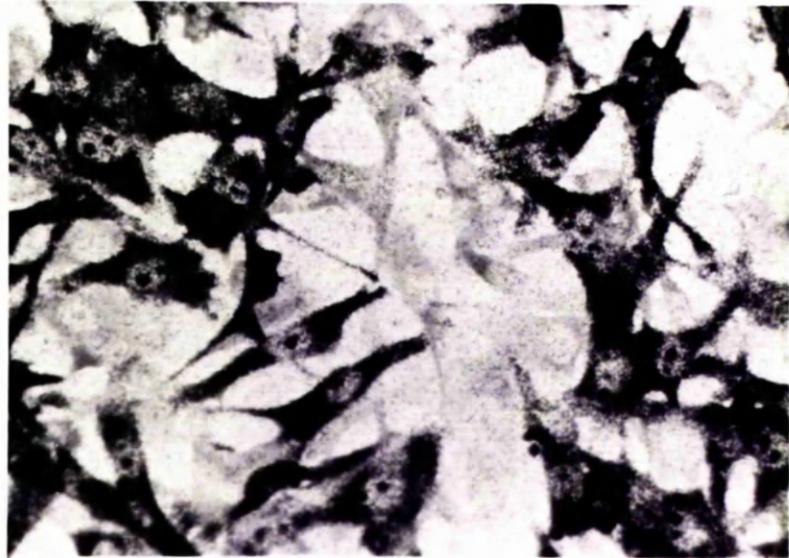
(b)

(c)

to a 1:1 mixture of donor and recipient cells, there is a much lower
background of grains, which is important for quantitation of
small amounts of label present in the recipient cells.

3.1.3.2. Effect of actinomycin D on a co-culture of a few donor cells with many recipient cells.

A large excess of unlabelled recipient cells, pretreated for
30 min with actinomycin D (0, 1 µg/ml), were co-cultured for 3h,
in the presence of actinomycin D (0, 1 µg/ml) and excess
unlabelled uridine, with a few donor cells, prelabelled with
[³H]uridine for 4h. Autoradiographs of the untreated mixed
population of cells (Fig. 6a,b) show a gradient of label running
from the heavily labelled donor cells to the unlabelled recipient
cells. The autoradiograph of the actinomycin D treated mixed
population of cells (Fig. 6c) shows that the extent of transfer
has been dramatically reduced. This result strongly suggests that
prelabelled RNA is not transferred, and that the label in recipient
cells shown in Fig. 6(a,b,d) must be due, predominantly at least,
to the transfer of a labelled acid soluble pool.

The transfer of label from donor cells to recipient cells, in
the presence and absence of 1 µg/ml actinomycin D during co-culture,
was quantitated to obtain an exact estimate of the proportions of
acid soluble pool, and of RNA, transferred from donor to recipient
cells. Fig. 7(a,c) shows the distribution of grains/cell in
untreated and actinomycin D treated donor cells respectively after
a 3h co-culture. The difference in the mean values (100.0 for
untreated; 83.8 for actinomycin D treated) is caused by two
different effects of actinomycin D. First, the incorporation of
the labelled acid soluble pool into RNA during co-culture, which
occurs in untreated cells, is inhibited by actinomycin D. Second,
there is a net loss in the actinomycin D treated cells of labelled

Fig. 6

Effect of actinomycin D on a co-culture between a few donor cells and many recipient cells.

2.5×10^5 B_1 cells (recipients) were plated out in 50mm dishes.
1×10^4 TG_2 cells (donors) were plated out in 50mm dishes.
Cultures were grown for 16h. Co-cultures (few donors to many
recipients) were formed as in Section 2.2.3. Donor cells were
labelled for 4h with $[^3H]$uridine (5µCi/ml). Recipient cells
were incubated with actinomycin D (0, 1µg/ml) for 30min
before the trypsinisation and co-culture step. Donor and
recipient cells were co-cultured together for 3h in the presence
of 0.04mg/ml uridine and actinomycin D (0 or 1µg/ml). Donor and
untreated recipient cells were also co-cultured together for 3h
in the absence of either uridine or actinomycin D (d). Cells
were processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 30 days. Magnification:
a) x 440;   b,c,d) x 700

a,b) Control untreated co-culture (+ uridine);

  c) Actinomycin D treated co-culture (+ uridine);

  d) Untreated co-culture (no uridine).
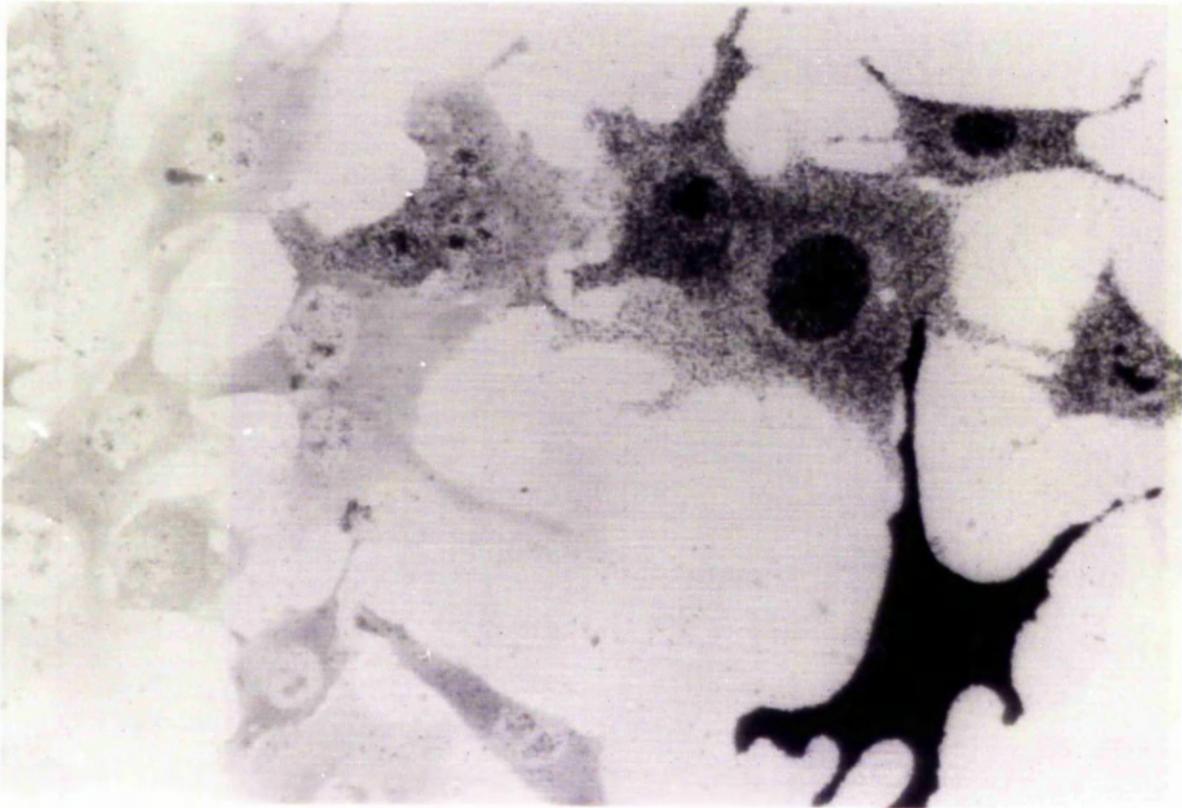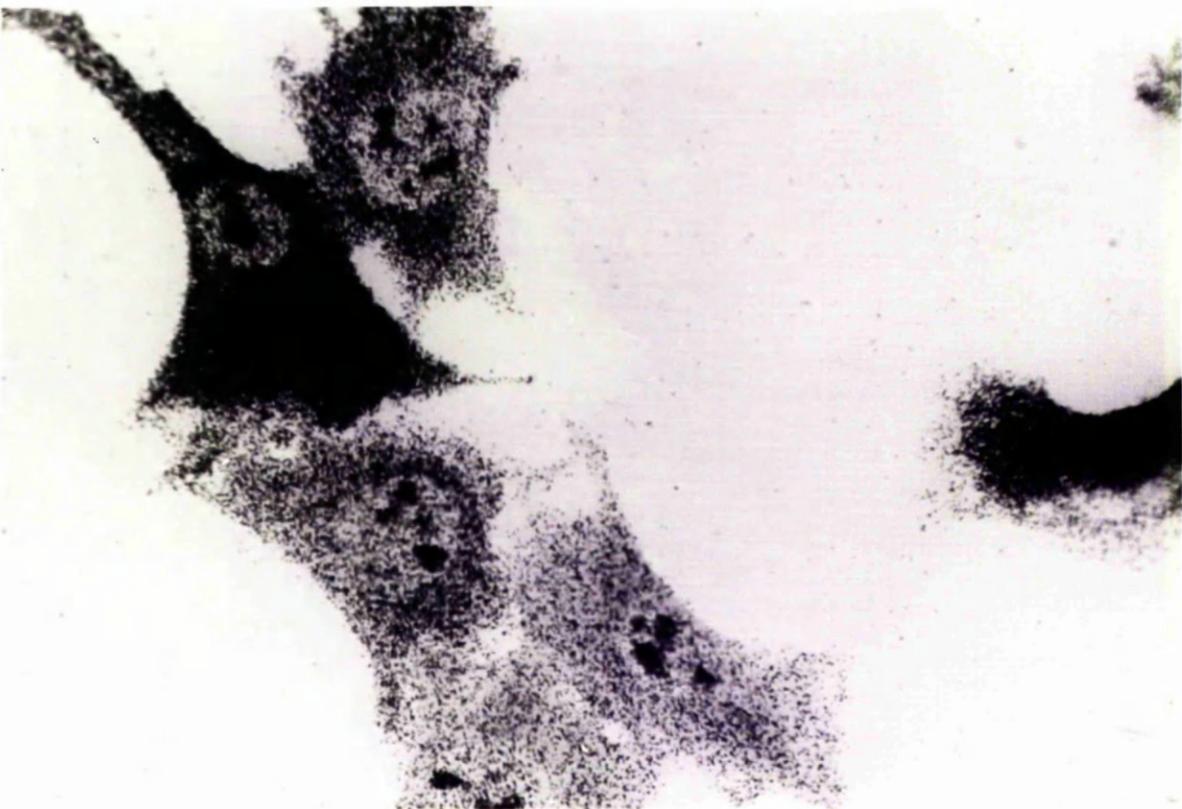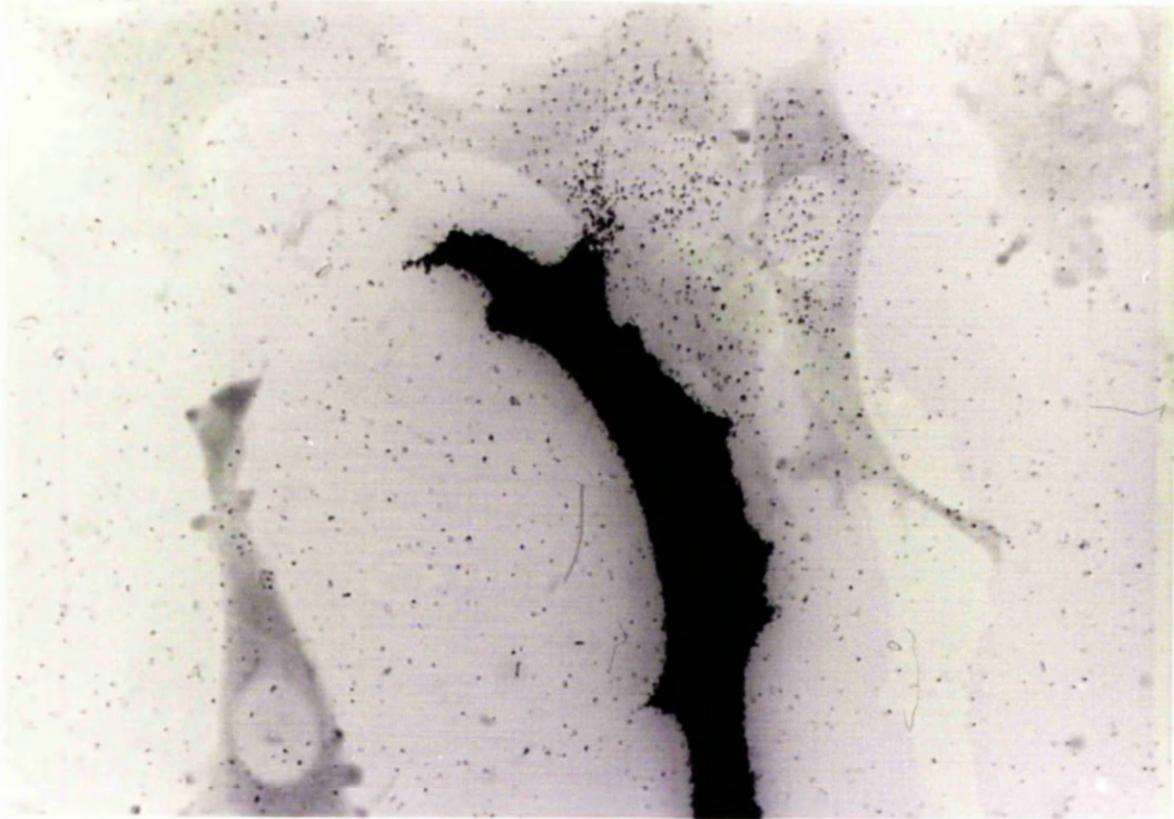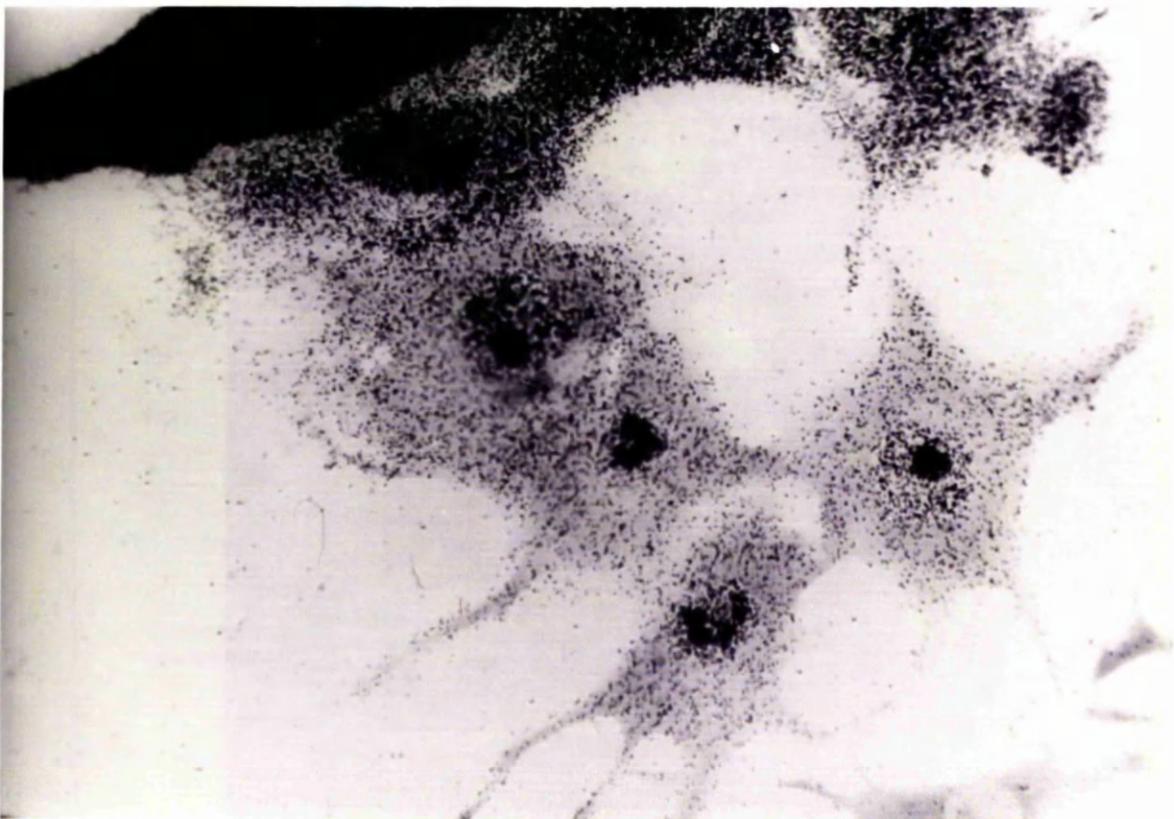
Fig. 7

Quantitation of the effect of actinomycin D on a co-culture
of a few donor cells with many recipient cells

5×10^5 B_1 cells (recipients) were plated out in 50mm plates.
24h later, 3×10^4 TG_2 cells (donors) were plated out in
50mm dishes and the cultures were grown overnight. Co-cultures
(few donor cells to many recipients) were formed as in Section
2.2.3. Donor cells were labelled for 4h with $[^3H]$uridine
(0.17µCi/ml). Recipient cells were incubated with actinomycin D
(0, 1µg/ml) for 30 min before the trypsinisation and
co-culture step. Donor and recipient cells were co-cultured
for 3h in the presence of 0.04mg/ml uridine and actinomycin D
(0 or 1µg/ml). Cells were processed for grain counting.
Autoradiographs were developed after 22h for grain counting
on donor cells (a,c) and after 232h and 1608h for grain
counting on untreated recipient cells (b) and actinomycin D
treated recipient cells (d) respectively. Mean number of
grains/actinomycin D treated recipient cell (d) was corrected
to 232h development time.
Only recipient cells in contact with donor cells were counted.
200 donor cells were counted; ⊁350 recipient cells were counted.

    a) untreated donor; b) untreated recipient;

    c) actinomycin D treated donor; d) actinomycin D treated
                                      recipient.

Mean number of grains/cell: a)100.0; b)14.6; c)83.8; d)0.83.

Donor cells

Cells

30
20
10

100

200

↓

(a)

Recipient cells

Cells

30
20
10

100

200

(c)

+ Act. D.

131
81
106
62

30
20
10

50

↓

(b)

+ Act. D.

(d)

50
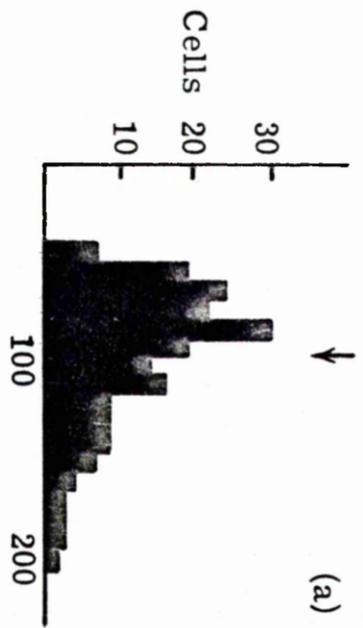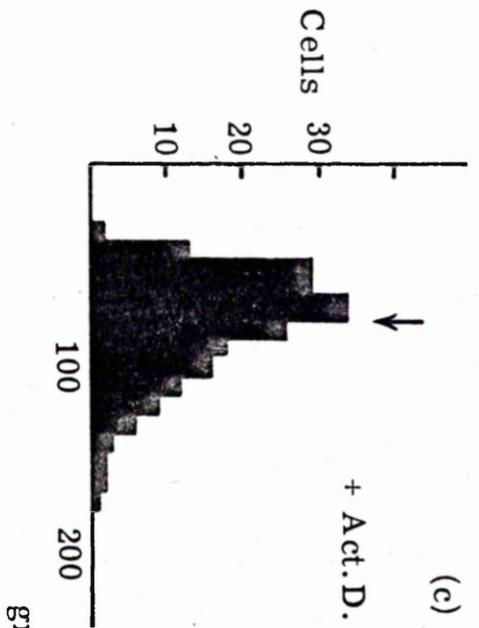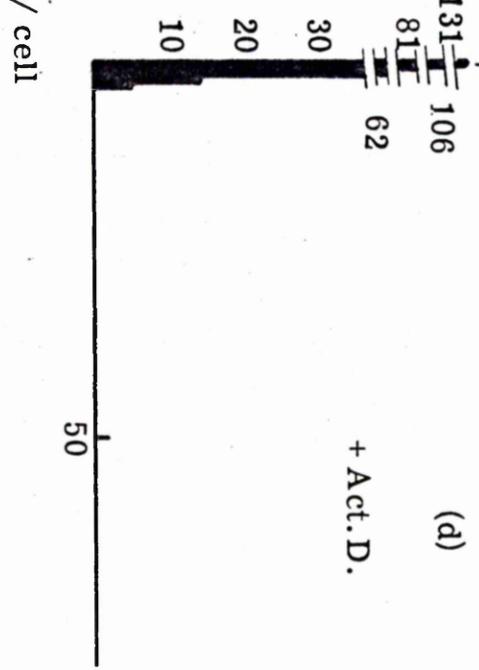
grains / cell

heterogeneous nuclear RNA, since this turns-over rapidly with a half-life of 1h (Penman et al., 1968). However, the major cytoplasmic species already labelled (rRNA and tRNA) are stable in the presence of actinomycin D. Fig. 7(b,d) shows the distribution of grains/cell in untreated and actinomycin D treated recipient cells in direct contact with donor cells. The mean number of grains/untreated cell is 14.6 and the equivalent mean number of grains/actinomycin D treated cell is 0.83. This shows th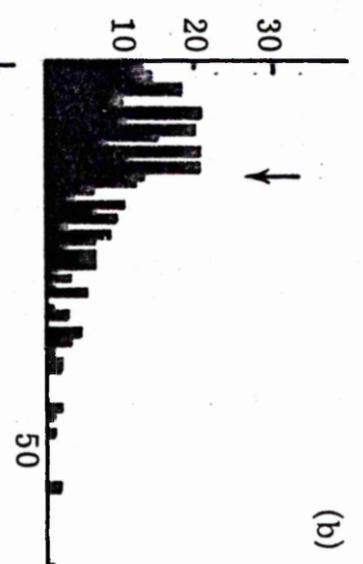at the presence of actinomycin D during co-culture reduces the transfer of label by 94.3%. Since 1 µg/ml actinomycin D inhibits cellular RNA synthesis by only 95%, the residual label in the actinomycin D treated recipient cells could therefore be due to labelled acid soluble pool transfer, with subsequent incorporation at the reduced rate into recipient cell nucleic acid. Therefore, there appears to be no evidence for significant transfer of RNA from donor to recipient cells.

The possibility that actinomycin D inhibits transfer of label to the recipient cells, by interfering with the mechanism of junction formation, was ruled out on two grounds :- 1) residual label in recipient cells (Fig. 6c) and, 2) by studying metabolic co-operation between BHK-TK^{+} and BHK-TK^{-} cells in the presence of actinomycin D.

3.1.3.3. Effect of actinomycin D on metabolic co-operation between BHK-TK^{+} and BHK-TK^{-} cells.

Pitts (1971) has shown that when BHK-TK^{+} and BHK-TK^{-} cells are grown together in the presence of $\left[^{3}H\right]$ thymidine, label is transferred from the wild-type cells to the variant cells in contact.

Cultures of BHK-TK^{-} cells, pretreated for 30 min with actinomycin D (0, 1 µg/ml), were co-cultured for 5h, in the presence of actinomycin D (0, 1 µg/ml), excess unlabelled uridine and $\left[^{3}H\right]$ thymidine, with unlabelled BHK-TK^{+} cells. Fig. 8(a,b) shows autoradiographs of untreated and actinomycin D treated cultures

Fig. 8

Effect of actinomycin D on metabolic co-operation between BHK-TK$^+$ and BHK-TK$^-$ cells

2.7×10^5 BHK-TK$^-$ cells (recipients) were plated out in 50mm dishes. 1×10^4 BHK-TK$^+$ cells (donors) were plated out in 50mm dishes and the cultures were grown overnight. The old medium in the recipient cells was replaced 4h prior to co-culture. Co-cultures (few donors to many recipients) were formed as in Section 2.2.3. BHK-TK$^-$ cells (recipients) were incubated with actinomycin D (0, 1μg/ml) for 30 min before the trypsinisation and co-culture step. Donor and recipient cells were co-cultured together for 5h in the presence of actinomycin D (0, 1μg/ml), 0.04mg/ml uridine and $[^3H]$ thymidine (6.7μCi/ml). Cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 13 days; magnification x 440.

a) untreated co-culture;   b) actinomycin D treated co-culture.

(a)

(b)

respectively. The level of incorporation of label in both BHK-TK$^+$
cells (donor) and BHK-TK$^-$ cells (recipient) in Fig. 8(b) is less

than Fig. 8(a) because actinomycin D at the level of 1 µg/ml

inhibits the incorporation of [^{3}H]thymidine into acid insoluble

material by 35% of untreated value. Therefore, it appears that

transfer of label clearly still occurs to a quantitatively

similar extent in the actinomycin D treated and untreated cultures.

3.1.4. Sites of labelling in recipient cells.

Analysis of the sites of the label in recipient cells, after

co-culture with [^{3}H]uridine labelled donor cells, shows that the

nucleoli are heavily labelled (e.g. Fig. 6a,b,d). Heavy nucleolar

labelling is also present in recipient cells in which the cytoplasm

is not well labelled (Fig. 9), suggesting that label appears in

the nucleoli first and later in the cytoplasm. This is consistent

with a labelled acid soluble pool being transferred to the

recipient cells, being incorporated into rRNA in the nucleoli and

subsequently appearing in the cytoplasm as labelled ribosomes. If

cytoplasmic RNA species were transferred from cell to cell, then

one would anticipate that there would be no particular nucleolar

labelling in recipient cells.

3.1.5. Effect of reduced labelled donor acid soluble pool on the

extent of labelling of recipient cells.

Since the results above suggest that RNA is not transferred

between cells, but that the acid soluble pool is, it is important

to show that transfer of label is reduced when the donor cells

contain labelled RNA but little or no labelled acid soluble pool.

[^{3}H]uridine labels stable RNA species such as rRNA and tRNA,

and unstable species such as heterogeneous nuclear RNA. Since

heterogeneous nuclear RNA is unstable and turns-over rapidly

Fig. 9

Analysis of the sites of label in [³H]uridine labelled donor
and recipient cells
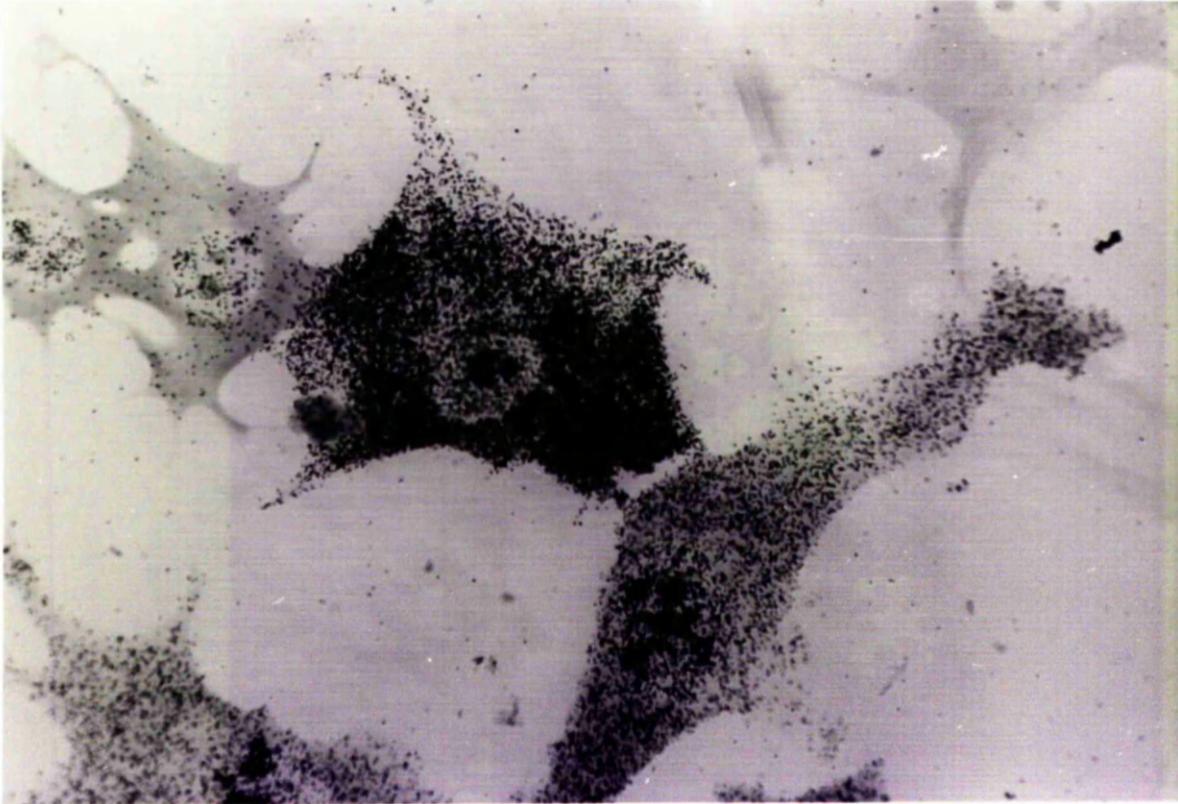
See Legend to Fig. 6a,b. Magnification x 700.

(Warner et al., 1966; Penman et al., 1968), there is a continual
flux of labelled molecules into and out of the acid soluble pool
and, therefore, depletion of the labelled acid soluble pool to
form stable RNA molecules requires a long chase.

Cultures, pulse labelled for 4h with $[^3H]$uridine, were chased
for various lengths of time in the presence of excess unlabelled
uridine. Table 12 shows that the labelled acid soluble pool is
reduced to 3% by 24h and remains constant at subsequent chase times.
Therefore donor cells, with heavily labelled RNA but with virtually
unlabelled acid soluble pools, can be prepared.

Donor cultures, pulse labelled for 4h with $[^3H]$uridine, were
chased for 24, 43 or 70h. These cultures were washed with BSS and
co-cultured for 3h, in the presence of excess unlabelled uridine,
with unlabelled recipient cells. Control (no chase) donor cultures,
prelabelled for 4h with $[^3H]$uridine, were co-cultured for 3h, in the
presence of excess unlabelled uridine, with unlabelled recipient
cells. Autoradiograph Fig. 10a shows the characteristic transfer
and gradient of label from donor to recipient cells in the control
culture. Fig. 10(b,c) shows that in the 43h chase and 70h chase
cultures respectively, the extent of transfer is drastically reduced.
Fig. 11(a,c) shows the distribution of grains/cell in the control
donor cells and 24h chase donor cells respectively. The difference
observed in the mean values (control cells: 96.1; chase cells:
64.1) is due to incorporation of labelled acid soluble pool and
cell division (donor cells doubling time is 17h under these
conditions) during the 24h chase. Fig. 11(b,d) shows the
distribution of grains/cell in control and 24h chase recipient
cells, in contact with donor cells. The mean number of
grains/control cell is 21.0 and the equivalent mean number of
grains/24h chase cell is 0.72. This shows that a 24h chase has
reduced the transfer by 96.6%. Therefore, it is the presence of

Table 12

Effect of chase on size of $[^3H]$uridine labelled acid soluble pool

Length of chase (h)	Acid soluble pool	
	c.p.m.	% zero chase value
0	206,408	100
3	87,544	42.4
24	6,528	3.16
48	10,020	4.86
72	9,738	4.72

Each determination an average of 3 dishes.

1×10^5 TG$_2$ cells were plated out in 35mm dishes and grown for 21h. Cultures were labelled for 4h with $[^3H]$uridine (2.5µCi/ml). The medium was removed and the cultures were washed x 3 with BSS (37°C). Three cultures were harvested at this time (zero time). The remaining cultures were chased in the presence of unlabelled uridine (0.04mg/ml) for 3, 24, 48 or 72h. The level of radioactivity present in the acid soluble pool at the end of these chase times was determined as in Section 2.2.9.

Effect of reduced $[^3H]$uridine labelled donor acid soluble

pool on the extent of labelling of recipient cells
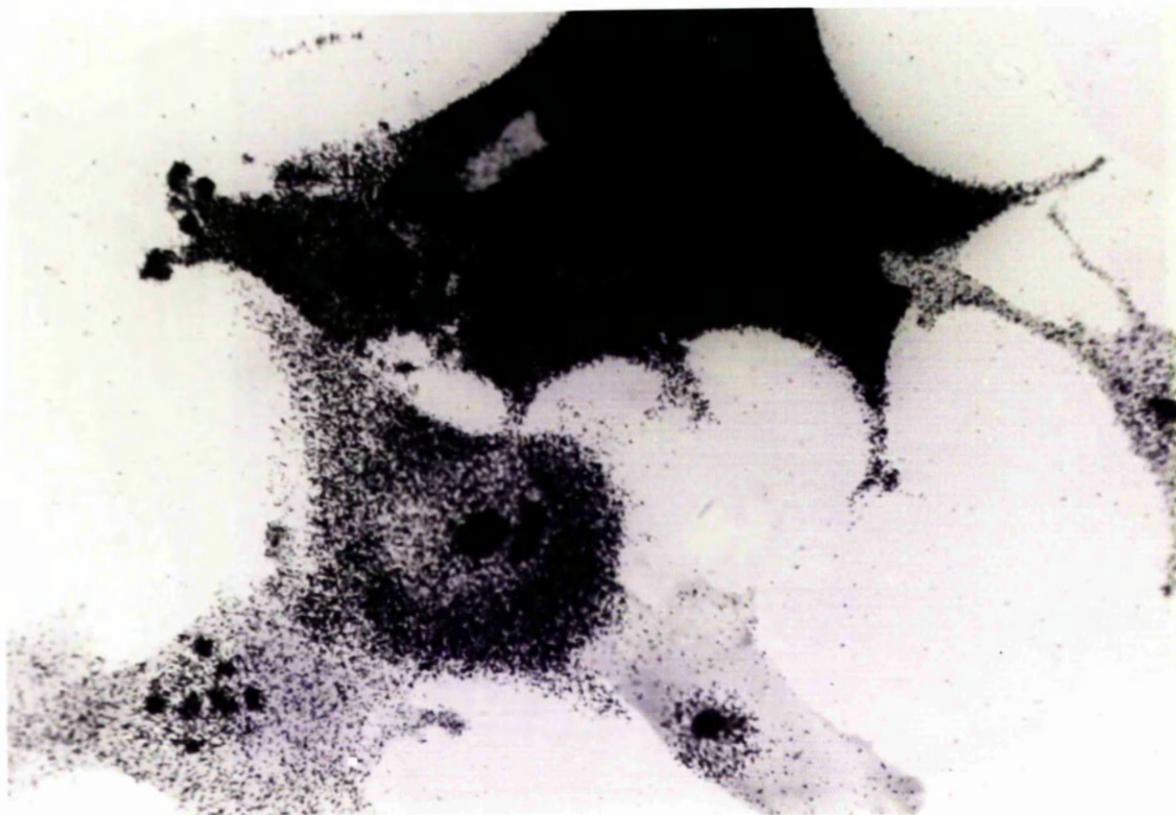
1×10^4 TG_2 cells (donors) were plated out in 50mm dishes and the

cultures were grown for 16h. At the same time, 7.5×10^5 B_1 cells

(recipients) were plated out in 50mm dishes and 48h later, 2.5×10^5

cells and 7.5×10^5 cells were plated out in 50mm dishes. Each

recipient culture was grown to confluence. Co-cultures were formed

as in Section 2.2.3. Donor cells were labelled for 4h with

$[^3H]$uridine (5μCi/ml). After the donor cultures were washed free of

label, one culture was co-cultured for 3h with unlabelled recipient

cells in the presence of 0.04mg/ml uridine (zero time). The

remaining donor cultures were cultured in 3ml EFC 10 for 43h or 70h,

washed x 3 in BSS (37°C) and then co-cultured for 3h with

unlabelled recipient cells in the presence of 0.04mg/ml uridine.

Cells were processed for autoradiography. Autoradiographs were

developed after 30 days. Magnification x 700.

a)4h label: no chase; b)4h label: 43h chase; c)4h label; 70h chase.

(b)

(c)

Fig. 11

Quantitation of the effect of reduced $[^3H]$uridine labelled

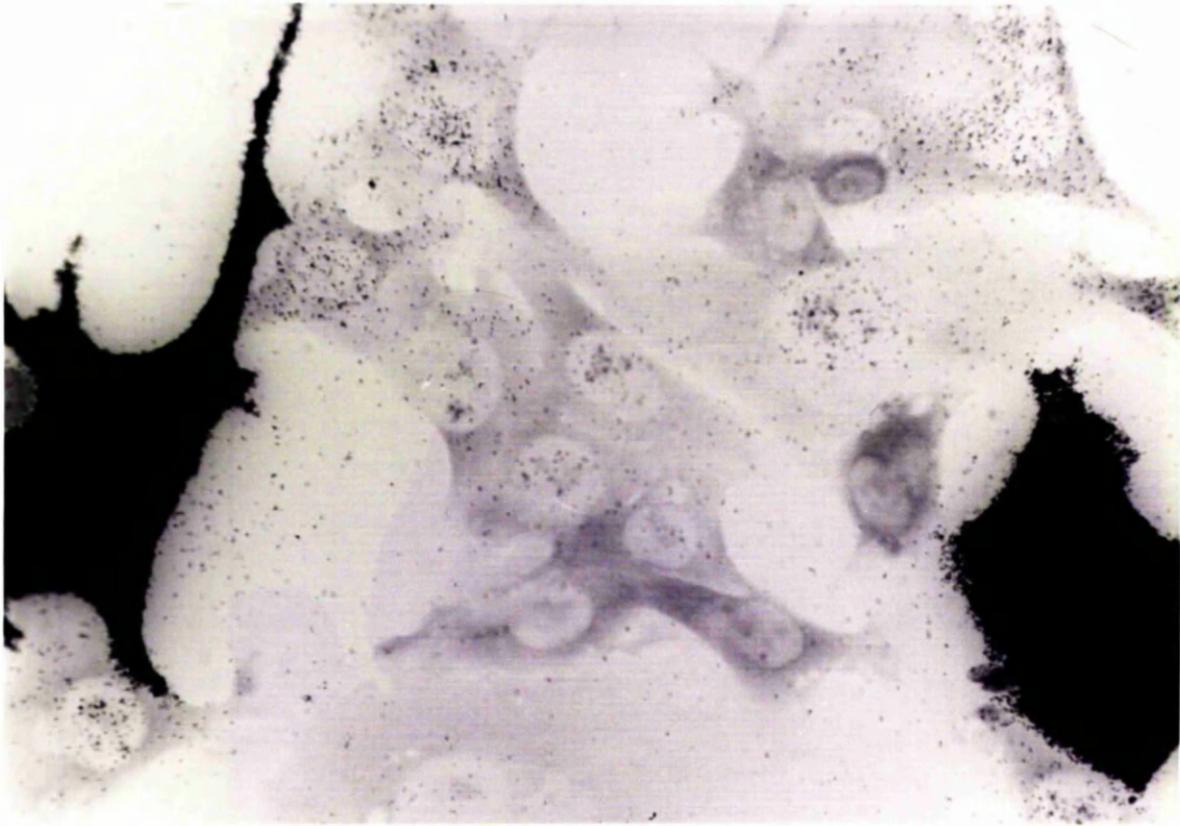donor acid soluble pool on the extent of labelling of

recipient cells

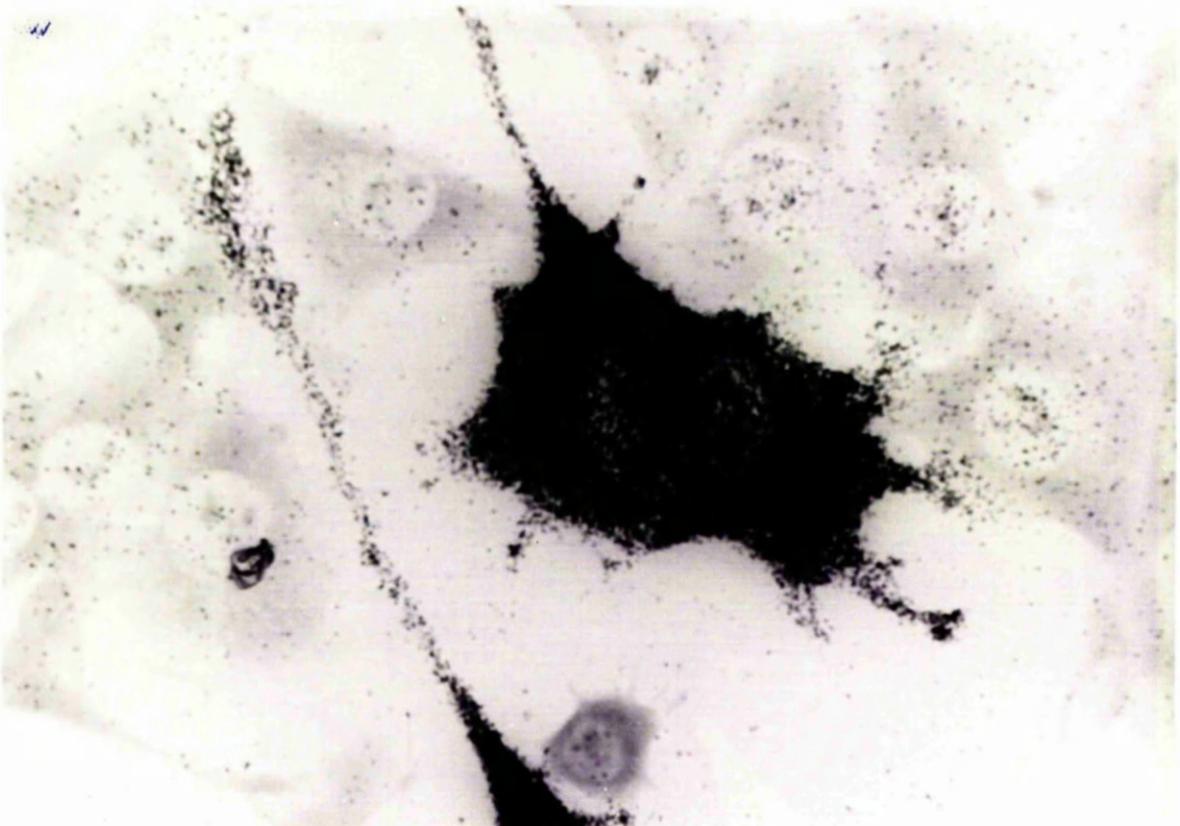3.5×10^5 and 7×10^5 B_1 cells were plated out in 50mm dishes

and the cultures were grown until confluent. 1×10^4 TG_2 cells

(donors) were plated out in 50mm dishes and the cultures were

grown for 24h. Co-cultures were formed as in Section 2.2.3.

Donor cells were labelled for 4h with $[^3H]$uridine (0.17μCi/ml).

After the donor cultures were washed free of label, one culture

was co-cultured for 3h with unlabelled recipient cells in the

presence of 0.04mg/ml uridine (zero time). The remaining donor

culture was grown in EFC 10 (3ml) for 24h, washed x 3 with

BSS (37°C) and then co-cultured for 3h with unlabelled

recipient cells in the presence of 0.04mg/ml uridine. Cells

were processed for grain counting.

Autoradiographs were developed after 22h for grain counting

on donor cells (a,c) and after 234h and 936h for grain counting

on control recipient cells (b) and chased recipient cells (d)

respectively. Mean number of grains/chased recipient cell (d)

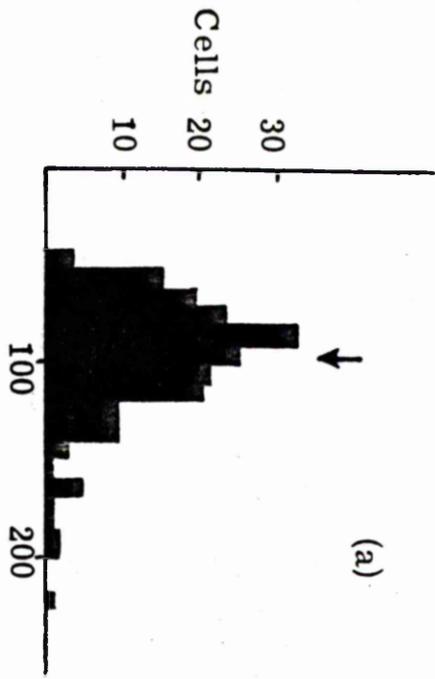was corrected to 234h development time.

Only recipient cells in contact with donor cells were counted.

200 donor cells were counted; >350 recipient cells were counted.

    a) donor: control; b) recipient: control;

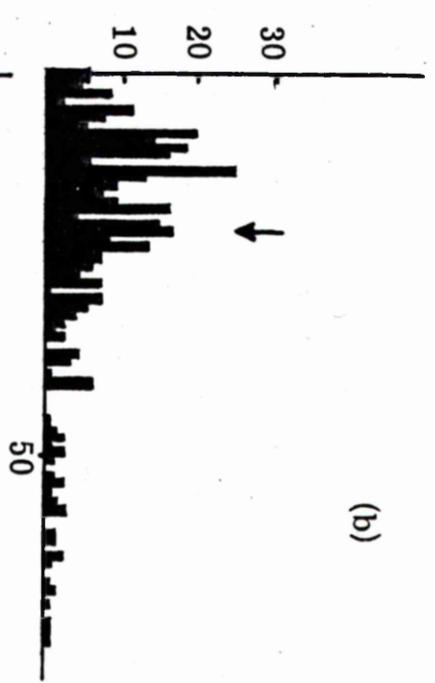   c) donor: chased;   d) recipient: chased.

Mean number of grains/cell: a)96.1; b)64.10; c)21.0; d)0.72.

Donor cells (a)

Recipient cells (b)
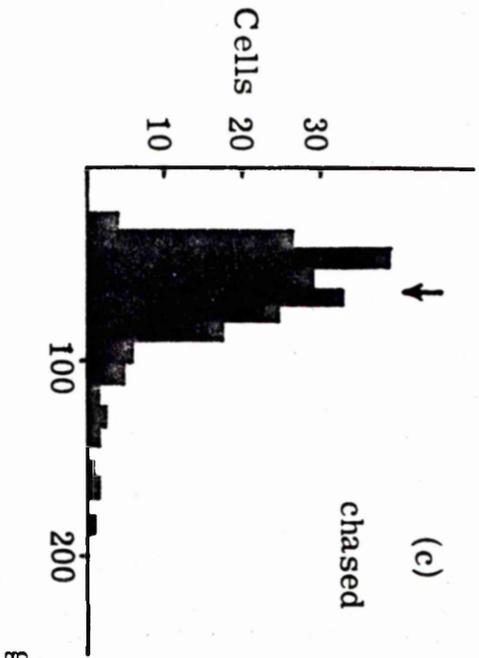
(c) chased

(d) chased
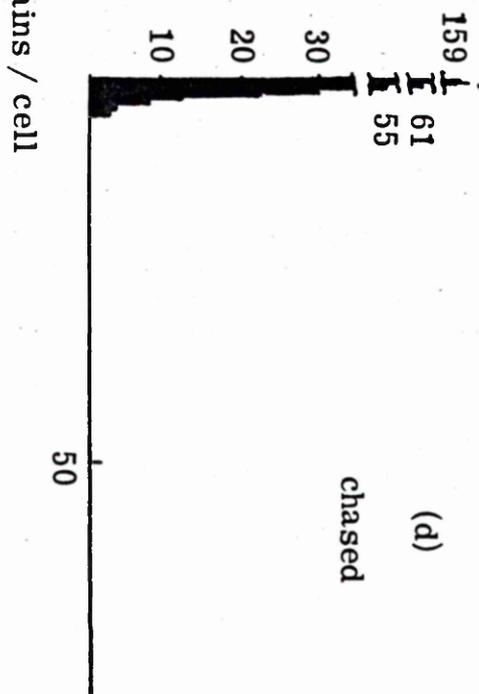
Cells

grains / cell

the labelled acid soluble pool in donor cells, and not labelled

RNA, which is required for the transfer of label from donor to

recipient cells, and the formation of the typical gradients of

label observed in autoradiographs.

It is possible that the reduction in the extent of transfer

in the experiment described above is due to some effect of the

radioactive label on the donor cells during the long chase.

However, when $[^3H]$ uridine prelabelled donor cells, chased for 20h

or 68h, were pulse labelled again with $[^3H]$ uridine for 4h, and

co-cultured for 3h, in the presence of excess unlabelled uridine,

with unlabelled recipient cells, extensive transfer was observed

(Fig. 12).

The results above show that the transfer of donor labelled

acid soluble pool to recipient cells, with its subsequent

incorporation into recipient RNA, explains the appearance of at

least the vast majority of label in recipient cells.

3.1.6. Estimation of the maximum extent of RNA transfer between
cells.

Analysis of the autoradiograph (Fig. 13a) of a 43h chase

co-culture, after a long exposure time, shows that the small

amount of label transferred to recipient cells is located in the

nucleoli, nucleus and cytoplasm of the cells. This suggests that

it is the transfer to recipient cells of part of the small residual

labelled acid soluble pool, still present in donor cells after the

chase (Table 12), which explains the light labelling of recipient

cells, and not the transfer of low levels of labelled RNA.

This result, and those obtained with actinomycin D (Section

3.1.3), show that little or no RNA is transferred between cells.

To estimate the maximum quantity of RNA which might be transferred

from donor to recipient cells, a combined chase and actinomycin D

Fig. 12

Effect of a long chase on the ability of labelled donor cells

to interact
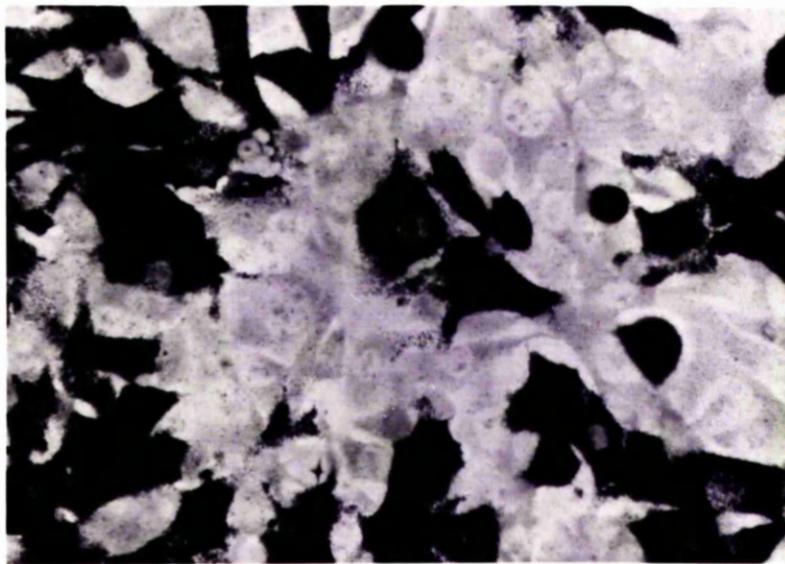
(a)

1×10^4 TG$_2$ cells (donors) were plated out in 35mm dishes and the cultures were grown for 16h. 4×10^5 B$_1$ cells (recipients) were plated out in 35mm dishes at the same time or 48h later and the cultures grown until confluent. Donor cells were labelled for 4h with $\left[^3\text{H}\right]$uridine (2.5µCi/ml). The cells were washed x 3 with BSS (37°C) and cultured in EFC 10 (2ml) for 20h or 68h. One donor culture at each chase time was pulsed again with $\left[^3\text{H}\right]$uridine (2.5µCi/ml). The other donor cultures were chased for a further 4h to give 24h and 72h chase control cultures. Donor and recipient cells were co-cultured together (Section 2.2.3.) for 3h in the presence of 0.04 mg/ml uridine. Cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 80 days. Magnification x 300 (a,b); x 480 (c,d).

a) 4h label: 24h chase; b) 4h label: 20h chase: 4h label;

c) 4h label: 72h chase; d) 4h label: 68h chase: 4h label.
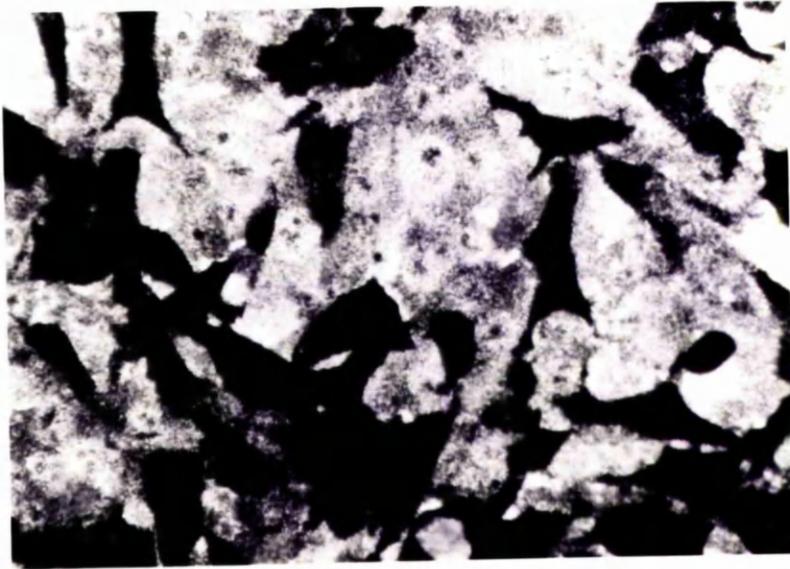
(b)

(c)

(d)

Fig. 13

Effect of a combined chase plus actinomycin D treatment on
transfer of [^{3}H]uridine labelled molecules between cells

For details, see Legend to Fig. 10(b). Recipient cells were
incubated with actinomycin D (0, 1µg/ml) for 30 min before
the trypsinisation and co-culture step. Donor and recipient
cells were co-cultured together for 3h in the presence of
0.04mg/ml uridine and actinomycin D (0, 1µg/ml). Cells were
processed for autoradiography. Autoradiographs were developed
after 69 days. Magnification x 480.

    a) 4h label: 43h chase control;

  b,c) 4h label: 43h chase + actinomycin D.
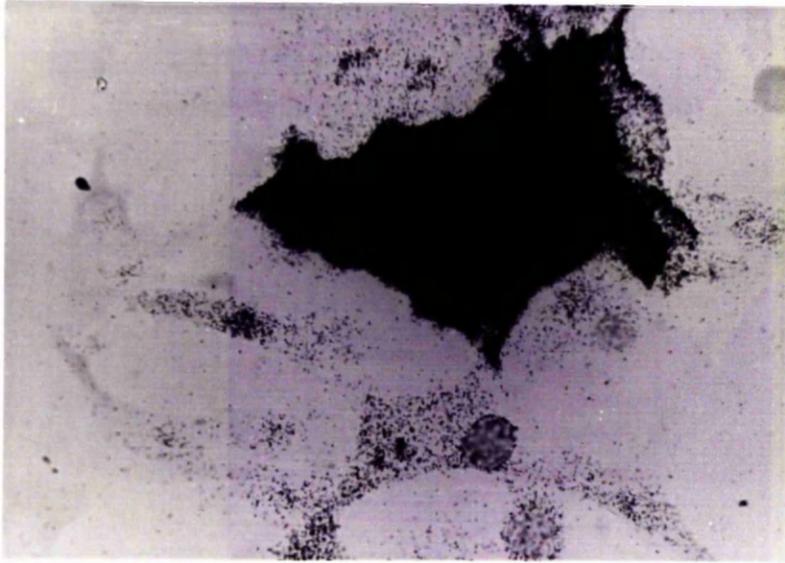
(a)
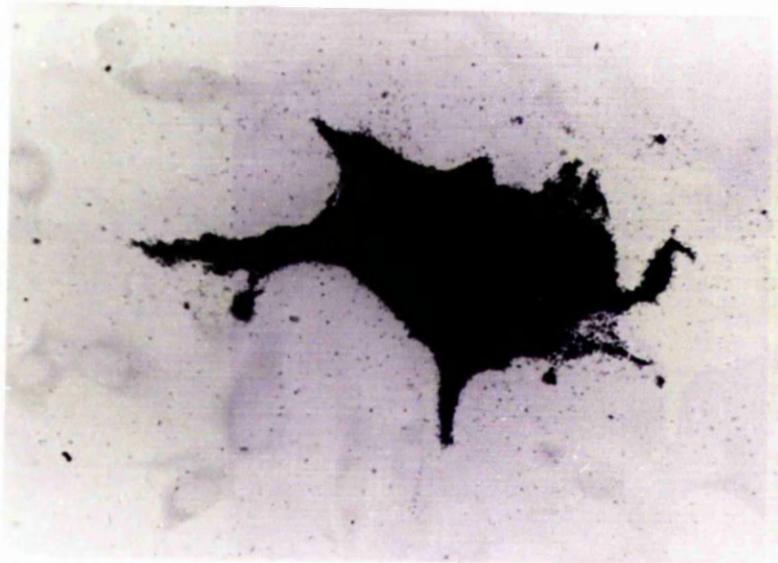
(b)
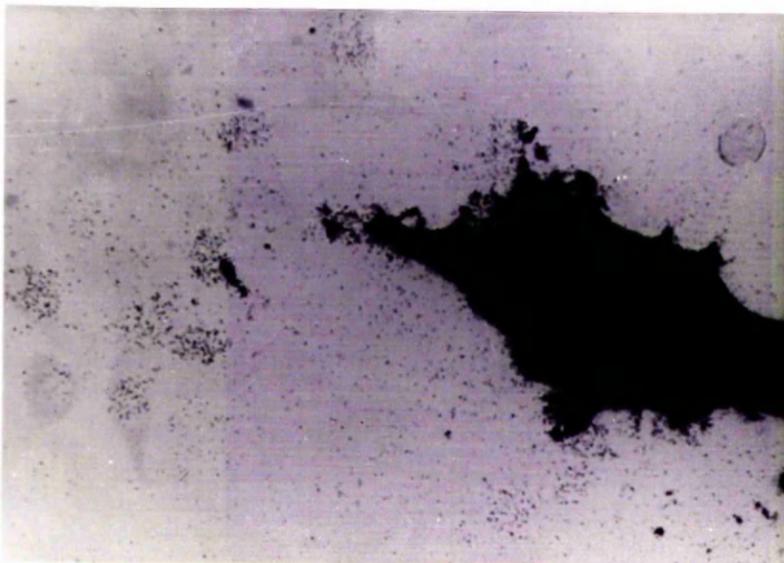
(c)

treatment were used.

Donor cells, pulse labelled with $[^3H]$uridine for 4h, were
chased to deplete the labelled acid soluble pool, and then
co-cultured for 3h, in the presence of actinomycin D (0, 1 µg/ml)
and excess unlabelled uridine, with unlabelled recipient cells,
pretreated for 30 min with actinomycin D (0, 1 µg/ml). The
autoradiographs (Fig. 13) show that the small amount of label,
transferred from donor to recipient cells in the control chased
co-culture without actinomycin D (Fig. 13a), is virtually
eliminated in the actinomycin D treated mixed culture (Fig. 13b).
However, Fig. 13(c) shows that very small amounts of label can
still be transferred from donor cells to some recipient cells,
in the actinomycin D treated co-culture. This label is located
in the nuclei of recipient cells and evidence (Section 3.2.3.)
suggests that the label is in DNA. The extent of transfer of
labelled RNA from donor to recipient cells was estimated by grain
counting to be no greater than 0.4% of the total label transferred
from donor to recipient cells, and this 0.4% includes
incorporation of labelled acid soluble pool, transferred from
donor cells, into DNA in recipient cells.

3.1.7. Transfer of $[^3H]$uridine labelled molecules between 3T3 cells.

Since metabolic co-operation has been shown between 3T3 cells
and BHK cells (Pitts, 1972), it is unlikely that a different class
of labelled molecules is transferred to or from 3T3 cells, compared
to that transferred to or from BHK cells. However, since Kolodny's
results were obtained using 3T3 cells, it was of interest to
confirm that a labelled acid soluble pool is also transferred
between 3T3 cells.

A 1:1 mixture of 3T3 donor cells, prelabelled with $[^3H]$uridine,
and unlabelled 3T3 recipient cells were co-cultured together in the

presence of excess unlabelled uridine for 3h. The autoradiograph

(Fig. 14) shows that transfer of label between the 3T3 cells

could be detected. The nucleoli in the recipient cells are heavily

labelled, suggesting that labelled acid soluble pool is indeed

being transferred to recipient cells, and is being incorporated

into rRNA.

To distinguish between this suggested labelled acid soluble

pool transfer, and the transfer of labelled RNA (as suggested by

Kolodny), donor cells, pulse labelled with $[^3H]$ uridine for 4h, were

chased 20h to deplete the labelled acid soluble pool. The donor

cells were then co-cultured for 3h, in the presence of excess

unlabelled uridine, with unlabelled recipient cells.

Autoradiographs show that the transfer of label from donor cells

to recipient cells is considerably reduced in the chase

co-culture (Fig. 15b), compared to the quantity of label transferred

in the control co-culture, without a chase (Fig. 15a).

This therefore suggests that, as with BHK cells, there is

no significant transfer of RNA between 3T3 cells, and that

transfer of labelled acid soluble pool from donor cells to

recipient cells, with its subsequent incorporation into recipient

cells, probably accounts for all the label present in the

recipient cells.

3.1.8. Comparison of the characteristic features of $[^3H]$ uridine
labelled acid soluble pool transfer and metabolic co-operation.

Available evidence from experiments on metabolic co-operation

suggests that nucleotides are transferred between cells (see

section 1.2.7.). Evidence presented above also shows that the

labelled uridine acid soluble pool, but not labelled RNA, is

transferred between cells. The characteristics of these two types

of transfer between cells will be examined.

Fig. 14

Transfer of $[^3H]$ uridine labelled molecules between 3T3 cells

3.5×10^6 3T3 cells (donors and recipients) were plated out in
35mm dishes, and the cultures were grown until they were
confluent. A 1:1 co-culture of donor and recipient cells was
formed as in Section 2.2.2. Donor cells were labelled for 4h
with $[^3H]$ uridine (5µCi/ml). Donor and recipient cells were
co-cultured together for 5h in the presence of 0.04mg/ml uridine.
Cells were processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 43 days. Magnification x 300.

Fig. 15

Effect of reduced [3H]uridine labelled donor acid soluble

pool on the transfer of label between 3T3 cells

1×10^4 3T3 cells (donors) were plated out in 35mm dishes.
1×10^6 3T3 cells (recipients) were plated out in 35mm dishes
at the same time and also 24h later. The donor cultures were
grown for 16h. Co-cultures were formed as in Section 2.2.3.
Donor cells were labelled for 4h with [3H]uridine (2.5μCi/ml).
After the donor cultures were washed free of label, one
culture was co-cultured for 3h with unlabelled recipient cells
in the presence of 0.04mg/ml uridine (zero time control). The
remaining donor culture was cultured in EFC 10(2ml) for 20h,
and co-cultured for 3h with unlabelled recipient cells in the
presence of 0.04mg/ml uridine. Cells were processed for
autoradiography as in Section 2.2.5.1. Autoradiographs were
developed after 60 days. Magnification: a) x 480; b) x 300.

a) 4h label: no chase;   b) 4h label: 20h chase.
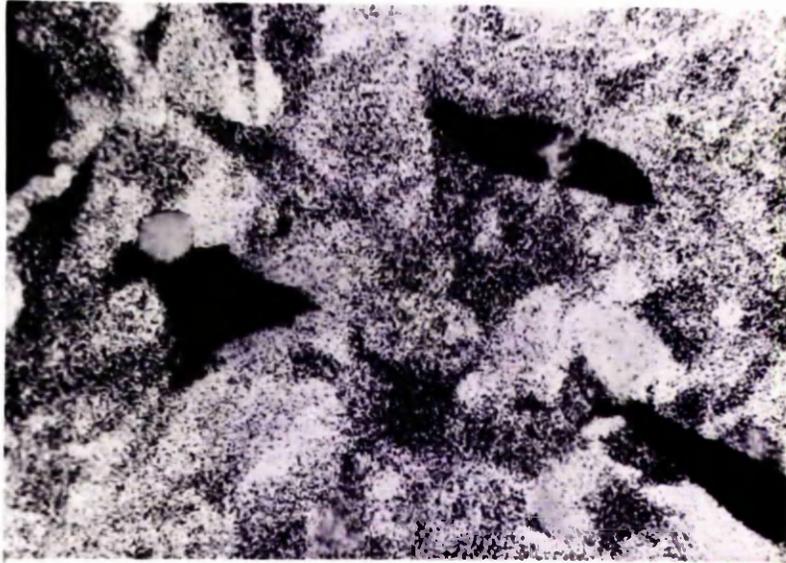
(a)
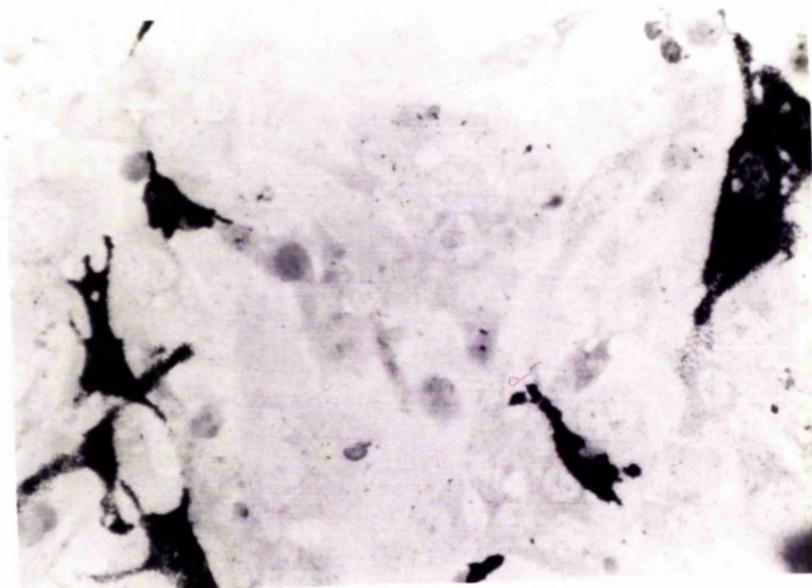
(b)

3.1.8.1. Extent of BHK, HeLa and L cell interactions.

Pitts (1972), studying metabolic co-operation between
BHK/BHK, HeLa/BHK and L/BHK cells, reported that there are
quantitative differences in the extent of interaction between cells
(Table 3).

Transfer of label between BHK/BHK, HeLa/BHK and L/BHK cells
was studied by co-culturing cells (either BHK, HeLa or L),
prelabelled with $[^3H]$uridine, with unlabelled BHK cells.
Autoradiographs (Fig. 16a,b,c) were prepared. Quantitation of the
extent of interaction (Table 13) shows that there is good transfer
between BHK/BHK cells, intermediate transfer between HeLa/BHK
cells and virtually no transfer between L/BHK cells. Therefore, the
extent of interaction between cells due to labelled uridine acid
soluble pool transfer is similar to that due to metabolic
co-operation.

The inability of L cells to show metabolic co-operation is
dominant in mixed cultures (Pitts, 1971). Fig. 16(a,c) also shows
that the inability of L cells to transfer the $[^3H]$uridine labelled
acid soluble pool is dominant in mixtures of L cells and BHK cells.

3.1.8.2. Direct transfer of label from cell to cell.

Evidence has been presented (see Section 1.2.2.) to show that
direct contact between the cells is required for metabolic
co-operation. Direct contact between donor and recipient cells is
also apparently required for the transfer of $[^3H]$uridine labelled
acid soluble pools to occur, as shown in many of the figures.
Fig. 17(a) shows that where a recipient cell is connected to a
donor cell by only a thin cytoplasmic bridge, transfer can still
occur quite efficiently. In the absence of apparent direct contact,
transfer does not occur (Fig. 17b).

Therefore, all the available evidence suggests that the

Fig. 16

Extent of BHK, HeLa and L cell interactions

1×10^4 donor cells (BHK (TG$_2$), HeLa, L) were plated out in 35mm dishes and the cultures were grown for 18h. A confluent culture of TG$_2$ cells, in a 8oz bottle, was used as recipient cells. Co-cultures were formed as in Section 2.2.3. Donor cells were labelled for 4h with [^{3}H]uridine (2.5μCi/ml). Donor and recipient cells (4×10^5) were co-cultured together for 3h. Cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 20 days. Magnification x 170.

a) BHK/BHK; b) HeLa/BHK; c) L/BHK.

(a)
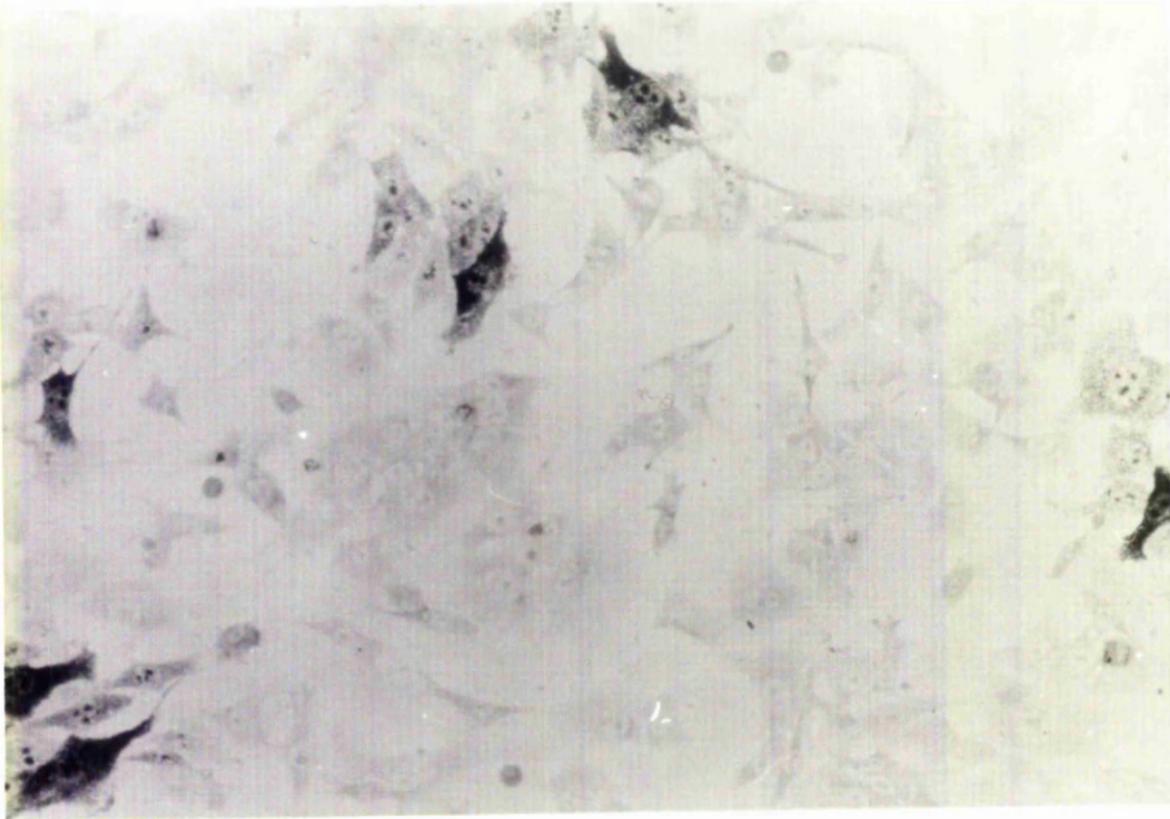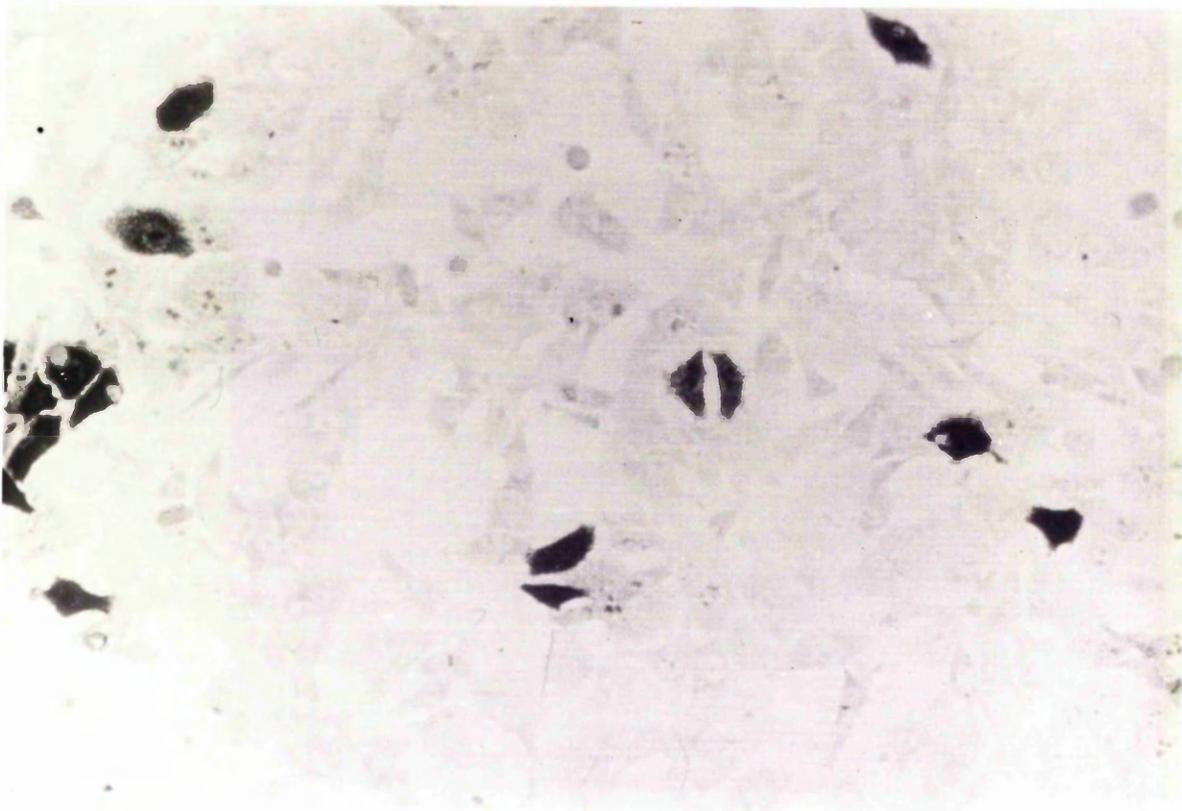
Table 13

Quantitation of the extent of interaction of various cell lines

(donors) with BHK cells (recipients)

Donor cells	Pattern of label in recipient cells (%)		
	Heavy	Light	Background
BHK	99	1	—
HeLa	75	22	3
L	—	3	97

See Legend to Fig. 16 for details of preparation of the

autoradiographs. The extent of interaction of donor cells was

quantitated as follows:-

Heavy: heavily labelled recipient cells

in contact with donor cells. cf. Fig. 33(b);

Light: lightly labelled recipient cells

in contact with donor cells. cf. Fig. 33(c);

Background: background grains only in

recipient cells in contact with donor cells. cf. Fig. 33(a,d).

At 'least 300 donor cells were counted for each cell line.

Fig. 17

Requirement of direct cell-to-cell contact for transfer of
label between cells

(a)

(b)
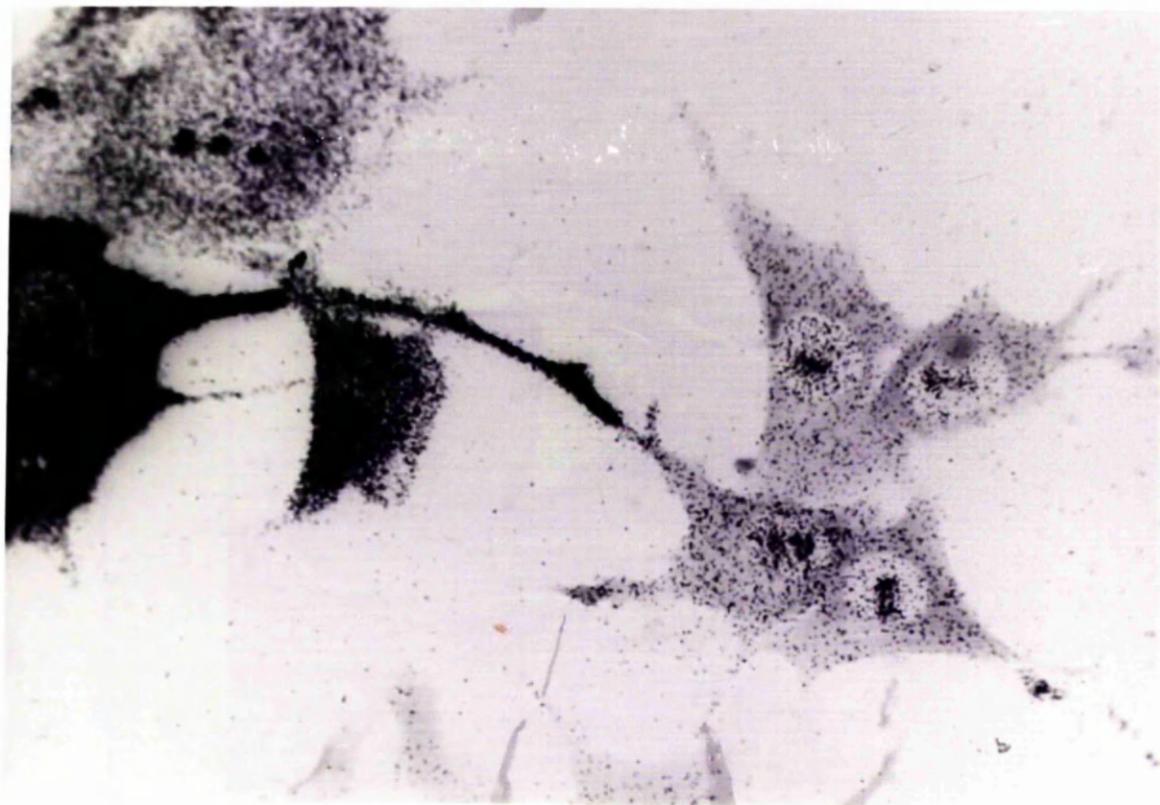
See Legend to Fig. 6a,b. Magnification x 700.

characteristic features of metabolic co-operation between cells are very similar to those shown by $\left[^3H\right]$uridine labelled acid soluble pool transfer between cells. This strongly suggests that the two phenomena are mediated by the transfer of the same types of molecules.

3.1.9. Nature of the molecules transferred between cells.

Considerable evidence has been presented above to show that it is the acid soluble pool which is transferred between cells. This pool consists of nucleosides and nucleotides. Nucleotides cannot pass from cell to cell without either loss of the phosphate group (Leibman & Heidelberger, 1955; Subak-Sharpe, 1969), or using some "special transfer mechanism".

Since excess unlabelled uridine did not significantly decrease the quantity of label transferred to recipient cells (Section 3.1.1.), this eliminates medium transfer of $\left[^3H\right]$uridine between cells as the source of recipient cell label (also, see Section 3.1.8.2.). Also, excess unlabelled uridine probably dilutes any intracellular nucleoside pools in the cells, so that direct transfer of $\left[^3H\right]$uridine between cells is unlikely to be the basis for the label observed in recipient cells.

Further evidence against direct transfer of labelled nucleosides (and bases) between cells could be obtained from the study of the transfer of a $\left[^3H\right]$hypoxanthine labelled acid soluble pool, present in BHK-IPP$^+$ cells, to BHK-IPP$^-$ cells. However, it was necessary first to show that such a pool could be transferred to, and incorporated into, the BHK-IPP$^-$ cells.

BHK-IPP$^+$ cells, prelabelled with $\left[^3H\right]$hypoxanthine for 4h, were co-cultured for 3h, in the presence of actinomycin D (0, 1 μg/ml), with unlabelled BHK-IPP$^-$ cells, pretreated with actinomycin D (0, 1 μg/ml) for 30 min. Autoradiographs were prepared. Transfer

of label to recipient cells in the untreated co-culture occurs

(Fig. 18a), although the extent of transfer is small, compared

to the $[^3\text{H}]$ uridine level, due to the low specific activity of the

$[^3\text{H}]$ hypoxanthine. Fig. 18(b) shows that very little label is

transferred between cells in the actinomycin D treated co-culture.

Therefore, the label present in recipient cells (Fig. 18a) is due

to transfer of labelled acid soluble pool from BHK-IPP$^+$ cells,

and its incorporation into BHK-IPP$^-$ cells, and is not due to

labelled polynucleotide transfer.

The presence of label in IPP$^-$ cells (Fig. 18a) must be due

to either a) transfer of labelled nucleotides from IPP$^+$ cells,

and their incorporation into recipient cells, or

b) transfer of both labelled nucleosides (or base) and

IPP$^+$ enzyme (or information to make the enzyme) from IPP$^+$ cells

to IPP$^-$ cells.

Available evidence suggests that a functional IPP$^+$ enzyme

is not present in IPP$^-$ cells, cultured in contact with IPP$^+$ cells.

(Section 1.2.6.). This shows that nucleotides are the molecules

which are transferred between cells.

A "special mechanism" must therefore be invoked for

nucleotide transfer between cells. Gap junctions have been

suggested to be the site of transfer of molecules between cells

and these could provide this "special mechanism".

Fig. 18

Transfer of $[^3H]$hypoxanthine labelled acid soluble pool from

donor cells to recipient cells
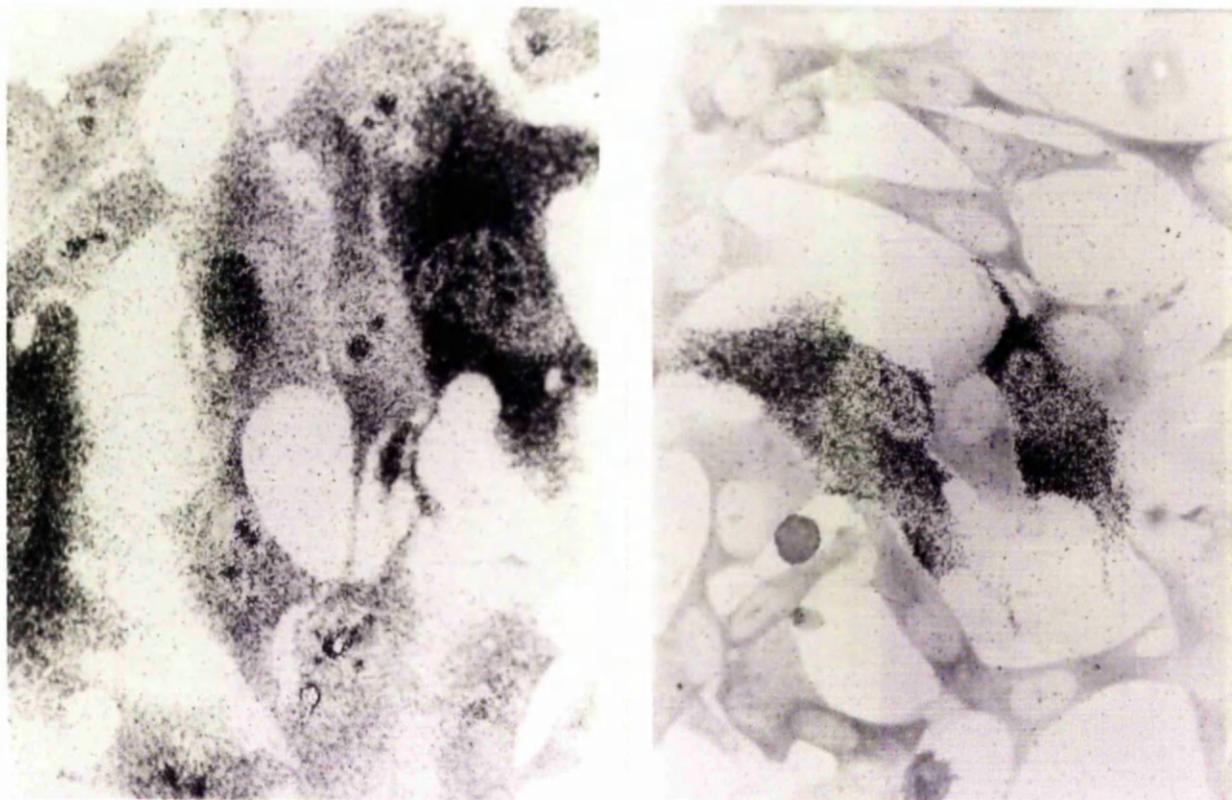
(a)   (b)

1×10^4 BHK-IPP$^+$ cells (B_1; donors) were plated out in 35mm dishes
and the cells were grown for 18h. 4×10^5 BHK-IPP$^-$ cells (TG_2;
recipients) were plated out in 35mm dishes. Co-cultures were
formed as in Section 2.2.3. BHK-IPP$^+$ cells (donors) were labelled
for 4h with $[^3H]$hypoxanthine ($5\mu Ci/ml$). BHK-IPP$^-$ cells (recipients)
were incubated with actinomycin D (0, $1\mu g/ml$) for 30 min before
the trypsinisation and co-culture step. Donor and recipient cells
were co-cultured together for 3h in the presence of actinomycin D
(0, $1\mu g/ml$). Cells were processed for autoradiography as in
Section 2.2.5.1. Autoradiographs were developed after 26 days.
Magnification x 480.

    a) Untreated culture;   b) actinomycin D treated culture.

3.2. Transfer of DNA precursors between cells.

3.2.1. Is DNA transferred between cells?

Although it is generally accepted that DNA is not commonly transferred between cells (Section 1.2.7.), there is no direct evidence against such transfer, and various reports have presented evidence for the movement of DNA and chromosomes from cell to cell (Lindholm & Britton, 1967; Bendich et al., 1967). Therefore, the suggestion that the nuclear label found in BHK-TK⁻ cells, growing in contact with BHK-TK⁺ cells in the presence of $[^3H]$thymidine, is due to labelled thymidine nucleotide transfer, and not labelled DNA transfer, requires critical examination.

Since $[^3H]$thymidine labels the acid soluble thymidine nucleotide pool, as well as the DNA, the labelled acid soluble pool must be depleted by further culture in the absence of $[^3H]$thymidine, before DNA transfer, in the absence of possible nucleotide transfer, can be studied. It was found that the pool decays rapidly, and after a 2h chase, it falls to 6% of the original value at the start of the chase (Table 14).

Donor cells were prelabelled with $[^3H]$thymidine for 20h which allowed most cells to pass through the S phase (doubling time of donor cells under these conditions is 17h). A 10h chase depleted the label in the acid soluble pools, and the cells were then co-cultured for 3h with unlabelled recipient cells. There is no label present in the nuclei of the recipient cells adjacent to the heavily labelled donor cells (Fig. 19), showing that there is no detectable transfer of DNA between cells. This is consistent with the earlier suggestion that thymidine nucleotide transfer between cells is the basis of metabolic co-operation between TK⁺/TK⁻ cells. However, direct evidence for thymidine nucleotide transfer between cells is still required.

Table 14

Effect of a chase on the size of $[^3H]$ thymidine labelled acid soluble pool

Length of chase (h)	Acid soluble pool	
	d.p.m.	% zero chase value
0	61,316	100
0.5	10,418	17
1	5,137	8.4
2	3,599	5.9
10	981	1.6

Each determination an average of 2 dishes.

1×10^5 TG$_2$ cells were plated out in 35mm dishes and grown for 16h. Cultures were labelled for 1h with $[^3H]$ thymidine ($5\mu Ci/ml$; $1.25 \times 10^{-6}M$). The medium was removed and each culture was washed x 3 with BSS ($37^\circ C$). Two cultures were harvested at this time (zero time). The remaining cultures were chased for 0.5, 1, 2 or 10h. The level of radioactivity present in the acid soluble pool at the end of these chase times was determined as in Section 2.2.8.

Fig. 19

No transfer of DNA between BHK cells
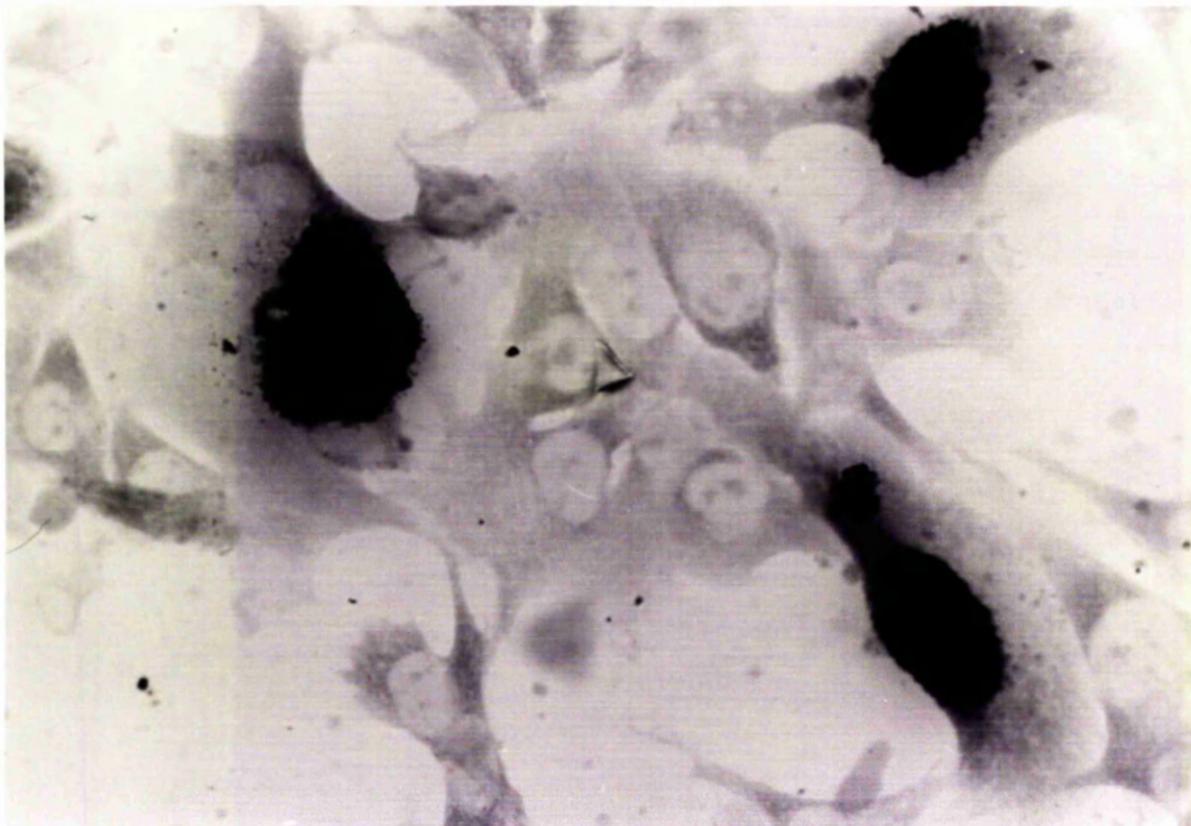
7×10^5 BHK-TK$^-$ cells (B$_1$) were plated out in a 50mm dish. .

1×10^4 BHK-TK$^+$ cells (TG$_2$) were plated out in a 50mm dish and

the culture was grown for 17h. BHK-TK$^+$ cells were labelled for

20h with [^{3}H]thymidine (3.3μCi/ml; 8×10^{-7}M). The BHK-TK$^+$ cells

were washed x 3 with EFC 10 (3ml; 37°C) and the cells incubated

in 3ml EFC 10 for 10h. A co-culture between BHK-TK$^+$ cells (donors)

and BHK-TK$^-$ cells (recipients) was formed as in Section 2.2.3.

BHK-TK$^+$ cells and BHK-TK$^-$ cells were co-cultured together for 3h.

The cells were processed for autoradiography as in Section 2.2.5.1.

Autoradiographs were developed after 15 days. Magnification x 700.

3.2.2. Thymidine nucleotide transfer between cells.

The possible transfer to recipient cells of $[^3H]$thymidine labelled acid soluble nucleotide pools, present in donor cells, was investigated.

Donor cells (BHK-TK$^+$) were grown in the presence of $[^3H]$thymidine for 22h to mark the cells and to label the acid soluble nucleotide pool of the S phase cells. The donor cells were washed with BSS to remove the unincorporated $[^3H]$thymidine, and then co-cultured with unlabelled recipient cells (BHK-TK$^-$) for 30, 60 and 120 min. The cells were prepared for autoradiography by a method which allows detection of acid soluble material (Section 2.2.5.2.), to increase the sensitivity of the experiment. Fig. 20 shows labelled recipient cells around a donor cell after 30 min co-culture. The label present in recipient cells could be due to labelled thymidine nucleotide transfer, labelled thymidine transfer or labelled DNA transfer from donor cells. However, labelled DNA is not transferred from donor to recipient cells (Section 3.2.1.). Also, labelled thymidine transfer does not occur because 1) thymidine should pass as easily out of the cells, as between cells. Therefore, recipient cells distant from donor cells should be labelled. This is not found.   2) if labelled thymidine is transferred to recipient cells (BHK-TK$^-$), it will not be incorporated into DNA, unless the TK$^+$ enzyme is also transferred. However, there is no evidence for the transfer of specific enzymes (se Section 1.2.6.) or of proteins (Section 3.3.1.) between cells. On the other hand, labelled thymidine nucleotides transferred to the BHK-TK$^-$ recipient cells would be incorporated into DNA.

Therefore, it appears that thymidine nucleotides are transferred from donor cells to recipient cells and are incorporated into recipient cell DNA.

Fig. 20

Transfer of $[^3H]$ thymidine labelled acid soluble pool between

BHK cells
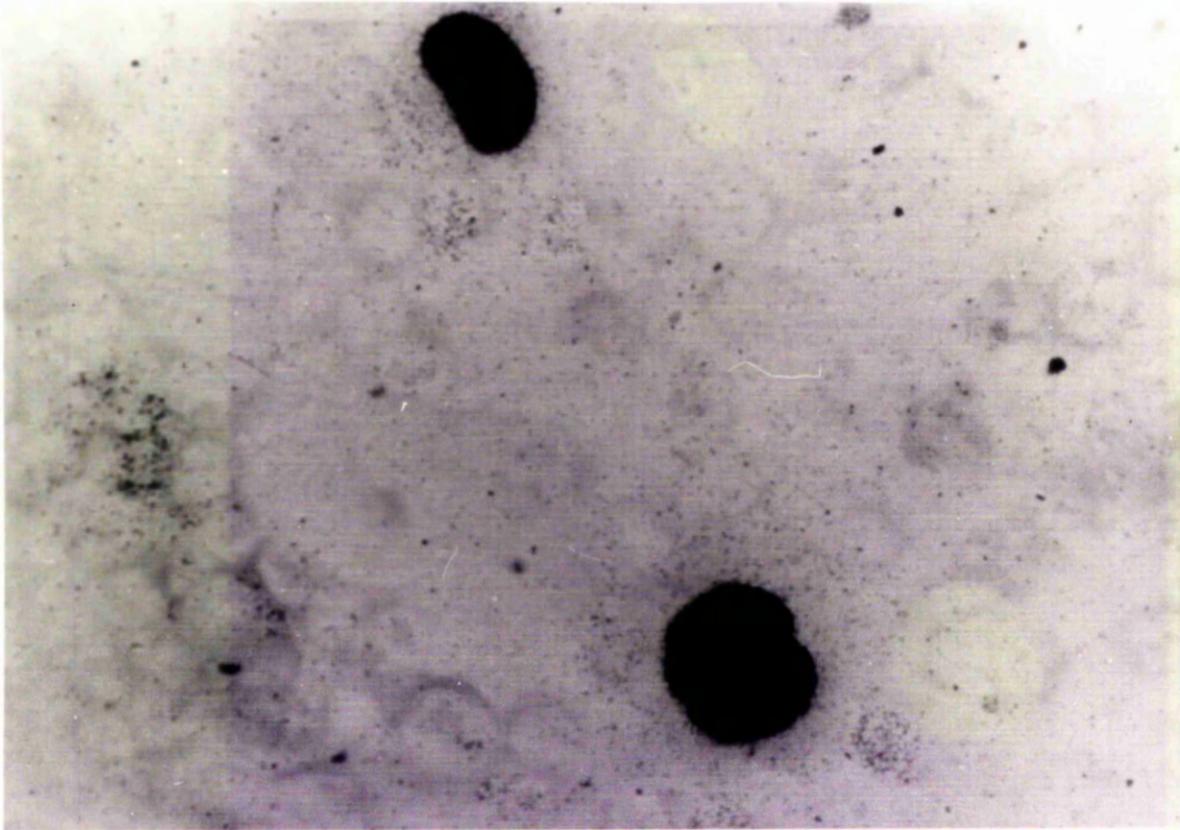
1×10^4 BHK-TK$^+$ cells (TG$_2$; donors) were plated out in 35mm

dishes and the cultures were grown for 16h. A subconfluent

culture of BHK-TK$^-$ cells (B$_1$), growing in a 8oz bottle, was used

as recipient cells. BHK-TK$^+$ cells were labelled for 22h with

$[^3H]$ thymidine (2μCi/ml; 1.25×10^{-6}M). Trypsinisation of the

recipient cells, and co-culture of the donor and recipient cells

$(1.25 \times 10^6$/dish) was as in Section 2.2.3. Co-culture was

continued for 30 min, 60 min and 120 min. Cells were processed

for autoradiography as in Section 2.2.5.2. Autoradiographs were

developed after 62 days. Magnification x 440.

Fig. 20; 30 min co-culture.

3.2.3. Labelling of recipient cell DNA by nucleotide transfer
from $[5\text{-}H^3]$ uridine labelled donor cells.

An anomalous incorporation into recipient cell nuclear
acid insoluble material is observed (e.g. Fig. 13c) when donor
cells, prelabelled for 4h with $[5\text{-}H^3]$ uridine, are co-cultured,
in the presence of actinomycin D (1 µg/ml) and excess unlabelled
uridine, with unlabelled recipient cells, pretreated for 30 min
with actinomycin D (1 µg/ml). Since only some recipient cells
contain predominant nuclear label (Fig. 21b), this suggests that
the label may be in DNA as only some recipient cells would be
present in the S phase of the cell cycle during co-culture. A
similar geometry of labelled cells, to that above, has already
been described for metabolic co-operation between BHK-TK$^+$/BHK-TK$^-$
cells (Section 3.1.3.3.), and for thymidine nucleotide transfer
between cells (Section 3.2.2.). Therefore, the nature of the
molecules labelled by $[5\text{-}H^3]$ uridine was examined. It was shown
that label was incorporated into RNA and a little into DNA.

Cultures of cells were labelled for 4h with $[5\text{-}H^3]$ uridine.
After removal of the acid soluble pool, followed by washing with
distilled water, the cells were incubated with RNase (0, 10 µg/ml)
for 48h at 37°C. Autoradiographs were prepared. Fig. 22(a) shows
an untreated culture with heavily labelled cells, containing
prominent nucleolar labelling. Fig. 22(b) shows that after RNase
digestion, heavily labelled nuclei still remain in some cells,
although label in the cytoplasm and nucleoli has been reduced
to background level. This is in agreement with Comings (1965),
who labelled human fibroblasts with $[5\text{-}H^3]$ uridine, and showed
that RNase digestion left label only in the nucleus (not in
nucleolus) of 25% of the cells. This remaining label was sensitive
to DNase digestion. Therefore, he concluded that $[5\text{-}H^3]$ uridine
could label DNA.

Fig. 21

Presence of label, transferred from [3H]uridine labelled donor cells, in the nuclei of some recipient cells.

(a)

(b)
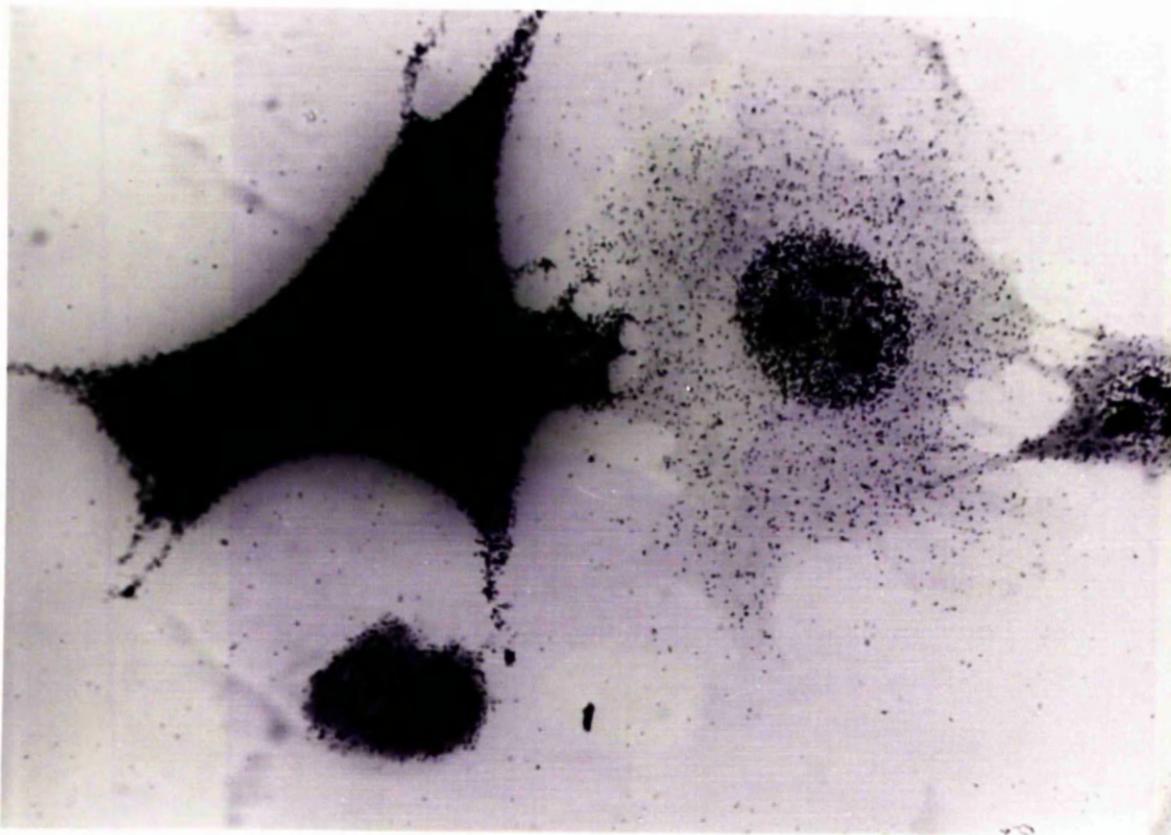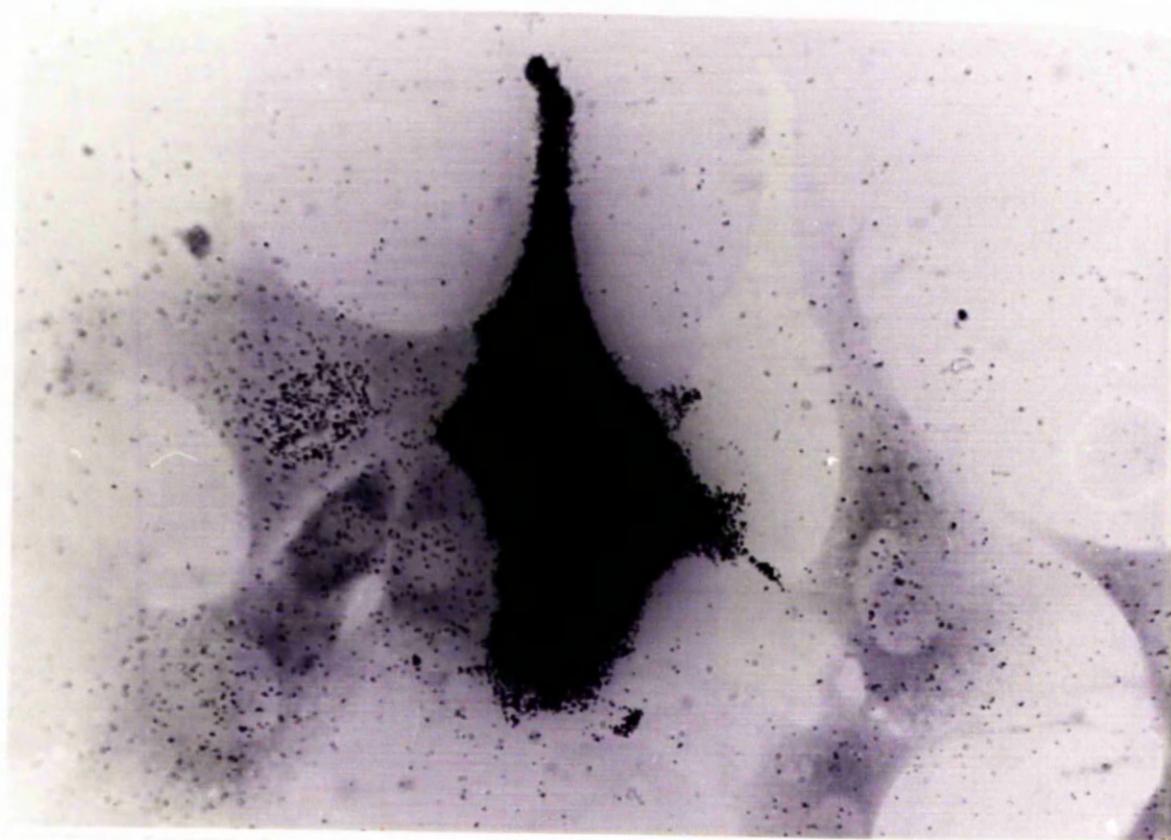
See Legend to Fig. 6(a,b,c). Magnification x 700.

a) control untreated co-culture (+ uridine);

b) actinomycin D treated co-culture (+uridine).

Fig. 22

Effect of RNase on $[5-H^3]$ uridine labelled cells
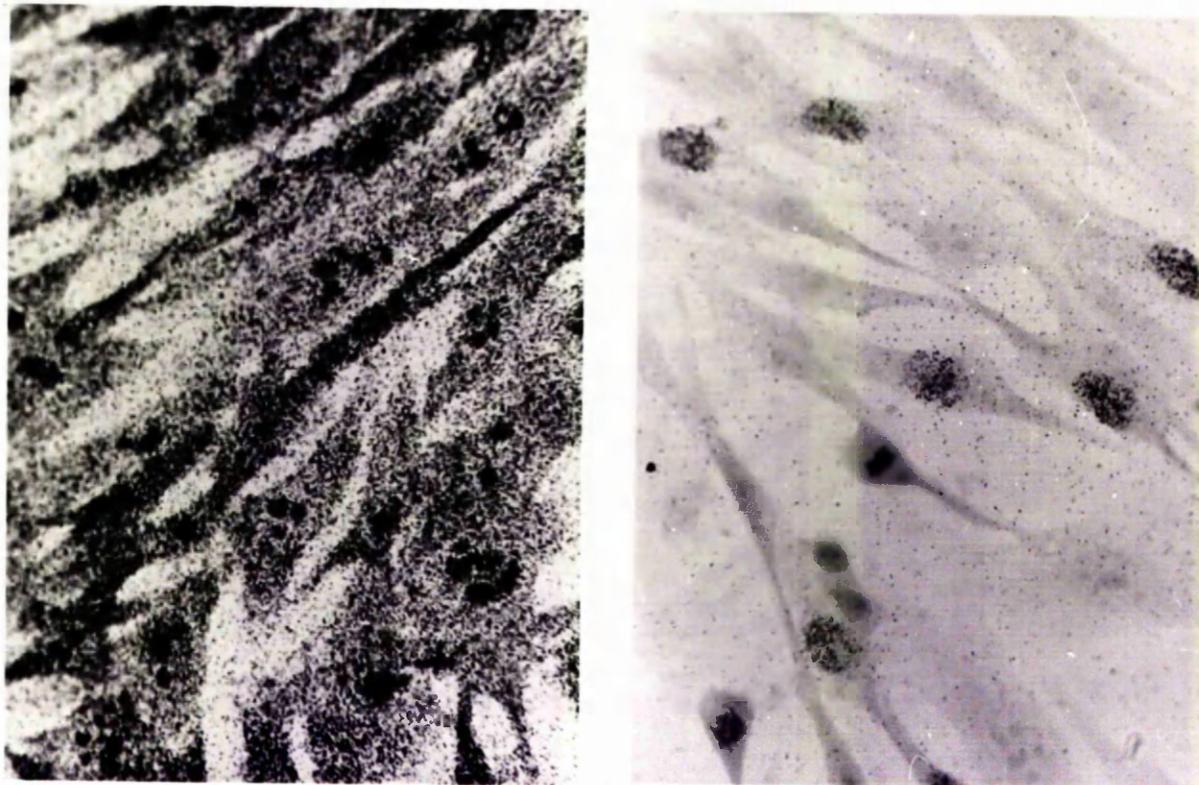
(a)   (b)

1×10^5 TG$_2$ cells were plated out in 35mm dishes, containing

3 coverslips, and the cultures grown for 18h. The cultures were

labelled for 4h with $[^3H]$ uridine (2.5μCi/ml). The cells (on coverslips)

were immersed in formol saline, and washed in 10% TCA and

distilled water, as in Section 2.2.5.1. Coverslips were placed

in vials containing RNase (0, 10μg/ml) and the vials (sealed)

were incubated at 37°C for 48h. The coverslips were washed in

10% TCA and distilled water, dipped in ethanol and dried at 37°C

(see Section 2.2.5.1.). Autoradiographs were prepared and

developed after 14 days. Magnification x 480.

   a) 0μg/ml RNase;   b) 10μg/ml RNase.

Uridine is probably converted to cytidine nucleotides
(Lieberman, 1956; Hurlbert & Kammen, 1960) in the cell, and these
can be incorporated into DNA. Therefore, $[5\text{-}H^3]$ uridine could be
incorporated into DNA by this pathway.

Although the nuclear labelling is much easier to observe
in the actinomycin D treated cultures (Fig. 21b), autoradiographs
of co-cultures in the absence of actinomycin D show nuclear
labelling in recipient cells (Fig. 6a, 21a). Since DNA is not
transferred between cells (Section 3.2.1.), this further shows
that labelled nucleotides are transferred from the donor cells to
the recipient cells and that these are incorporated into DNA.

3.2.4. An attempt to obtain a simplified quantitative system to
       measure the extent of label transferred between cells.

The method of grain counting is slow and laborious for
the quantitation of varying degress of interaction occurring
between different cell types. An alternative method would
facilitate the quantitation of nucleotide transfer between cells.

A quantitative assay could in theory be developed by
measuring the incorporation of prelabelled donor nucleotide pools
into the nucleic acid of mixed cultures of the donor and
unlabelled recipient cells, providing the incorporation of
labelled nucleotides into the nucleic acid of the donor cells
could be specifically inhibited. Then, the difference between
the incorporation of label into nucleic acid of the mixed cell
population (inhibited donor cells plus recipient cells) and
into the nucleic acid of a control population (inhibited donor
cells alone) would give a direct measure of nucleotide transfer
between these cells.

Hydroxyurea was selected as a inhibitor for the following
reasons:-

1) it is an effective inhibitor of DNA synthesis, while permitting continued synthesis of RNA and protein (Adams et al., 1966);

2) a site of action is known. It interfers with the enzyme ribonucleoside diphosphate reductase, which reduces ribonucleotides to deoxyribonucleotides (Adams et al., 1966);

3) it increases the size of the $[^3H]$dTTP pool compared to untreated cells (Adams & Lindsay, 1966; Shoog & Nordenskjöld, 1971);

4) although inhibition by hydroxyurea is reversible after removal of the hydroxyurea, the recovery of DNA synthesis is slow for the first 2h (Shoog & Nordenskjöld, 1971).

It may be predicted therefore, that after labelling hydroxyurea treated cells with $[^3H]$thymidine, they should contain a highly labelled acid soluble pool, and that during a subsequent chase in the absence of hydroxyurea, cellular DNA synthesis should recover slowly. Such cells could be used as donor cells for the quantitative assay of nucleotide transfer.

Cultures of donor cells were treated with hydroxyurea (6mM) for 3h (conditions which give 98% inhibition of DNA synthesis, measured by $[^3H]$thymidine uptake into labelled acid insoluble material). The donor cells were pulsed with $[^3H]$thymidine during the last 10 min of the hydroxyurea treatment. The hydroxyurea and unincorporated label were removed by washing the cultures with BSS. Two cultures were harvested to measure the $[^3H]$thymidine incorporation into DNA in the donor cells at this time (zero time). To 6 cultures, recipient cells in fresh medium were added and to a further 6 cultures, medium alone was added, and incubation continued. Duplicate control cultures and duplicate mixed cultures were harvested at ½h, 1h and 1½h. Label present in acid insoluble and acid soluble material was estimated as in Section 2.2.8.

Fig. 23(a) shows that the level of label incorporated into acid
insoluble material is the same at each time for both control and
mixed cultures. This suggests that after removal of hydroxyurea,
DNA synthesis in the donor cells recovered rapidly, and that
label is incorporated into both mixed and control cultures at a
similar rate. Fig. 23(b) shows that the labelled acid soluble pool
disappears at a similar rate in both mixed and control cultures.
However, a considerable fraction of the labelled acid soluble pool
(60%) is not incorporated into DNA, but is lost from the cell. A
similar loss (65%) of labelled acid soluble pool was observed from
L cells treated with hydroxyurea (Adams et al., 1971).

Another inhibitor of DNA synthesis, mitomycin C, which is
not rapidly reversible, also caused a considerable loss (92%)
of the prelabelled nucleotide pool from donor cells during the
chase. Therefore, although the two inhibitors used were
unsatisfactory, the method in principle is sound, if an effective
inhibitor, which does not cause a loss of labelled nucleotides
from the donor cells, can be obtained.

Fig. 23

Incorporation of a $\left[^3H\right]$ thymidine prelabelled acid soluble pool, present in hydroxyurea treated donor cells, in the absence of

hydroxyurea

2.3×10^5 TG_2 cells (BHK-TK$^+$; donors) were plated out in 50mm dishes and the cultures were grown for 18h. A subconfluent culture of BHK-TK$^-$ cells (B_1), growing in a 8oz. bottle, was used as recipient cells. Each donor culture was incubated for 3h in the presence of hydroxyurea (6mM). The donor cultures were pulsed with $\left[^3H\right]$ thymidine (2µCi/ml: 5×10^{-7}M) during the last 10 min of hydroxyurea treatment. The cultures were washed x 3 with EFC 10. Two cultures were washed x 5 with 5ml BSS (0^oC) at this time (zero time). Recipient cells (4.4×10^5) in 5ml EFC 10 were added to one half of the remaining cultures and 5ml EFC 10 alone was added to the other half of the cultures. Duplicate cultures of donors + recipients and donors alone were harvested after 30, 60 and 90 min incubation by washing x 5 BSS (0^oC). Label present in acid insoluble material and acid soluble pool was estimated as in Section 2.2.8.

a) Acid insoluble material; b) Acid soluble material.

— ———◎——— — : Donor cells alone.

————————◖——————— : Donor + recipient cells.

(a)

d.p.m.

50,000

40,000

30,000

20,000

10,000

0   0.5   1   1.5

Length of chase (h)

(b)

d.p.m.

100,000

80,000

60,000

40,000

20,000

0   0.5   1   1.5

Length of chase (h)
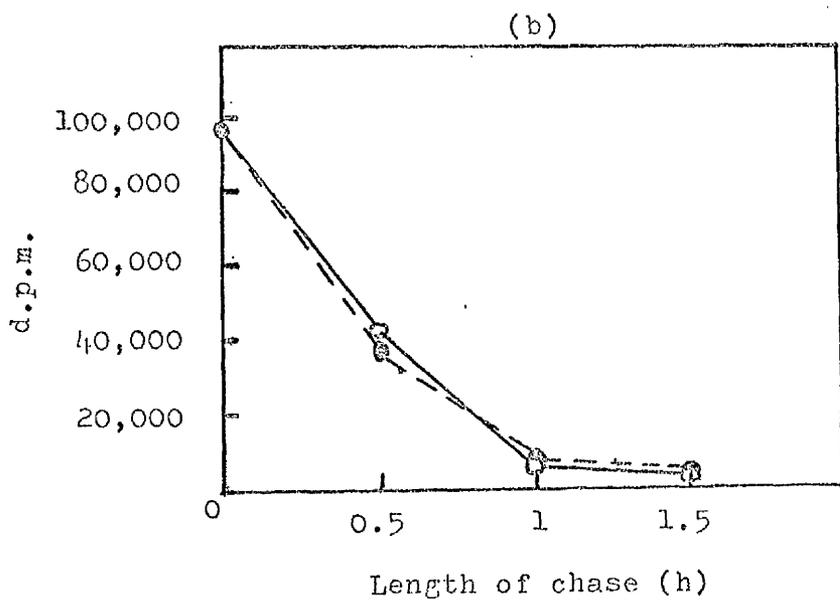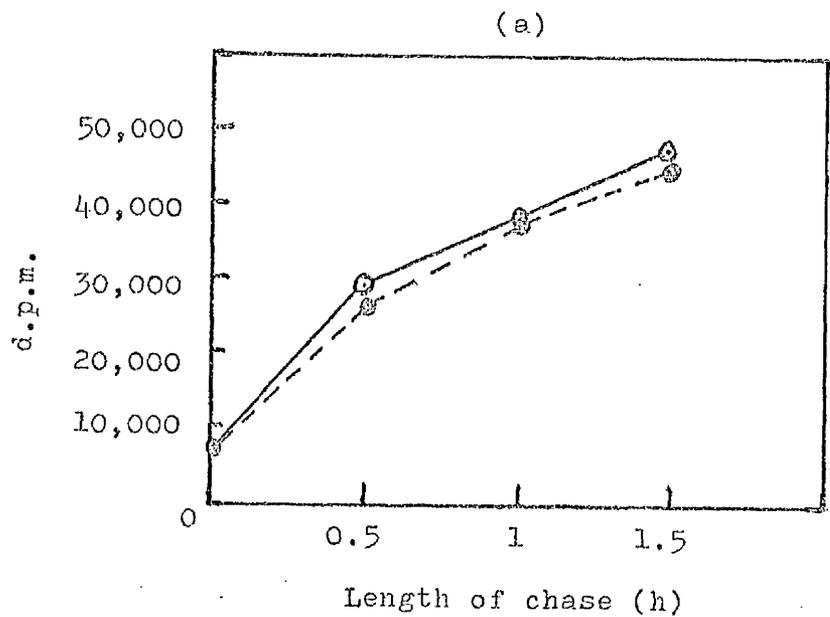
3.3. Transfer of other molecules between cells.

The available evidence (Sections 1.1.2., 3.1. & 3.2.) suggests that small molecules, such as dyes and nucleotides, can be transferred between cells, but that macromolecules such as RNA and DNA are not. Therefore, the possible transfer between cells of other small molecules (glucose labelled acid soluble pool) and other macromolecules (proteins) was investigated.

3.3.1. Is protein transferred between cells?

Although detectable levels of specific enzymes are not transferred between cells (Section 1.2.6.), it is possible that there is a general transfer of low levels of many proteins between cells.

Donor cells, prelabelled with $[^3H]$leucine for 4h (1% normal leucine medium), 12h (10% normal leucine medium) and 21h (normal medium) were co-cultured for either 5h or 24h, in the presence of x10 normal leucine medium, with unlabelled recipient cells. Even after a long autoradiographic exposure (91 days), no detectable transfer of label from donor cells to recipient cells was observed (Fig. 24a,b,c). Therefore, this suggests that there is no detectable (<0.02%; quantitated by grain counting) transfer of proteins between cells.

$[\text{Methyl-H}^3]$-methionine will also label proteins of the cell. However, since it can donate its methyl group for methylation of other molecules in the cell, it will also label to a minor extent DNA (Burdon & Adams, 1969), and methylated RNA molecules such as rRNA and tRNA.

Donor cells, grown for 18h in the presence of formate (2 mg/ml) to prevent methyl group incorporation into the purine ring skeletons, were prelabelled with $[\text{methyl-H}^3]$-methionine for 4h in the presence of 1% normal methionine medium and formate (2 mg/ml),

Fig. 24

No transfer of protein (^{3}H leucine labelled) between BHK cells

5×10^3 TG$_2$ cells (donors) were plated out in 35mm dishes and the cultures grown for 16h. 3.6×10^5 B$_1$ cells (recipients) and 2×10^5 B$_1$ cells (recipients) were plated out in 35mm dishes and grown for 24h and 48h respectively. Co-cultures were formed as in Section 2.2.3. Donor cells were labelled for 4h (in 1% normal leucine medium), 12h (in 10% normal leucine medium) and for 21h (in normal leucine medium) with ^{3}H leucine (2μCi/ml). Donor and recipient cells were co-cultured together for either 5h or 24h in the presence of x 10 normal leucine medium. Cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 91 days. Magnification x 480.

    a) 4h label: 5h co-culture;   b) 12h label: 24h co-culture;

    c) 21h label: 5h co-culture.

(a)
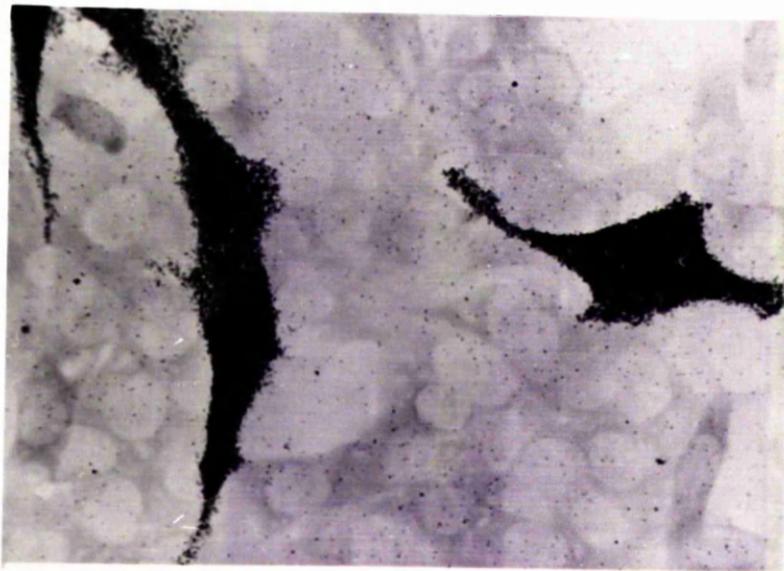
(b)

(c)
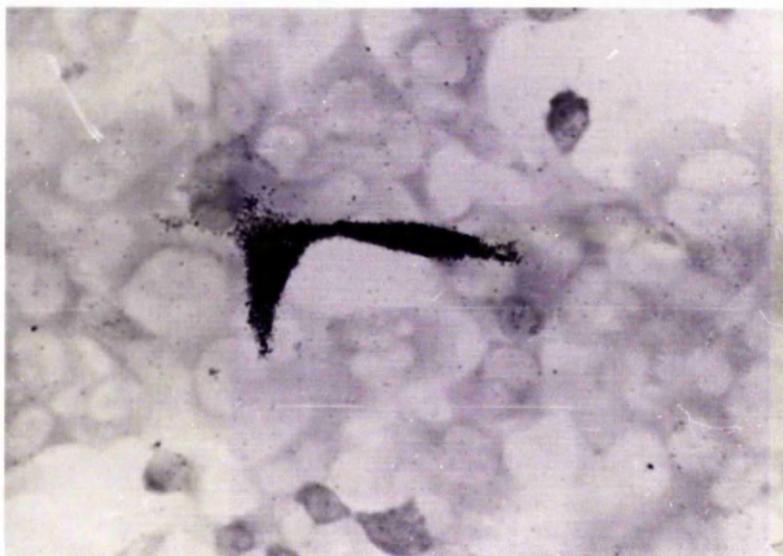
and then co-cultured for 5h, in x10 normal methionine medium with added formate (2 mg/ml), with recipient cells. The autoradiograph (Fig. 25) shows that there is no detectable transfer of label ($<$0.05%) from donor cells to recipient cells.

Therefore, there appears to be no transfer of proteins, labelled with either $[^3H]$leucine or $[methyl-H^3]$-methionine between cells.

3.3.2. Transfer between cells of molecules labelled with D-glucose-2-H^3.

Results presented in Sections 3.1. & 3.2. show that nucleotides can be transferred between cells. The possibility that other small phosphorylated molecules, such as phosphorylated sugars, may be transferred from cell to cell was studied. Labelled glucose was selected; however, since glucose can be metabolised in the cell by the glycolytic pathway to form many cellular intermediates, and since interest was centred on glucose phosphates or related compounds, D-glucose-2-H^3 was chosen. The metabolism of this labelled glucose molecule results in the loss of the tritium label during the reaction catalysed by phosphoglucoisomerase, an enzyme present in the glycolytic pathway.

Cultures of donor cells were prelabelled for 6h with glucose-2-H^3 in glucose free medium and co-cultured for 3h, in either glucose free medium or normal medium, with unlabelled recipient cells. Autoradiographs show that label is transferred from donor cells to adjacent recipient cells, whether unlabelled glucose is present (Fig. 26a) or absent (Fig. 26b) during co-culture.

The labelled material, present in recipient cells, could be due to either incorporated labelled acid soluble pool transferred from donor cells and/or labelled glycoproteins and glycolipids transferred from donor cells. However, transfer of glycogen, which

Fig. 25

No transfer of protein (methyl-H^3-methionine labelled)

between BHK cells
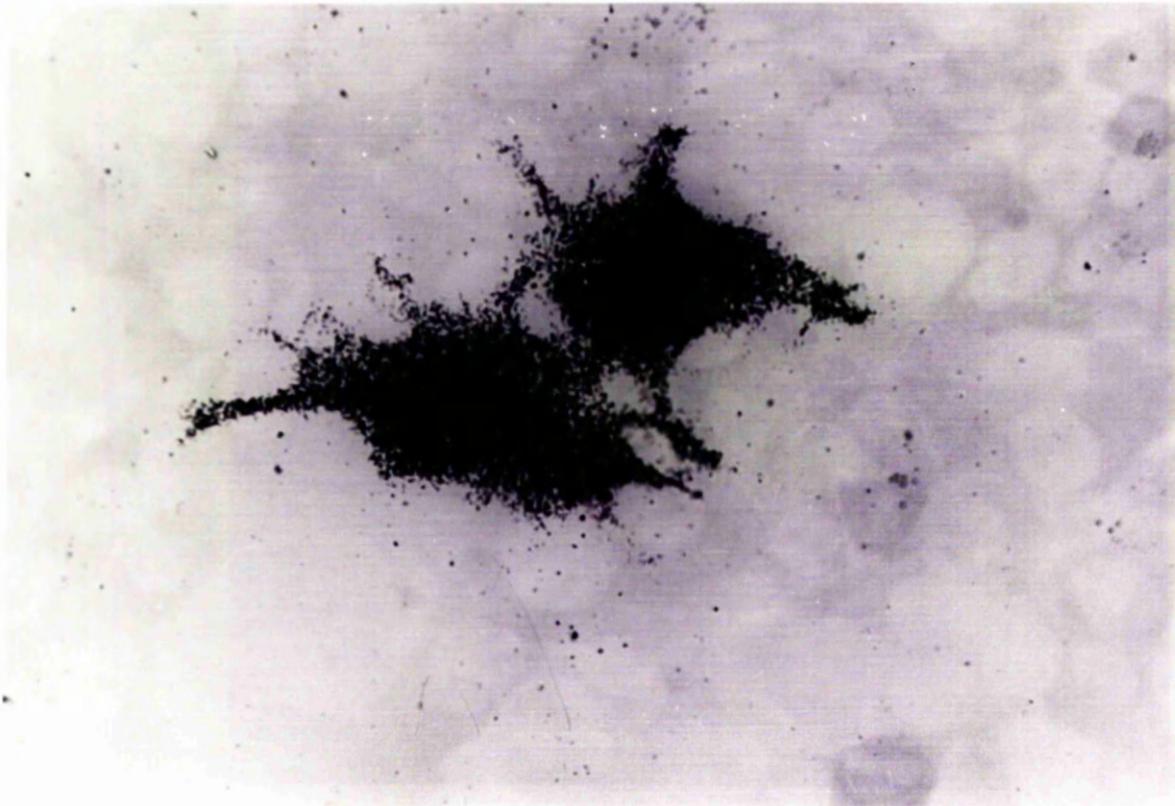
4×10^5 B_1 cells (recipients), in 2ml EFC 10 containing
2mg/ml formate, were plated out in 35mm dishes. 1×10^4 TG_2
cells (donors), in 2ml EFC 10 containing 2mg/ml formate,
were plated out in 35mm dishes and the cultures grown for
18h. Co-cultures were formed as in Section 2.2.3. Donor cells
were washed x 3 with BSS (2ml; $37^{\circ}C$). Medium containing 1%
normal methionine and 2mg/ml formate was added to both donor
and recipient cultures. Donor cells were labelled for 4h
with methyl-H^3-methionine ($2\mu Ci/ml$). Donor and recipient
cells were co-cultured together for 5h in the presence of x 10
normal methionine medium and formate (2mg/ml). Cells were
processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 63 days. Magnification
x 700.

Fig. 26

Transfer of D-glucose-2-H^3 labelled molecules from donor cells

to recipient cells

1×10^4 TG$_2$ cells (donors) were plated out in 35mm dishes.
1.5×10^5 B$_1$ cells (recipients) were plated out in 35mm dishes
at the same time and also 24h later. The donor cultures were
grown for 20h. Co-cultures were formed as in Section 2.2.3.
Donor cultures and one recipient culture were washed x 3 BSS
(2ml; 37°C), which is glucose free, and the cultures were
incubated in 0% glucose normal medium. Donor cultures were
labelled for 6h with glucose-2-H^3 (5μCi/ml). Donor cells were
washed x 3 with BSS. One donor culture had 0% normal glucose
medium added and was incubated for 18h.
Donor and recipient cells were co-cultured together for 3h,
either in the absence of glucose (0% normal glucose medium
using the appropriate recipients) or in the presence of glucose
(normal glucose medium). The chased donor cells were co-cultured
for 3h with unlabelled recipient cells, in the presence of
normal glucose medium. Cells were processed for autoradiography
as in Section 2.2.5.1. Autoradiographs were developed after 79 days.
Magnification x 480.

a) 4h label: + glucose in co-culture;   b) 4h label: no glucose in
co-culture;   c) 4h label: 18h chase; + glucose in co-culture.

(a)
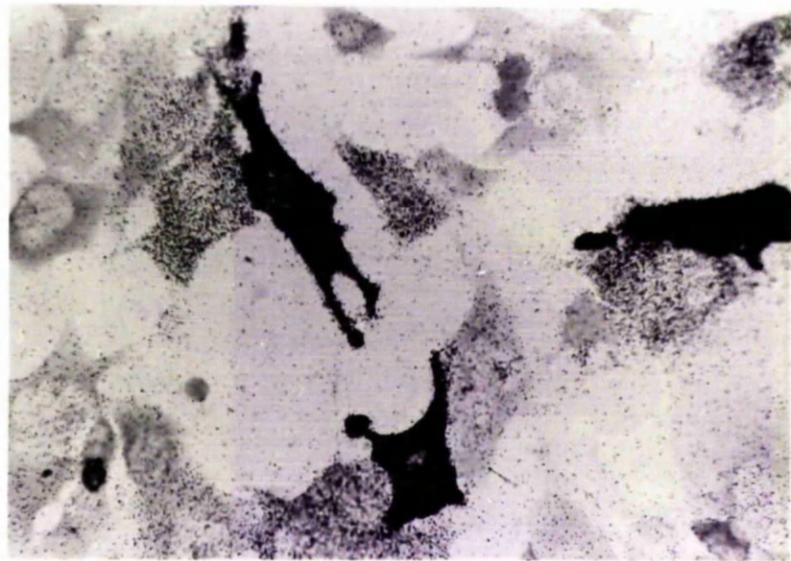
(b)
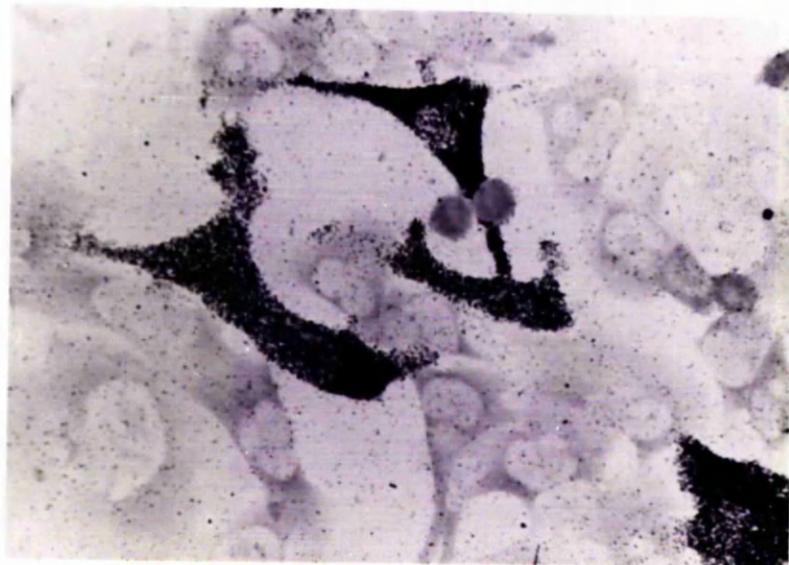
(c)

is also labelled in the cell by glucose-2-H^3, would not be detected, because it is acid soluble and will be extracted by the acid washes during the preparation of the cells for autoradiography (see Section 2.2.5.1.).

A chase, prior to co-culture, will deplete the labelled acid soluble pool in the donor cells. After these donor cells are co-cultured with unlabelled recipient cells, the presence or absence of label in recipient cells will provide information about the classes of labelled molecules transferred.

Cultures of donor cells, prelabelled for 6h with glucose-2-H^3 in glucose-free medium, were co-cultured, either immediately or after a 18h chase, for 3h in normal medium, with unlabelled recipient cells. Autoradiographs (Fig. 26a,c) show that the chase, which reduces the labelled acid soluble pool to 16% of its original pulsed value, considerably reduces the extent of label transferred to recipient cells. This suggests that labelled precursors (acid soluble), but not labelled macromolecules (acid insoluble), are transferred between cells. Since glucose-2-H^3 will label glycolipids, glycoproteins, RNA and DNA, presumably precursor molecules such as sugar phosphates and nucleotides are the types of molecules transferred from donor to recipient cells.

3.4. Studies on molecules which may inhibit the formation or the

   function of junctions required for metabolic co-operation.

The transfer between cells of molecules such as nucleotides,

which cannot cross the cellular membrane, requires direct contact

and junction formation.

The study of inhibitors of junction formation and function

would contribute to the biochemical analysis of junctions and the

mechanism of transfer of molecules between cells respectively.

Such inhibitors could also lead to an understanding of the

importance of such junctions between cells.

3.4.1. Inhibitors of RNA and protein synthesis.

Inhibitors of RNA and protein synthesis were used to examine

the nature and metabolic stability of the junctional components.

The effect of actinomycin D on interaction between cells has

already been examined for different reasons (Section 3.1.3.3.)

and the data showed that normal levels of RNA synthesis

immediately prior to, or during, co-culture are not required for

interaction between cells.

Cycloheximide, an inhibitor of protein synthesis, was used

to examine the role of protein synthesis in junction formation.

Protein synthesis in BHK cells is inhibited by cycloheximide

(Table 15). Final concentrations of 10 µg/ml and 100 µg/ml, which

inhibit protein synthesis by 88% and 91% respectively, were used

to study the effect of cycloheximide on cell-cell interactions.

Cultures of BHK-TK$^+$ cells, growing in dishes, were labelled

with $[^3H]$thymidine for 16h to mark the cells for autoradiography.

Incubation was continued for $6\frac{1}{2}$h, in the absence of $[^3H]$thymidine,

to deplete the labelled acid soluble pool. Cultures of BHK-TK$^-$

cells were cultured in suspension for 1h in spinner flasks.

Cycloheximide was then added to cultures of BHK-TK$^-$ cells and

Table 15

Effect of cycloheximide on protein synthesis in BHK cells

Cycloheximide conc. (µg/ml)	[³H] leucine incorporation	
	d.p.m.	% untreated value
0	23,543	100
0.1	8,422	35.8
1	3,768	16.0
10	2,787	11.8
100	2,012	8.6

Each determination an average of 2 dishes.

2×10^5 TG$_2$ cells were plated out in 35mm dishes and grown 16h. Cells were cultured for 1h in the presence of cycloheximide (0, 0.1, 1, 10 and 100µg/ml). [³H] leucine (2µCi/ml) was then added to each culture and incubation of the cultures continued for 1h. The medium was removed from the cultures, and each was washed x 5 with BSS (2ml; 0°C). The cells were solubilised by incubating them for 3 min at 37°C in 0.3M-NaOH (1ml; room temperature). 0.5ml of this solution containing the solubilised cells was placed in a vial and evaporated to dryness at 60°C (~7h). Hyamine hydroxide (0.5ml) was added to each vial and incubation continued at 60°C for 15 min. Toluene based scintillation fluid was added to each vial. The radioactivity in each sample was then determined with a Philips liquid scintillation spectrometer.

BHK-TK$^+$ cells (0, 10, 100 µg/ml). After a 1h incubation with cycloheximide, the medium was removed from the BHK-TK$^+$ cells, and BHK-TK$^-$ cells were added in the suspension medium, which contained the appropriate cycloheximide concentrations. [^{3}H]thymidine was added and incubation was continued for 4h.

Autoradiographs were prepared. Fig.27(a) shows interaction between BHK-TK$^+$ and BHK-TK$^-$ cells in the untreated culture. Interaction still occurs in the presence of 10 µg/ml and (Fig. 27b) 100 µg/ml cycloheximide, although the extent of labelling of donor and recipient cells is reduced. Co-culture of donors and recipients, in the absence of [^{3}H]thymidine (Fig. 27c), shows that labelled DNA is not transferred due to the presence of cycloheximide. The reduction in the level of label, in donor and recipient cells in cycloheximide treated cultures (Fig. 27b), is due to the inhibition of thymidine nucleotide incorporation into DNA in the treated cells (100 µg/ml cycloheximide reduces the incorporation of [^{3}H]thymidine into acid insoluble material (DNA) by 85%). Identical results were obtained from a study on the effect of cycloheximide (10 µg/ml, 100 µg/ml) on metabolic co-operation between cells, which were already in co-culture before cycloheximide was added.

These results show that de novo protein synthesis is not required for either the formation of junctions, the maintenance of junctions or the transfer of nucleotides through junctions. This suggests that the proteins, required for junction formation (structural junctional proteins or the enzymes synthesising junctional components) and for nucleotide transfer, must already be present in the cell, or cell membrane, prior to cell-cell contact, and that they do not significantly decay in 1h.

Fig. 27

The effect of cycloheximide on metabolic co-operation

BHK-TK$^-$ cells were seeded into 8oz bottles and grown for 48h.
1 x 10^4 BHK-TK$^+$ cells were plated out in 35mm dishes (containing
3 coverslips) and the cultures grown for 8h. BHK-TK$^+$ cultures
were prelabelled with [^{3}H]thymidine (5µCi/ml) for 16h. The
cultures were then washed x 3 with BSS and incubation was
continued in fresh EFC 10. These cultures were chased for 6½h.
1h before the end of the chase, BHK-TK$^-$ cells were trypsinised
and grown in suspension culture in spinner flasks (50ml capacity).
Each flask contained BHK-TK$^-$ cells (2 x 10^5/ml) in 20ml EFC 10,
gassed with 5% CO_2. These flasks were left at 37°C for 1h.
Cycloheximide (0, 10, 100µg/ml) was added to both BHK-TK$^+$
cultures and BHK-TK$^-$ cultures and incubation was continued for
1h. BHK-TK$^+$ cultures were washed x 1 with BSS (37°C; 2ml), and
2ml of BHK-TK$^-$ cells (containing the appropriate cycloheximide
concentration) were added to the appropriate BHK-TK$^+$ cultures.
Co-culture was for 4h in the presence of [^{3}H]thymidine (10µCi/ml).
Cells were processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 6 days. Magnification x440.

    a) co-cultured in the presence of [^{3}H]thymidine;

    b) co-cultured in the presence of [^{3}H]thymidine and
cycloheximide (100µg/ml);

    c) co-cultured in the presence of cycloheximide (100µg/ml).
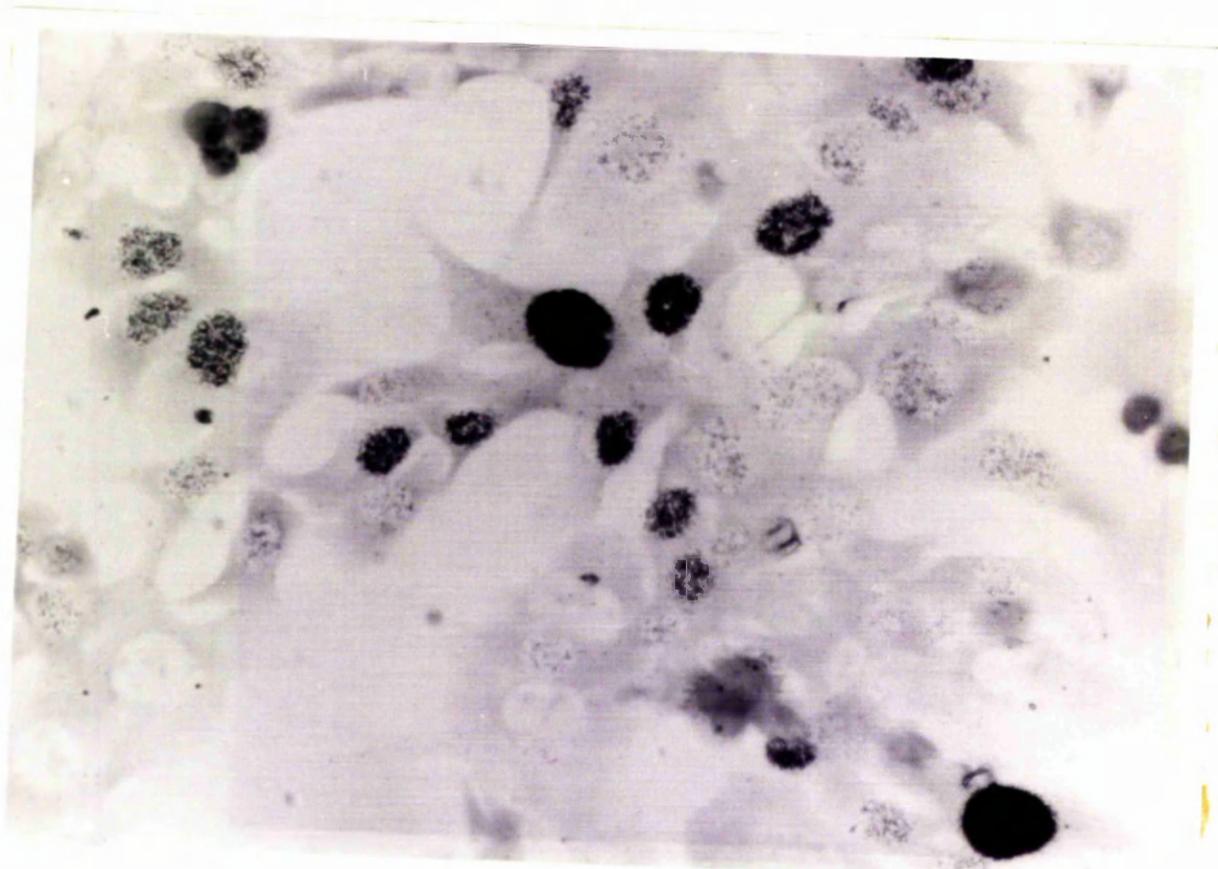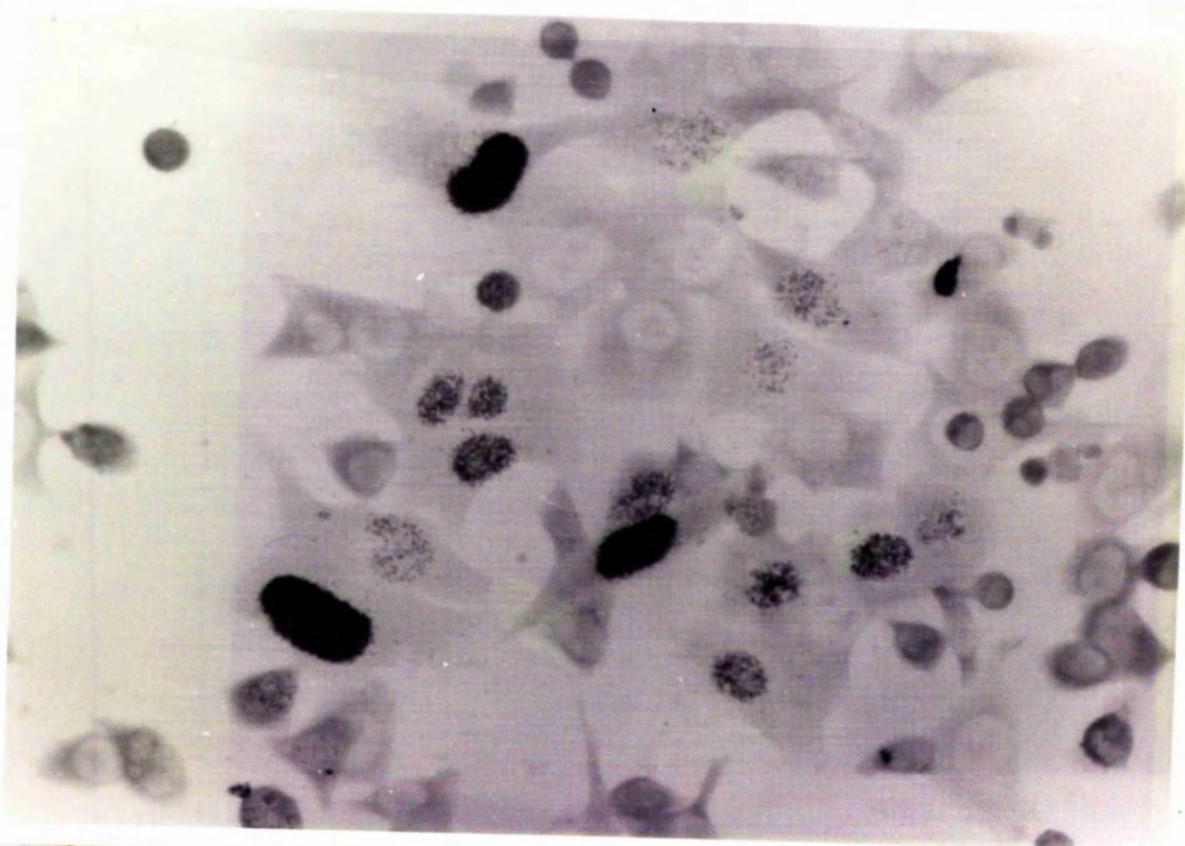
(c)
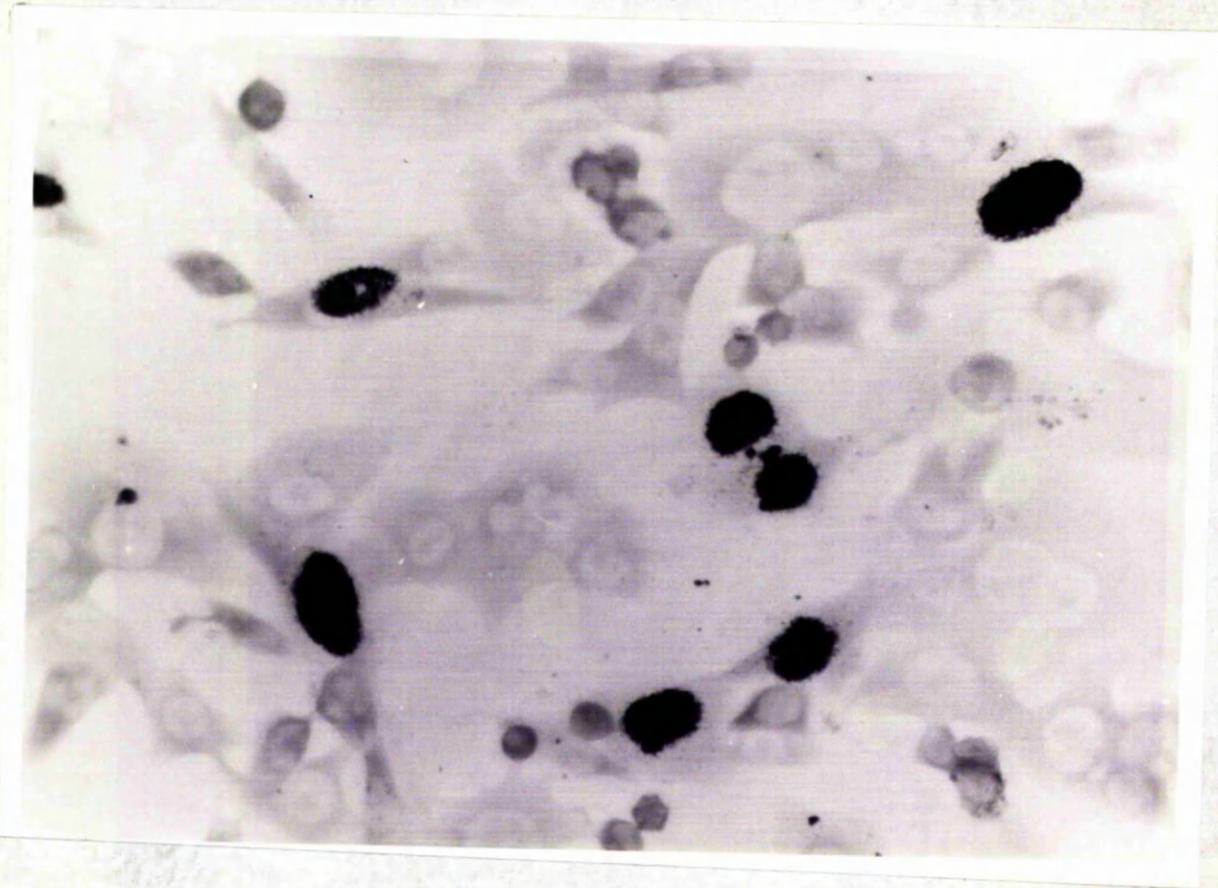
3.4.2. Effect of various reagents which interact with membranes.

McCargow and Pitts (1971) have suggested, from studies of a L/BHK hybrid cell (Section 1.2.4.), that L cells do not possess some membrane determinant, essential for the formation of junctions between cells. Since all $[^3H]$uridine labelled donor cells interact with all recipient cells in co-culture, any such membrane determinant must be present on all cells in a cell population.

Therefore reagents, which interact with membranes, were used in an attempt to define the nature of the determinants required for cell-cell interaction.

3.4.2.1. Effect of PHA and Con A on cell-cell interaction.

Phytohaemaggutinin (PHA), isolated from Phaseolus vulgaris, binds to proteins on the cell surface (Allan et al., 1971; Allan & Crumpton, 1973), and it is therefore possible to study the role of such membrane components in junction formation.

Mixed cultures (1:1) of BHK-IPP$^+$ cells and BHK-IPP$^-$ cells were plated out in the presence of $[^3H]$hypoxanthine and PHA, to allow the PHA to interact with membrane components before junction formation occurred. The cultures were incubated for 16h and then processed for autoradiography. It appears that interaction occurred at all concentrations of PHA used (0, 0.0025 ml PHA per ml of culture medium, 0.025 ml per ml, 0.25 ml per ml; Fig. 28a,b,c,d), even though some cells were clumped at 0.025 ml PHA per ml, and all cells were clumped and rounded up, but still attached to the plate, at 0.25 ml PHA per ml.

PHA binding sites do not, therefore, appear to play a role in junction formation.

Concanavalin A (Con A), which interacts with specific sugar residues (α-D-mannopyranosides, α-D-glucopyranosides and α-N-acetyl-D-glucosamides) of cell surface glycoproteins (Sharon

Fig. 28

The effect of PHA on metabolic co-operation

3.5×10^5 BHK-IPP$^+$ cells and 3.5×10^5 BHK-IPP$^-$ cells were plated out in 35mm dishes (+ 3 coverslips) in the presence of PHA (0, 0.0025, 0.025, 0.25ml PHA/ml culture medium)[*] and [^{3}H]hypoxanthine (5μCi/ml). Incubation was continued for 16h. The cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 6 days. Magnification x480. Not all cells are in focus simultaneously at this magnification because PHA caused the cells to clump.

[*]For use, 5ml sterile water was added to each vial of dehydrated PHA.

a) control untreated culture;

b) 0.0025ml PHA/ml culture medium;

c) 0.025ml PHA/ml culture medium;

d) 0.25ml PHA/ml culture medium.

(a)

(b)

(c)

(d)
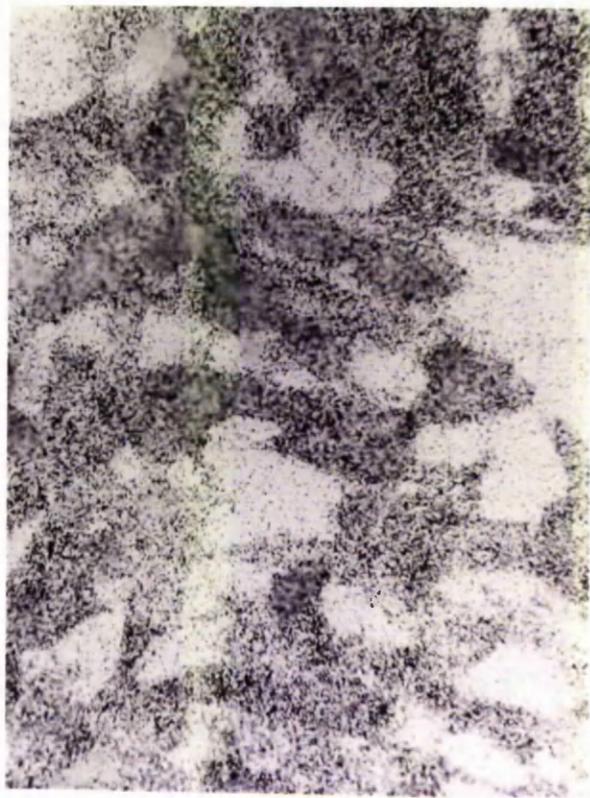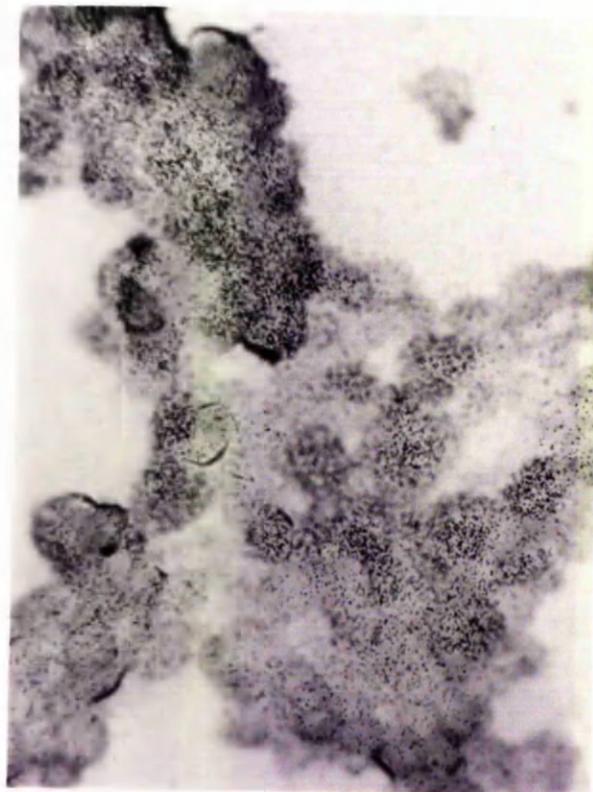
& Lis, 1972), was also used to examine the role of its binding
sites in junction formation.

Cultures of donor cells, prelabelled with $[^3H]$ uridine for
4h, were incubated with Con A (0, 10, 25, 50 μg/ml) for 15 min.
They were then co-cultured for $1\frac{1}{2}$h, in the presence of Con A
(concentrations as above), with unlabelled recipient cells.
Autoradiographs were prepared. These show that interaction
occurred at all concentrations of Con A used (Fig. 29a,b,c,d).
However, 50 μg/ml Con A inhibits the attachment of most recipient
cells, although a few do attach and interact with donor cells
(Fig. 29d).

It appears that concanavalin A binding sites are also not
involved in junction formation.

3.4.2.2. Effect of nupercaine on cell-cell interaction.

Phospholipase A_2, which is present in biological membranes,
such as mitochondrial (Scherphof et al., 1966) and plasma (Waite
& Van Deenen, 1967) membranes, is inhibited by nupercaine
(Scarpa & Lindsay, 1972). Phospholipase A_2 releases fatty acids
from phospholipids, and therefore the possible involvement of
phospholipid metabolism in junction formation can be investigated
using nupercaine. Scarpa & Lindsay (1972) reported that a
concentration of 150 μM nupercaine inhibited purified porcine
pancreas phospholipase A_2.

Cultures of both BHK-TK$^+$ cells and BHK-TK$^-$ cells were
incubated with nupercaine (0, 40, 150 μM) for 2h. The BHK-TK$^-$
cultures were then co-cultured for $2\frac{1}{2}$h, in the presence of
nupercaine (concentration as above) and $[^3H]$ thymidine, with the
appropriate nupercaine treated BHK-TK$^+$ cultures. Autoradiographs
were prepared. These show (Fig. 30a,b,c) that interaction occurred
at both concentrations of nupercaine used. 400 μM final

Fig. 29

Effect of Con A on $[^3H]$uridine labelled nucleotide transfer

between cells

1×10^5 TG$_2$ cells (recipients) were plated out in 35mm dishes.
5×10^3 TG$_2$ cells (donors) were plated out in 35mm dishes
and grown for 16h. Co-cultures were formed as in Section 2.2.3.
Donor cultures were labelled for 4h with $[^3H]$uridine (2.5μCi/ml).
The donor cultures were washed x 3 with BSS (37°C; 2ml). 2ml
EFC 10 containing Con A (0, 10, 25, 50μg/ml) was added to each
culture and incubation was continued for 15 min. Donor and
recipient cells were then co-cultured together for 90 min in
the presence of Con A (conc. as above). Cells were processed
for autoradiography as in Section 2.2.5.1. Autoradiographs
were developed after 27 days. Magnification x480.

a) Untreated control;

b) 10μg/ml Con A;

c) 25μg/ml Con A;

d) 50μg/ml Con A.

(a)

(b)

(c)

(d)

Fig. 30

Effect of nupercaine on metabolic cooperation

(a)
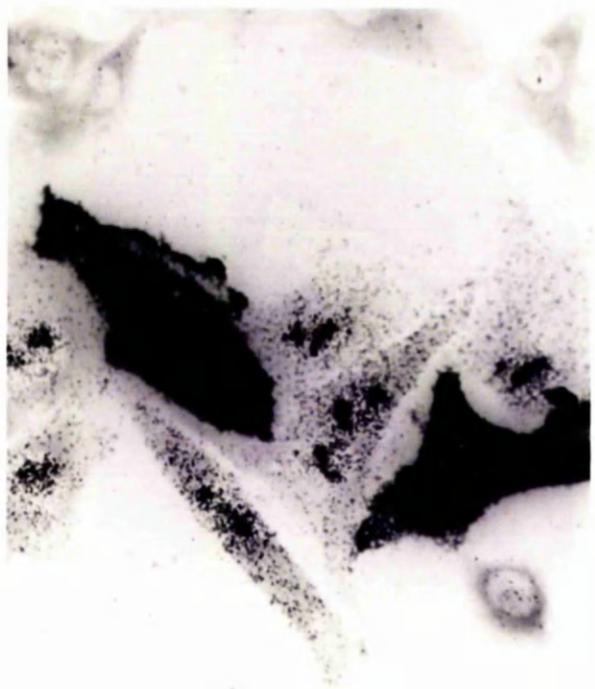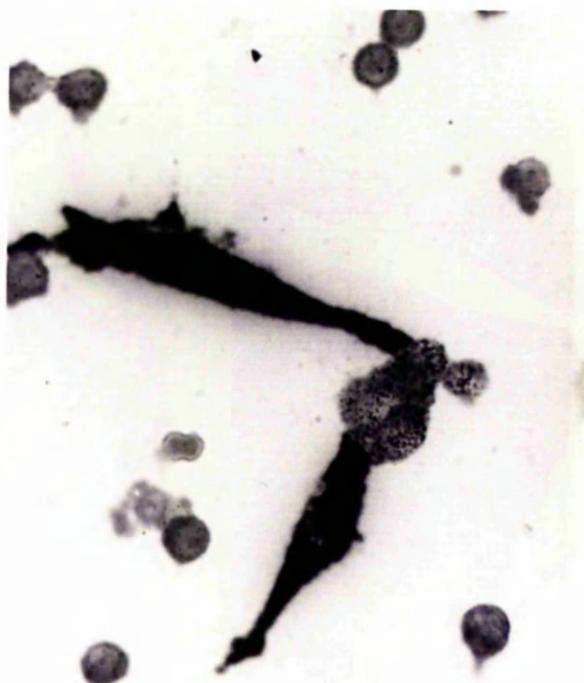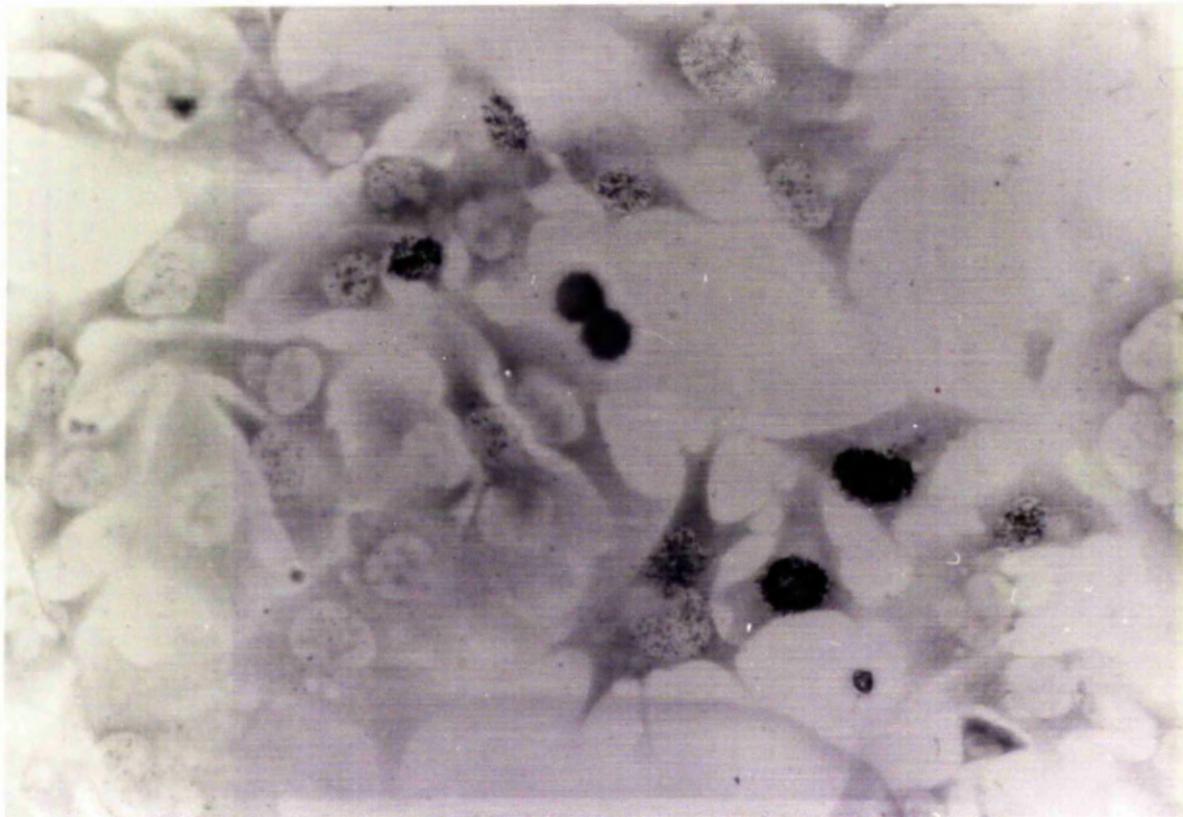
5×10^3 BHK-TK$^+$ cells were plated out in 35mm dishes (+ 3 coverslips) and grown for 20h. 1×10^5 BHK-TK$^-$ cells were plated out in 35mm dishes and grown for 20h. Nupercaine (0, 40, 150µM) was added to the BHK-TK$^+$ cultures for 2h. The medium was removed from the BHK-TK$^+$ cells and the BHK-TK$^-$ cells were trypsinised and added to the BHK-TK$^+$ cells. The cells were co-cultured together for 2.5h in the presence of nupercaine (conc. as above) and $\left[^3H\right]$ thymidine (5µCi/ml). Cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 9 days. Magnification x440.

    a) Untreated culture; b) 40µM nupercaine;
    c) 150µM nupercaine.
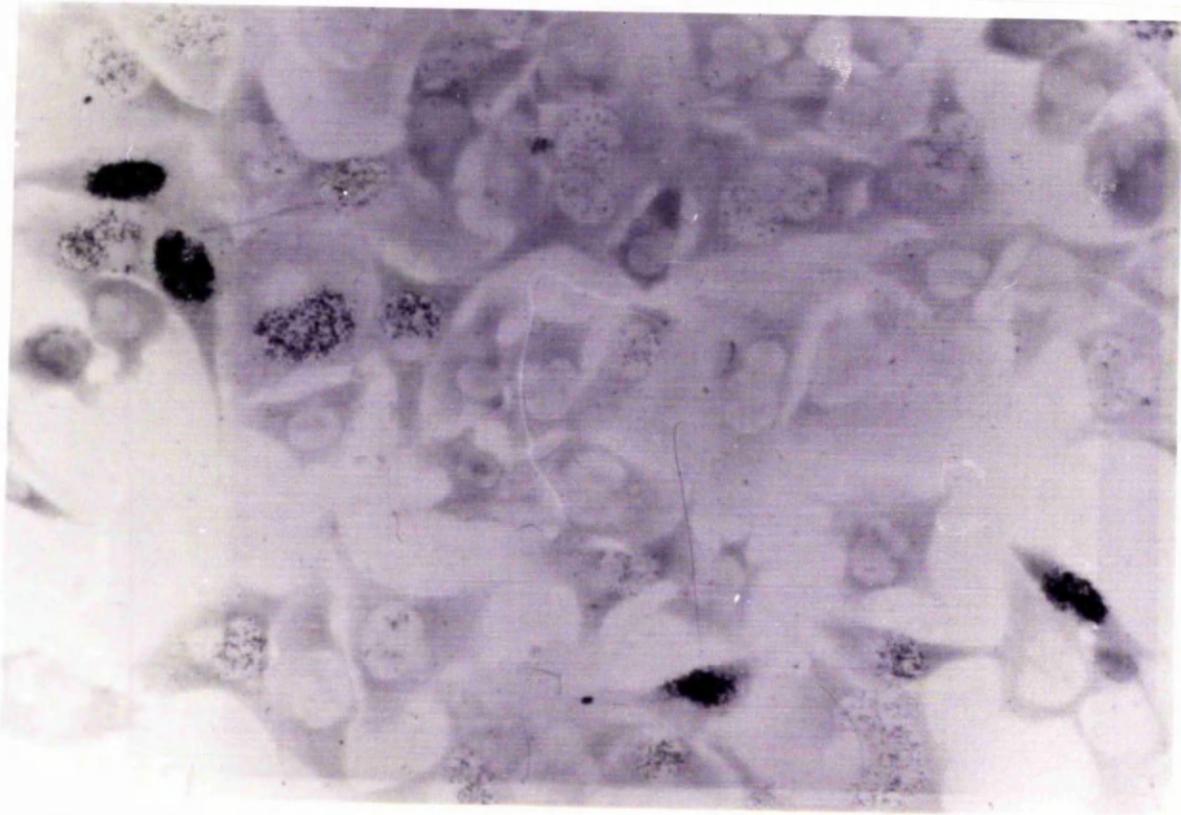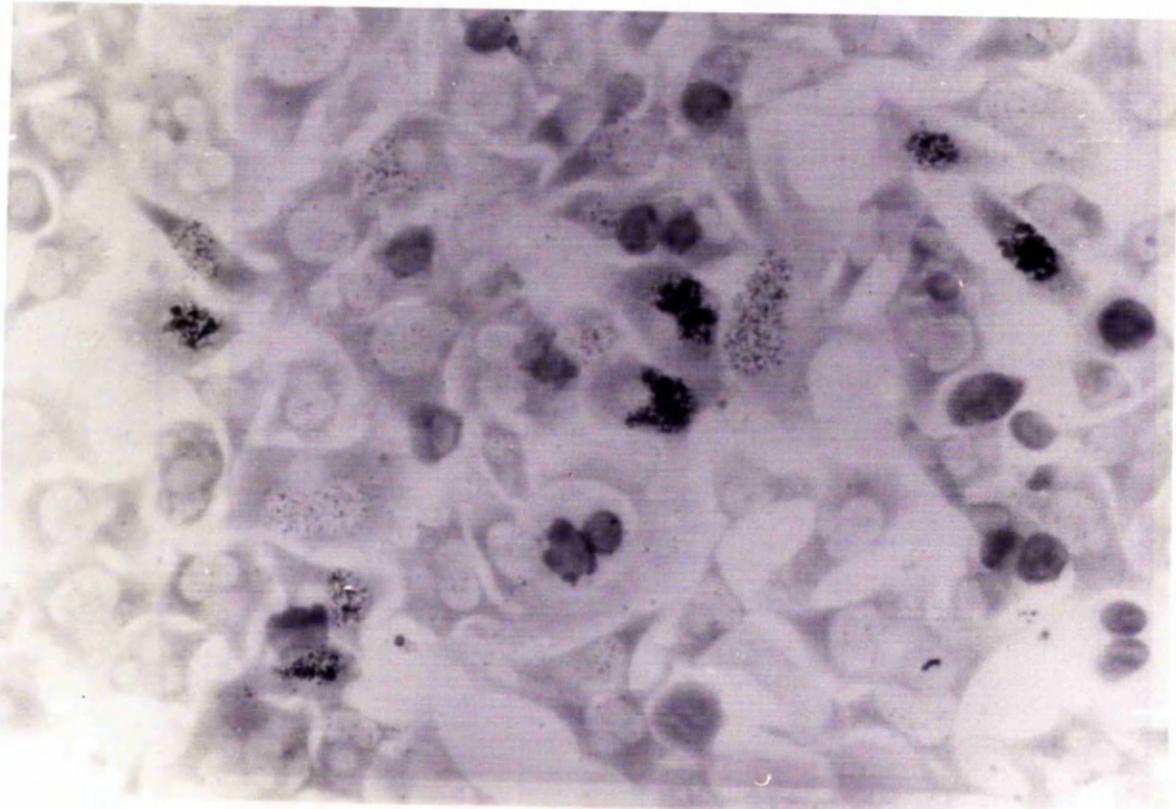
concentration nupercaine caused the cells to round up and detach after a 2h incubation.

Therefore, it appears that removal of fatty acids from phospholipids in the membrane of phospholipase A_2 is not necessary for junction formation.

3.4.2.3. Effect of p-hydroxymercuribenzoate on cell-cell interaction.

p-Hydroxymercuribenzoate (PHMB), which reacts with thiol groups, was used to study the possibility that thiol containing proteins may be involved in junction formation.

Cultures of donor cells, prelabelled with $\left[^3H\right]$uridine for 4h, were incubated with PHMB (0, 0.025, 0.05, 0.1 mM) for 30 min. They were then co-cultured for $1\frac{1}{2}$h, in the presence of PHMB (at the above concentrations), with unlabelled recipient cells. Autoradiographs were prepared. The quanity of label present in recipient cells in the 0.025 mM PHMB treated co-culture (Fig. 31b) was similar to that present in untreated recipient cells (Fig. 31a), although some cells had started to round up. However, in the 0.05 mM PHMB treated co-culture (Fig. 31c), while most recipient cells had spread after 1h co-culture, a considerable number had rounded up and detached again after $1\frac{1}{2}$h co-culture. Also, most of the donor cells in this culture had rounded up and detached. No interaction was observed in this culture. In the 0.1 mM PHMB culture, it was noted that even after the 30 min preincubation of the donor cells with PHMB, these were rounding up and detaching. Therefore, it appears that PHMB has a rapid toxic effect on BHK cells at these concentrations. However, since interaction between cells was observed at 0.025 mM PHMB, it appears that proteins, containing reactive thiol groups, are not required for junction formation.

Fig. 31

Effect of PHMB on $[^3H]$uridine labelled nucleotide transfer

between cells

1×10^5 B$_1$ cells (recipients) were plated out in 35mm

dishes. 5×10^3 TG$_2$ cells (donors) were plated out in 35mm

dishes and grown for 16h. Co-cultures were formed as in

Section 2.2.3. Donor cultures were labelled for 4h with

$[^3H]$uridine (2.5μCi/ml). The donor cells were washed x 3

with BSS (37°C; 2ml). 2ml EFC 10 containing PHMB (0,0.025,

0.05mM) was added to each culture and incubation was

continued for 30 min. Donor and recipient cells were then

co-cultured together for 90 min in the presence of PHMB

(conc. as above). Cells were processed for autoradiography

as in Section 2.2.5.1. Autoradiographs were developed after

82 days. Magnification: x 480.

a) untreated culture;   b) 0.025mM PHMB;   c) 0.05mM PHMB.

(a)

(b)

(c)
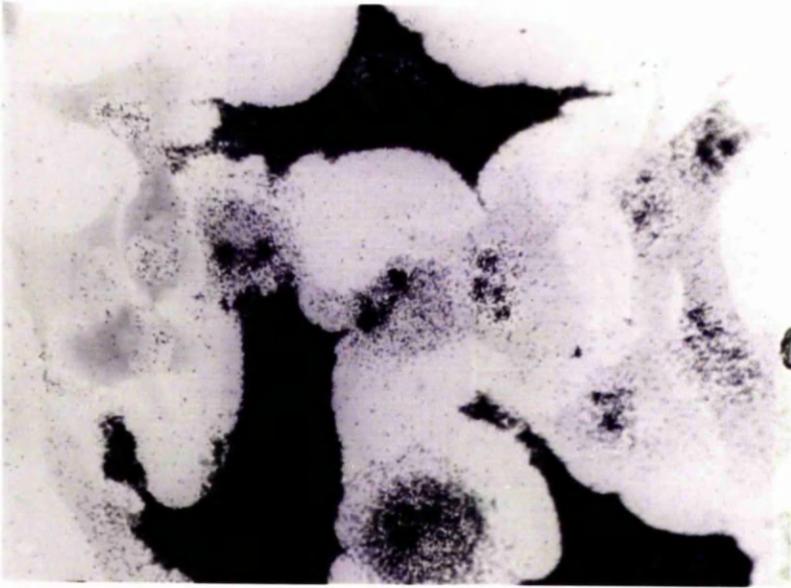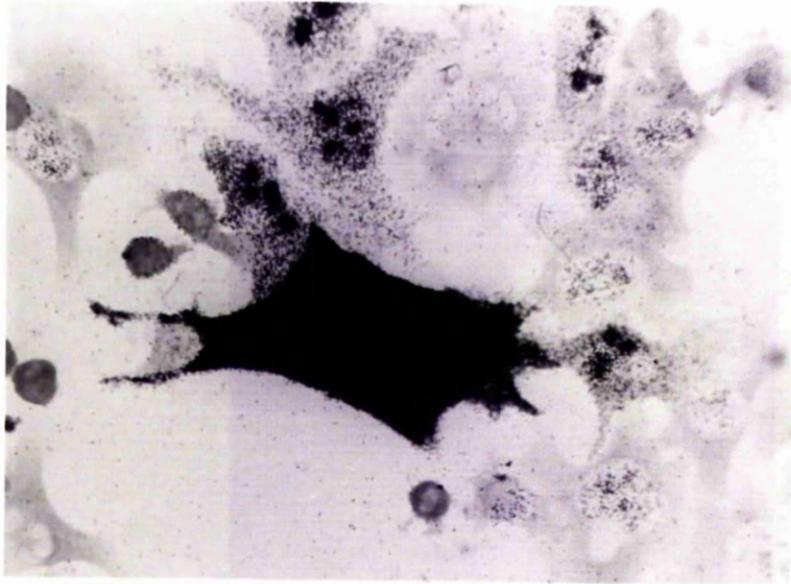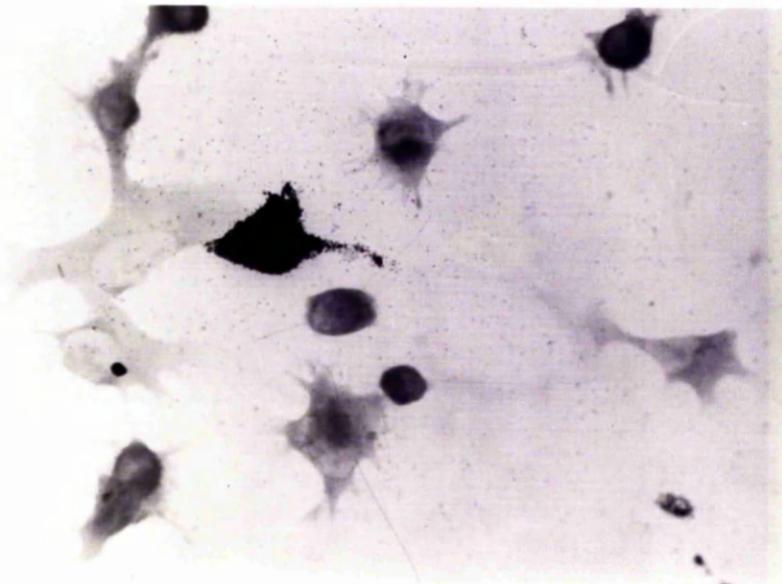
The conclusions drawn from the effect of these reagents on cell-cell interaction require that:-

1) all PHA and Con A binding sites are blocked.

2) nupercaine inhibits all phospholipase A_2 activity.

3) PHMB blocks all thiol groups.

However, it should be noted that a differential effect between the extent of interaction of an inhibitor with its target sites and the viability of the cells is required, if the inhibitor is to be useful for studies on cell-cell interactions. Also, it would only have been informative to have characterised the extent of blocking or inhibition of any of the reagents studied above, if inhibition of cell-cell interaction had been observed in the absence of toxic effects.

3.5.1. Location of genetic locus (loci) required for interaction between cells.

The location of the genetic locus required for interaction between cells is being studied. The method selected requires the fusion of a cell, which can interact (int$^+$ cells), with a cell, which cannot interact (int$^-$ cells), to form an int$^+$ cell hybrid. By correlating the loss of chromosomes from such hybrids with the ability of the hybrids to interact, it should be possible to locate the chromosome carrying the locus for the protein required for interaction. McCargow & Pitts (1971) have shown that an int$^+$ hybrid can be isolated from fusion of a BHK-int$^+$ cell line with a L-int$^-$ cell line. Since there is only a limited loss of chromosomes (10-20%) in some interspecific hybrids (Weiss & Green, 1967), and since chromosomes of both parental types are lost, the use of a hybrid where there is preferential loss of one set of the parental chromosomes (the int$^+$ parent) is clearly an advantage. Weiss & Green (1967) reported that in human-mouse hybrids, at least 75% and in some cases more than 95% of the human chromosomes were lost, while very few of the mouse chromosomes were lost. Therefore, a hybrid between a human embryo lung fibroblast line (H116) and an L cell line (A9), which lacks the enzyme inosinic pyrophosphorylase (Littlefield, 1966) was used. McCargow and Pitts (1971) have reported that the A9 cell line does not interact. The H116 cell line was shown (Fig. 32a,b) to interact with BHK-IPP$^-$ cells.

Two different hybrid clones isolated from the fusion of H116 cells and A9 cells were assayed for interaction. Cultures of clone 201 pass 10 and clone 3 pass 14, prelabelled for 4h with [^{3}H]uridine, were co-cultured for 3h with unlabelled BHK recipient cells. Autoradiographs show that, in general, clone 201.10 cells do not interact (Fig. 33a), although a few interact poorly.

Fig. 32

Interaction between H116 cells and BHK-IPP⁻ cells

(a)   (b)

1×10^4 H116 pass 18 cells and 2×10^5 BHK-IPP⁻ cells were
plated out in 35mm dishes (+ 3 coverslips) and grown for
23h in the presence of $\begin{bmatrix}3\\H\end{bmatrix}$ hypoxanthine (5μCi/ml). Cells
were then processed for autoradiography as in Section 2.2.5.1.
Autoradiographs were developed after 26 days. Magnification:
x 480.

a) a field showing an H116 cell;

b) a control field without an H116 cell.

Fig. 33

Interaction between Cl 201.10 or Cl 3.14 cells and PHK cells

(a)

4×10^5 B_1 cells (recipients) were plated out in 35mm dishes.
5×10^3 Cl 201.9 or Cl 3.13 cells (donors) were plated out in
35mm dishes and grown for 16h. Co-cultures were formed as in
Section 2.2.3. Donor cells were labelled for 4h with $[^3H]$uridine
(2.5µCi/ml). Donor and recipient cells were co-cultured
together for 3h. Cells were processed for autoradiography as in
Section 2.2.5.1. Autoradiographs were developed after 20 days.
Magnification: x 700 (a); x 480 (b,c,d).

a) Cl 201.10: background grains in recipient cells;

b) Cl 3.14: heavily labelled recipient cells;

c) Cl 3.14: lightly labelled recipient cells;

d) Cl 3.14: background grains in recipient cells.

(b)

(c)

(d)
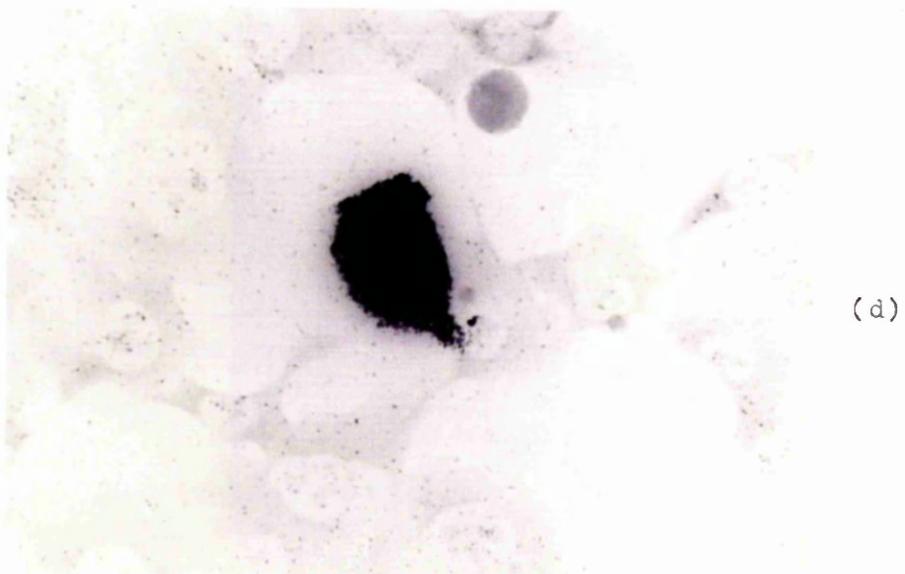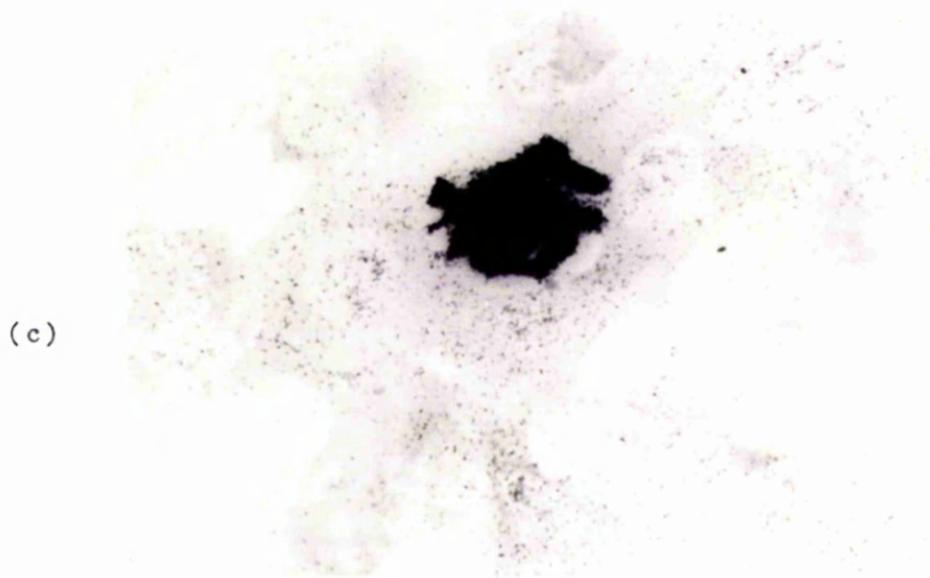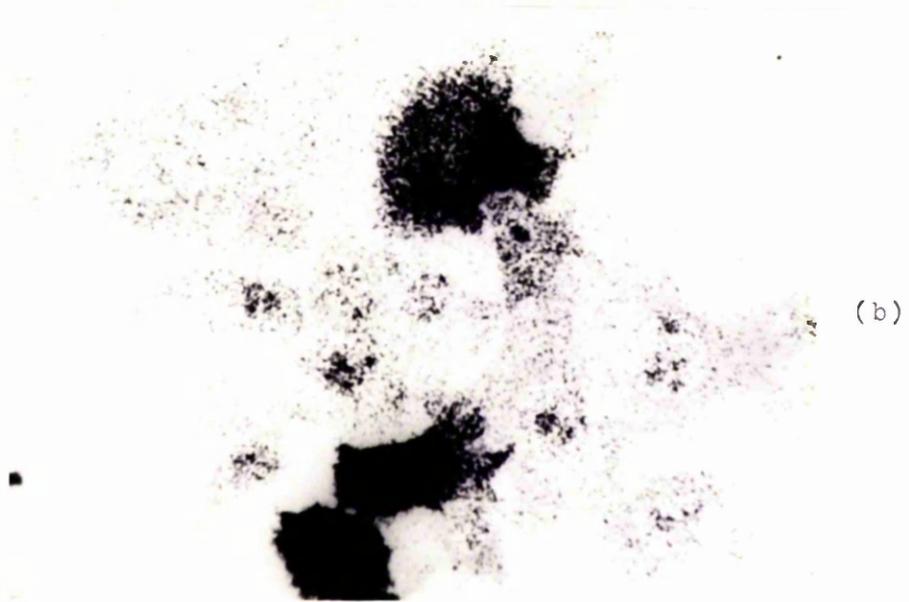
However, clone 3.14 shows a range of interaction ability from good interaction to no interaction (Fig.33b,c,d).

The extent of interaction of the two hybrids at different pass numbers was quantitated. Cultures of the appropriate cells, prelabelled for 4h with $[^3H]$ uridine, were co-cultured for 3h with unlabelled BHK recipient cells. Autoradiographs were prepared. Interaction between donor and recipient cells was classified on the basis of the pattern and extent of label in the recipient cells as follows (Fig. 33b,c,d respectively):-

1) heavily labelled recipient cells,

2) lightly labelled recipient cells, and

3) recipient cells with only background level of grains.

The data are presented in Table 16. This shows that, whereas 92% of clone 3.14 cells can still interact, 86% of the clone 201.10 cells do not interact. Table 17 shows the human chromosomes or chromosome markers (data kindly provided by Dr. Gormley), which could be detected in clone 3.11 and clone 201.6. The only human chromosome markers, present in clone 3.11 (int^+) and absent in clone 201.6 (int^-), are lactate dehydrogenase-A and esterase-A, which are both located on chromosome 11. This is consistent with the possibility that human chromosome 11 contains the locus for the protein required for interaction in H116 cells.

3.5.2. Morphology of interacting and non-interacting cells.

Analysis of Table 3 suggests that there may be a possible correlation between the ability of a cell to interact and the morphology of the cell. Int^+ cells appear to be spindle-shaped, whereas int^- cells are epithelioid. However, although the morphology of clone 201.10-int^- (Fig. 33a) is similar to that of the L-int^- parental cell, the morphology of clone 3.14-int^+

Table 16

Quantitation of the extent of interaction of various passes of

clones 3 and 201 (donors) with BHK cells (recipients)

Donor cells	Pattern of label in recipient cells (%)		
	Heavy	Light	Background
201.10	1	13	86
201.12	1	11	88
201.28	—	6	94
3.14	64	28	8
3.20	12	14	74
3.27	15	10	75
3.32	—	3	97

5×10^3 donor cells were plated out in 35mm dishes and grown for
16h. Autoradiographs were prepared as in the Legend to Fig. 33
and developed after 20 days. See Legend to Table 13 for the
details on the quantitation of the extent of interaction of
donor cells.

Table 17

Human chromosome analysis of clone 3.11 and clone 201.6

Clone 3.11	Clone 201.6	Chromosome assignment[*]
X	X	X
14	14	14
Isocitrate dehydrogenase	Isocitrate dehydrogenase	2
APRT (weak)	APRT (weak)	16
Pyruvic kinase (?)	Pyruvic kinase	7
LDH-A	—	11
Esterase-A	—	11

Data kindly provided by Dr. Gormley.

[*]Ruddle (1973)

(Fig. 33b,c,d) was also similar to L cells, rather than the

H116-int$^+$ parental cell (Fig. 32a). This suggests that there is

no direct relationship between the morphology of a cell and the

ability of the cell to interact.

3.5.3. Partial isolation of an interacting L cell.

A detailed study of the effect of interaction on the growth

behaviour and morphology of cells requires isolation of cells

from the same cell line differing only in their ability to

interact. Pitts (personal communication) has consistently failed

to isolate BHK-int$^-$ cells, perhaps suggesting that more than a

single step mutation is required. However, Table 13 shows that a

few L cells may interact weakly, suggesting that it might be

possible to isolate an interacting L cell.

Pitts (1971) has shown that a thick 1:1 mixed population

of BHK-IPP$^-$ and BHK-TK$^-$ cells can grow together in the presence

of HAT medium, by mutual exchange of nucleotides (Section 1.2.7.).

Therefore, if L-TK$^-$ cells and BHK-IPP$^-$ cells are co-cultured

together in HAT medium, growth in the culture will only result

from L-TK$^-$ cells interacting with BHK-IPP$^-$ cells.

A thick 1:1 mixed population of L-TK$^-$ cells and BHK-IPP$^-$

cells was formed and grown in the presence of HAT medium, which

was renewed after 2 days and 4 days co-culture. After 7 days

co-culture, although quite a few cells were still attached, most

of the cells appeared to be dead. Fresh medium, without HAT, was

added and replaced at intervals until the remaining viable cells

had grown to form a monolayer (21 days). The parallel morphology

of the monolayer suggested that most of the cells were BHK-IPP$^-$.

To test for the presence of interacting L-TK$^-$ cells, cells from

the monolayer were grown in the presence of [^{3}H]hypoxanthine and

autoradiographs were prepared. Fig. 34 shows that metabolic co-operation between cells was occurring in the culture, suggesting that interacting L cells were present. The possibility that these $\underline{int}^+$ cells are BHK-IPP$^-$ revertants is unlikely, at these concentrations of cells (5×10^6), and any revertants, if present, would be eliminated by the following step.

To isolate the L-TK$^-$ cells from BHK-IPP$^-$ cells, the monolayer was subcultured (1:15; 1:20; 1:30; 1:60) into medium containing 5-bromodeoxyuridine (50 µg/ml), which will kill BHK-IPP$^-$ cells ($\underline{TK}^+$), but not L-TK$^-$ cells (c.f. Littlefield, 1966). However, if any interacting L-TK$^-$ cells are in contact with BHK-IPP$^-$ cells, these will also be killed by metabolic co-operation (this is in agreement with the data in Table 18; only a few L-$\underline{int}^+$ cells are isolated from the 1:15 subculture), since the 5-bromodeoxyuridine will be transferred at the nucleotide level from BHK-IPP$^-$ cells to L-TK$^-$ cells.

Clones with the characteristic L cell morphology grew up in all cultures. In each culture, the clones were trypsinised and grown together until a monolayer was formed. All 4 cultures were assayed for the ability to

a) incorporate $\left[^3H\right]$ hypoxanthine to test for the presence of L-TK$^-$ cells,

b) incorporate $\left[^3H\right]$ thymidine to test for the presence of BHK-IPP$^-$ cells,

c) interact by prelabelling cells with $\left[^3H\right]$ uridine and co-culturing them for 3h with unlabelled BHK cells.

Autoradiographs were prepared. The data for each culture are presented in Table 18. In culture (2), there is no $\left[^3H\right]$ thymidine incorporating BHK-IPP$^-$ cells and 17% of the cells are interacting well (Fig. 35). Again, the morphology of the

Fig. 34

Metabolic co-operation between a possible L-IPP[+]-int[+] cell

and BHK-IPP^{-} cells
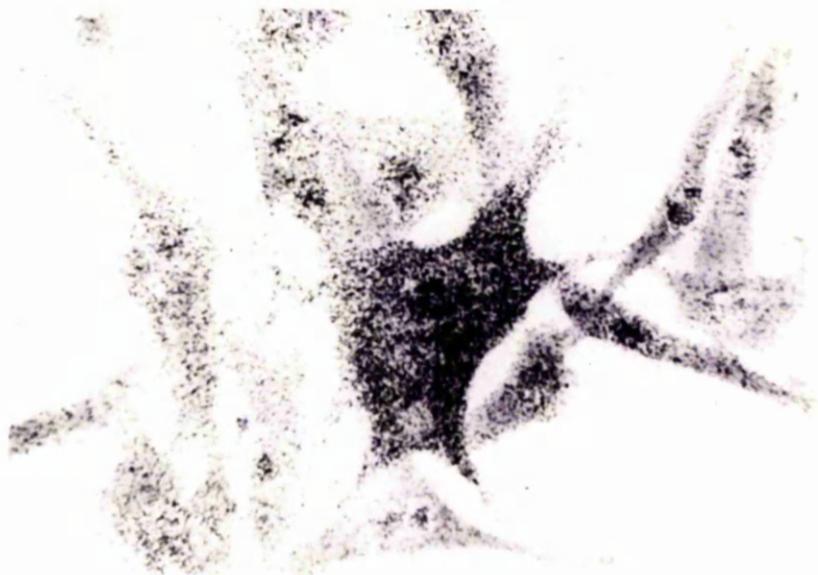
1 8oz. bottle BHK-IPP^{-} cells (thick) and 1 8oz. bottle
L-TK^{-} cells (thick) were combined in a fresh 8oz. bottle
and grown in HAT (6μg/ml thymidine, 6μg/ml aminopterin,
8μg/ml hypoxanthine) medium. The HAT medium was renewed
after 2 days and again after 4 days co-culture. After 7
days co-culture, fresh medium, without HAT, was added and
the surviving cells grown to form a monolayer. 2×10^5 cells
from this monolayer were plated out in 35mm dishes (+ 3
coverslips). The cells were incubated in the presence of
[3H] hypoxanthine (2.5μCi/ml) for 18h. The cells were processed
for autoradiography as in Section 2.2.5.1. Autoradiographs
were developed after 19 days. Magnification: x 480.

Table 18

Characterisation of cultures containing possible L-int$^+$ cells

See Legend to Fig. 34 for preparation of a monolayer of cells consisting of BHK-IPP$^-$ cells and L-TK$^-$ cells (including possible L-int$^+$ cells). This monolayer was subcultured (1:15, 1:20, 1:30, 1:60) into separate 8oz. bottles containing 50μg/ml 5-bromodeoxyuridine (Budr) in EFC 10 medium. At 7 day intervals (for 2 weeks), the old medium was replaced with fresh medium, containing Budr (50μg/ml).

Culture 1 (1:60): 3 clones grew up and these were trypsinised and grown in the same bottle in the presence of Budr for 14 days, and then in normal medium.

Culture 2 (1:30): 6 clones grew up and these were trypsinised and grown in the same bottle in the presence of Budr for 10 days, and then in normal medium.

Culture 3 (1:20): many clones grew up and growth of the clones was continued for a further 5 days in Budr medium.

Culture 4 (1:15): many clones grew up and growth of the clones was continued for a further 5 days in Budr medium.

Cultures 1-4 were then characterised as follows:-

1) $[^3H]$hypoxanthine incorporation: 2×10^5 cells (from each culture) were plated out in separate 35mm dishes (+ 3 coverslips) and grown for 20h in the presence of $[^3H]$hypoxanthine (5μCi/ml). The cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 20 days. All cells were heavily labelled.

2) $[^3H]$thymidine incorporation: 2×10^5 cells (from each culture) were plated out in separate 35mm dishes (+ 3 coverslips) and grown for 20h in the presence of $[^3H]$thymidine (5μCi/ml). The cells were processed for autoradiography as in Section 2.2.5.1. Autoradiographs were developed after 20 days. 1000 cells/autoradiograph were counted.

3) The extent of interaction of each culture was estimated as in the Legend to Table 13.

Cultures	Dilution at subculture	No. of clones /8oz. bottle	[3H]hypoxanthine incorporation	[3H]thymidine incorporation	% interaction		
					Heavy	Light	Background
1	1:60	3	++++	0%	6	39	55
2	1:30	6	++++	0%	17	31	52
3	1:20	many	++++	0.2%	6	23	71
4	1:15	many	++++	1%	0.5	5	94.5

Fig. 35

Interaction of possible L-int$^+$ cells with BHK cells
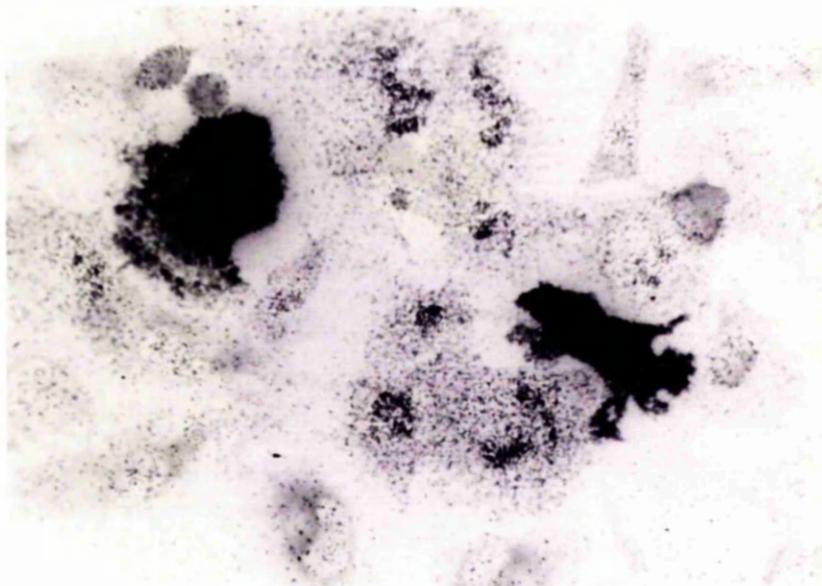
2×10^5 B_1 cells (recipients) were plated out in 35mm dishes.
5×10^3 cells (donors) from culture 2 (see Table 18) were
plated out in 35mm dishes and grown for 17h. Co-cultures
were formed as in Section 2.2.3. Donor cells were labelled
for 4h with [^{3}H]uridine (2.5μCi/ml). Donor and recipient
cells were co-cultured together for 3h. Cells were processed
for autoradiography as in Section 2.2.5.1. Autoradiographs
were developed after 70 days. Magnification: x 480.

L-int$^+$ cell does not appear to differ from that of the L-int$^-$ cell.

It should now be possible to obtain a clone of L-int$^+$ cells (L-TK$^-$ marker) from culture 2. Cultures of L-int$^+$ and L-int$^-$ would then be available for a detailed analysis of cell-cell interaction.

4. DISCUSSION

4. Discussion

Although direct transfer of molecules between cells may
be required to explain observations on control of cell movement
and cell growth, co-ordinated control of organ function and
development of multicellular organisms, the precise nature of
the molecules which can be transferred directly between cells,
has not been studied. This investigation of the classes of
molecules, which can be transferred between cells in tissue
culture, was therefore undertaken. Donor cells, containing
certain classes of molecules labelled with tritium, were
co-cultured with unlabelled recipient cells. The presence of
labelled molecules in recipient cells would then provide
information on the nature of the molecules, which can be
transferred directly between cells in tissue culture.

4.1. Transfer of $[^3H]$ uridine labelled nucleotides between cells.

Although $[^3H]$ uridine labels both nucleoside and nucleotide
pools, and RNA in the cell, the results (Section 3.1.) show
that labelled nucleotides (see Section 3.1.9.), but not labelled
nucleosides or labelled RNA, are transferred between cells. This
conclusion is based on the following observations.

1) When donor cells, prelabelled with $[^3H]$ uridine, are
co-cultured for 3h, in the presence of actinomycin D (1 µg/ml),
with unlabelled actinomycin D treated recipient cells, the level
of label in recipient cells is reduced to 5% of that in recipient
cells, cultured in the absence of actinomycin D (Section 3.1.3.).
Since actinomycin D (1 µg/ml) does not inhibit transfer of DNA
precursors between cells (Section 3.1.3.3.), this shows that the
label in untreated recipient cells must be due to labelled

nucleotide transfer from donor cells. If RNA transfer had occurred, 84% of the label present in untreated recipient cells should be present in the actinomycin D treated recipient cells (ratio of mean grains/cell of actinomycin D treated donor cells to untreated donor cells; Fig. 7a,c). The small amount of label (5%), transferred to actinomycin D treated recipient cells, is still due to incorporation of labelled nucleotides, since the level of actinomycin D (1 µg/ml) used causes only 95% inhibition of $[^3H]$ uridine incorporation into cellular acid insoluble material. Autoradiographs (Fig. 21b) show that while most of this residual label is located evenly distributed in the nucleus, it is present in only a proportion of the cells. This suggested that the label was in DNA, not RNA, and evidence from RNase digestion of $[^3H]$ uridine labelled cells supports this view (Section 3.2.3.). Heavy nuclear label is observed in a similar proportion of untreated recipient cells (Fig. 6a, 21a), presumably those which passed through the S phase of the cell cycle during co-cultivation. Since DNA is not transferred between BHK cells (Fig. 19), it is evident that the DNA labelling observed in both control and actinomycin D treated recipient cells must be due to nucleotide transfer and incorporation.

This is consistent with the concept that all the incorporation in recipient cells is from labelled nucleotide pools, transferred from donor cells to recipient cells.

2) When the labelled nucleotide pool, present in donor cells after exposure to $[^3H]$ uridine, was depleted by a chase, and these pulse-chased donor cells were co-cultured with unlabelled recipient cells, the level of label transferred to recipient cells was reduced to 3% of the unchased control value (Section 3.1.5.). This shows that the transfer of label to recipient cells is directly related to the amount of label in the donor cell

nucleotide pool. The transfer is clearly unrelated to the amount

of label in the donor cell RNA (pulse-chased donor cells

contained 67% of the label present in unchased donor cells;

Fig. 11a,c). This reduced level of label inthe RNA of the

pulse-chased cells is due to cell division during the chase

period, offset to some extent by the incorporation of the

labelled nucleotide pool present at the end of the pulse.

The label still present in recipient cells of the pulse

chase experiment (3%) can be accounted for by the transfer and

incorporation of labelled nucleotides (Fig. 13a), still present

in donor cells after the chase (Table 12).

This is again consistent with nucleotide transfer accounting

quantitatively for the label present in recipient cells.

The possibility, that donor cells after a chase fail to

transfer label, was ruled out (Fig. 12) by the observation that

chased donor cells, pulsed again with $[^3H]$ uridine to provide a

labelled acid soluble pool, transferred label to recipient cells.

3) When pulse-chased donor cells, with a depleted labelled

nucleotide pool, were co-cultured, in the presence of

actinomycin D, with unlabelled recipient cells, pretreated with

actinomycin D, the label present in treated recipient cells was

reduced to 0.4% of the control recipient value. This confirms the

conclusions drawn from 1) & 2) above that the small amounts of

label (5% & 3% respectively), still transferred to recipient cells,

were due to labelled nucleotide transfer. Autoradiographs

(Fig. 13c, 21b) show that the label (0.4%) remaining in recipient

cells after the combined chase/actinomycin D treatment is located

in the nucleus of only some cells. This suggests that the label is

in DNA, not RNA, as in 1) above. Since DNA is not transferred

(Fig. 19), this label must also be due to nucleotide transfer and

incorporation.

4) The labelling pattern (heavy nucleoli labelling) within recipient cells is consistent with nucleotide transfer from donor cells to recipient cells (Section 3.1.4.).

5) Transfer of labelled RNA between cells might be anticipated to result in a gradient of grain density at points of contact between donor and recipient cells due to the relatively slow diffusion rates of RNA. However, this is not observed (e.g. Fig. 17a. Note the sharp decrease in density of grains from donor cell to recipient cell in the long cytoplasmic processes connecting the two cells), again indicating that RNA is not transferred between cells.

In conclusion, it is quite clear that nucleotide transfer accounts quantitatively for the observed transfer of label between $[^3H]$uridine labelled donor cells and unlabelled recipient cells. However, the transfer of a few rare species of RNA cannot be ruled out, although, in general, no transfer of RNA is observed.

Kolodny (1971, 1972) has reported that labelled RNA, but not labelled nucleotides, is transferred between 3T3 cells and between SV3T3 cells. His conclusions are based on the following observations:-

1) Excess unlabelled uridine was added during co-culture in an attempt to deplete the label in the donor cell nucleotide pool. However, results (Section 3.1.1.) with BHK cells show that excess unlabelled uridine, present during co-culture, does not greatly reduce the amount of label transferred to recipient cells, but this is due to the failure of the excess unlabelled uridine to equilibrate with the labelled uridine nucleotide pool (Section 3.1.2.). Such a failure of unlabelled nucleosides to inhibit the incorporation of pre-existing labelled nucleotide

pools has been recorded by several authors (Sisken & Kinosita, 1961; Perry, 1963: Warner et al., 1966).

2) Kolodny (1971), however, noted that inhibition by excess unlabelled uridine may be unsatisfactory, and therefore used actinomycin D to inhibit labelled nucleotide incorporation during co-culture. He found that actinomycin D (12.5 µg/ml) did not reduce the extent of transfer of label from donor to recipient cells. However, this result could not be repeated using BHK cells, where it was consistently found, using BHK cells, that actinomycin D (1 µg/ml) drastically reduced the level of label present in recipient cells.

3) Kolodny (1971) co-cultured donor cells, prelabelled with $[\text{methyl-H}^3]$ methionine, with unlabelled recipient cells. He found that there was a 10-13% transfer of donor labelled RNA to recipient cells in 9h. Clason (1971), however, has noted that after pulse-labelling BHK cells for 2h with $[\text{methyl-H}^3]$ methionine, incorporation of label continues during the chase. Therefore, a labelled precursor pool must be present (possibly S-adenosyl methionine), which is not in equilibrium with the unlabelled methionine added at the beginning of the chase. Transfer of such a pool could, at least in part, account for Kolodny's observation.

4) Kolodny (1971) further ruled out nucleotide transfer on the basis of the following two points:- a) since $[^3\text{H}]$ uridine is converted to $[^3\text{H}]$ cytidine in the cell, the $[^3\text{H}]$ uridine to $[^3\text{H}]$ cytidine ratio of the RNA should decrease with increasing time of culture. Kolodny co-cultured donor cells, prelabelled with $[^3\text{H}]$ uridine for 4h, with unlabelled recipient cells for 20h. Analysis of donor and recipient RNA gave ratios of 1.1 and 0.9-1.0 respectively. Kolodny concluded that these ratios were very similar, and that, therefore, no labelled RNA was synthesised during the 20h co-culture. However, synthesis of labelled RNA

would continue during the 20h co-culture in both donor and
recipient cells, partly as a result of turnover of unstable
donor cell RNA, and although donor RNA would have a somewhat
higher ratio due to the stable labelled RNA synthesised during
the initial 4h label time, it would perhaps not differ
significantly from that of the recipient RNA ratio. A much more
critical test would have been to analyse the donor cell RNA
immediately after the 4h label time.

b) Kolodny (1971)
analysed the acid soluble nucleotide pools of the donor and
recipient cells after the 20h co-culture and found only
background levels of radioactivity. He concluded that it "seemed
unlikely that these pools could account for the labelled RNA
seen in recipient cells". However, the nucleotide pools were not
analysed at the beginning of the co-culture, when they should
have been extensively labelled (Table 12).

Since Kolodny's results were obtained with 3T3 cells,
whereas BHK cells were used in this work, the ability of 3T3
cells to transfer nucleotides and/or RNA was briefly
investigated (Section 3.1.7.). It was shown that 3T3 and BHK
cells behave similarly. Pulse chase experiments, similar to
those described above, showed that the amount of label present
in recipient 3T3 cells is related to the amount of label in the
acid soluble pool of the 3T3 donor cells, and not to the amount
of label in the RNA. Apparently, in 3T3 cells, as in BHK cells,
RNA is not transferred, but nucleotides are. This is confirmed
by the pattern of labelling in recipient 3T3 cells (exactly
similar to BHK cells), heavy nucleolar labelling being clearly
evident. Kolodny (1971) has further suggested that whole
ribosomes may be transferred between 3T3 cells. Since rRNA is
very rapidly complexed with proteins in the course of its

maturation (Vaughan et al., 1967), it would be probable that a ribonucleoprotein complex would have to be transferred between 3T3 cells. However, as shown in Section 3.3.1., no detectable protein (<0.05%) is transferred between cells.

It appears, therefore, that Kolodny's results are incorrect and that there is nucleotide transfer, but no RNA transfer or protein transfer, between cells in tissue culture.

4.2. Nature of the molecules transferred from wild-type cells

to variant cells during metabolic co-operation.

The five possibilities suggested (Section 1.2.6.), which could explain the modification of the phenotype of variant cells, growing in contact with wild-type cells, can be summarised as follows:-

a) transfer from wild-type to variant cells of ^{3}H labelled compounds beyond the enzyme block, present in variant cells (1 & 2; that is, labelled nucleotides; labelled nucleic acid).

b) transfer of a molecule from wild-type cells to variant cells which will give a functional wild-type enzyme in the variant cells (3,4 & 5; that is, enzyme, mRNA or gene coding for the enzyme; control molecule required for enzyme synthesis in variant cells).

b) was ruled out by the separation experiments discussed in the introduction (Section 1.2.6.), which showed that no functionally active enzyme was present in variant cells after co-culture and separation from wild-type cells. This suggested that no DNA (gene), mRNA or protein was transferred between the cells. This was confirmed by the results presented in Section 3.3.1. These results showed that there is no detectable protein transfer between cells.

Therefore a), the transfer of labelled nucleotides and/or labelled polynucleotides between cells, is suggested as the basis of metabolic co-operation.

However, uridine nucleotides, and not RNA, are transferred between cells in the system discussed earlier (Section 4.1.), and evidence for thymidine nucleotide transfer was obtained by co-culturing donor cells, prelabelled with $[^3H]$thymidine, with unlabelled recipient cells. In such a mixture, any label present in recipient cells at the end of co-culture must be due to thymidine nucleotide transfer, since there is no detectable DNA transfer between cells (Fig. 19). Label was detected in recipient cells, although only after a long exposure of the autoradiographs (Fig. 20). However, this difficulty in detecting label in recipient cells was not due to inefficient transfer of thymidine nucleotides, but to the rapid decay of the labelled thymidine pool in donor cells.

Metabolic co-operation has been studied in some detail between $\underline{IPP}^+/\underline{IPP}^-$ cell mixtures, between $\underline{APP}^+/\underline{APP}^-$ cell mixtures and $\underline{TK}^+/\underline{TK}^-$ cell mixtures (Sections 1.2. & 1.2.1.). $[^3H]$hypoxanthine and $[^3H]$adenine will label purine nucleotides, RNA and DNA in wild-type cells, whereas $[^3H]$thymidine will label only thymidine nucleotides and DNA. Since neither labelled DNA nor labelled RNA are transferred between cells, but nucleotides labelled by $[^3H]$uridine, $[^3H]$thymidine, or $[^3H]$hypoxanthine (Section 3.1.9.) are transferred between cells, this strongly suggests that the transfer of labelled nucleotides from wild-type cells to variant cells, and not the transfer of labelled polynucleotides, is the basis for metabolic co-operation between cells.

This conclusion is supported by several observations.

1) When a few BHK-TK$^+$ cells are cultured with many BHK-TK$^-$

cells, in the presence of $\left[^{3}H\right]$ thymidine, not all variant cells
in contact with a wild-type cell are labelled (e.g. Fig. 8).
This observation is not compatible with labelled DNA transfer
between cells (or all the variant cells should contain label),
but is compatible with transfer of labelled nucleotides from the
wild-type cells to variant cells, with their incorporation into
only those variant cells, which are present in the S phase of
the cell cycle.

2) Analysis of the characteristic features of uridine
nucleotide transfer between cells (Section 3.1.8.), namely the
varying extent of interaction of different cell types, the
dominance of the inability of L cells to interact in mixed
culture, and the requirement for cell-to-cell contact for transfer
of label, showed that they are very similar to those of
metabolic co-operation between cells (Sections 1.2.2.; 1.2.4.;
Table 3).

3) Transfer of D-glucose-2-H^{3} labelled acid soluble pool
was observed between cells, although transfer of macromolecules
labelled by D-glucose-2-H^{3} was not observed (Section 3.3.2.).
This labelled acid soluble presumably contains molecules such
as sugar phosphates and nucleotides.

Furthermore, transfer of several dyes between cells has
been observed (Section 1.1.2.). Some of these dyes resemble
nucleotides, both in molecular weight and charge (e.g. fluorescein).

There appears, therefore, to be considerable evidence
favouring the transfer of labelled nucleotides from wild-type
cells to variant cells, as the basis of metabolic co-operation
between cells.

4.3. Molecular nature of the junctions which mediate metabolic co-operation between cells.

Evidence presented in the introduction suggests that gap junctions may mediate ion transfer, dye transfer and metabolic co-operation between cells. Since nucleotide transfer between cells is the basis of metabolic co-operation, and nucleotides are similar to some dyes known to be transferred between cells, this further supports the idea that both types of molecules may use a similar mechanism for transfer between cells. Tables 1 & 2 show that there is a good correlation between ion transfer, dye transfer, metabolic co-operation and gap junctions between cells.

Electronmicrographs of freeze-cleaved cells show that gap junctions are composed of subunits (Revel & Karnovsky, 1967). It appears that these subunits extend across the cell membrane and protrude from both surfaces. Also, they may come in contact with those in the opposite membrane when cells come in contact, providing a continuous unit from one cytoplasm to the next (Peracchia, 1973). At the centre of the subunits, there appears to be a small depression, about $25A^{\circ}$ in diameter (McNutt & Weinstein, 1970; Peracchia, 1973), which has been suggested to be the mouth of a hydrophilic channel extending through the combined subunits from cytoplasm to cytoplasm (McNutt & Weinstein, 1970). This channel would then represent the morphological basis for the cell-to-cell transfer of ions and molecules, such as nucleotides and dyes.

Pitts (personal communication) and Cox et al. (1972) have studied the requirement of energy for metabolic co-operation between cells. They found that although energy inhibitors (e.g. cyanide, azide) reduced incorporation of precursors into both wild-type and variant cells during metabolic co-operation,

metabolic co-operation itself was not inhibited. This suggests
that the transfer of molecules between cells is less dependent
on energy than the incorporation of the precursor molecules,
but it does not elucidate whether energy is required for
transfer of molecules between cells, or whether energy is
required for the formation or continued maintainence of junctions
between cells required for metabolic co-operation. However,
Politoff et al. (1969), from studies with inhibitors of energy
metabolism (e.g. dinitrophenol, cyanide), have suggested that
energy is required to maintain low resistance junctions between
Chironomus cells.

From studies with cycloheximide, a protein inhibitor, it
was concluded that neither de novo nor continuous protein
synthesis are required for the formation of junctions between
cells, or for the transfer of nucleotides between cells
(Section 3.4.1.), showing that the required proteins are already
present in the cell.

Preliminary extraction and analysis of mouse hepatic gap
junctions has revealed the presence of one major protein band
(mol.wt.20,000) and possibly 2 minor protein bands, as well as
some neutral lipids and phospholipids (Goodenough & Stoeckenius,
1972). However, when Evans & Gurd (1972) isolated nexuses (gap
junctions) from mouse liver, they detected the presence of ~20
proteins (including glycoproteins) and neutral lipid, but found
that there was little phospholipid present. There are
discrepancies between these results, but this may reflect
different extraction procedures and efficiency of analysis of
the extracted material.

Attempts to identify the nature of the molecules required to
form junctions were unsuccessful (Section 3.4.2.). It appears
that neither the proteins which bind phytohaemaggutinin (PHA)

or concanavalin A, (Con A), nor phospholipid hydrolysis (to

form lysophospholipids, which may alter the fluidity of the

membrane; Lucy, 1970) are required for the formation or

maintainence of functional junctions between cells. However,

the presence of Con A or PHA does not sterically inhibit

junction formation indirectly.

Cox et al. (1972) reported that PCMB (100μM) did not

inhibit metabolic co-operation, when junctions had already

been formed between cells, prior to PCMB treatment. This

suggested that thiol proteins, which are exposed to PCMB, are

not required for the continued normal functioning of the

junctions. The results (Section 3.4.2.), obtained using PHMB

(25μM), confirm this conclusion and also suggest that thiol

proteins are not required for junction formation between cells.

Politoff et al. (1969) have reported that 100μM

p-chloromercuriphenylsulphonic acid, (PCMS) did not uncouple

ion flow between Chironomus salivary gland cells. However,

100μM N-ethyl maleimide (NEM), another thiol reagent, did

uncouple ion flow, suggesting that thiol proteins are involved

in maintaining junctional coupling. Politoff et al. (1969)

suggest that although PCMS is more reactive than NEM, the

cellular membrane is less permeable, and therefore, the groups

relevant to maintaining junctional coupling are intercellular.

In fact, NEM may be interacting with the energy metabolism

of the cell, which may be required for junction maintainence

(see page 81).

The failure of the chemical approach above suggests that,

at present, the direct approach of isolation and characterisation

of membranes and gap junctional complexes from membranes will be

more profitable. Alternatively, the availability of variant cell

lines, differing only in their ability to interact (int$^+$ or int$^-$),

may allow the characterisation of specific junctional elements required for interaction.

The selection procedure for such cell lines (L-int$^+$, L-int$^-$) is described in Section 3.5.3. Table 13 shows that 3% of the L-int$^-$ cells interact slightly and it is presumably these cells which were selected. If gap junctions are the morphological basis of cell-to-cell transfer, the L-int$^+$ cells should contain gap junctions. Some correlative evidence for this is suggested by Sheridan (personal communication), who finds that a few L-int$^-$ cells, presumably the 3% noted above, can form low resistance junctions.

Finally, the importance of a combined study of low resistance junctions, metabolic co-operation and gap junctions between cells of the same cell line, such as those by Gilula et al. (1972), should be emphasised in elucidating the correlative relationship between these three features. In particular, the use of related int$^+$ and int$^-$ cells, such as the L-int$^+$ and L-int$^-$ cells (Section 3.5.3.), for a combined study of low resistance junctions, dye transfer, metabolic co-operation and gap junctions between these cells could help to clarify the relationship between these types of direct cell-to-cell interactions between cells.

4.4. The biological functions of intercellular junctions.

The sizes of the molecules (Section 1.1.2.), shown to be transferred between cells, range in molecular weight up to about 2,000. These include physiological molecules, such as nucleotides, and non-physiological molecules, such as dyes. Since gap junctions, which probably mediate such transfer between cells, are found in virtually all tissues studied, this suggests that cell-cell interaction must be of basic importance to multicellular organisms.

The possible reasons for, and the possible effects of, the transfer of molecules directly between cells will be considered.

1) The distribution of nutrients and waste products between cells could occur. This would allow faster spread of molecules between cells, than by an extracellular route alone. It has been noted that in the squid, loss of electrical coupling between the yolk cell and embryonic cells occurs 1-2 days after the onset of the circulation (Furshpan & Potter, 1968).

2) Pitts (1971) has described an example of growth control between cells, based on nucleotide transfer between cells. BHK-TK⁻ and BHK-IPP⁻ cells, growing together in HAT medium, require the mutual exchange of nucleotides for growth. A 1:1 mixture of these cells can grow at the wild-type rate, if all cells are in contact. However, when a sparse 1:20 mixture is plated out, growth is initially slow, but then increases. Analysis of the confluent culture eventually formed shows that approximately equal numbers of the two variant cells are present. Presumably, two (or more) differentiated or variant cells could form such a symbiotic relationship in vivo.

3) Stoker (1967b) has suggested that transfer of an inhibitor molecule between normal cells in a confluent culture prevents

further growth of these cells. Although the inhibitor is
synthesised all the time, an inhibitory level only accumulates
in the cell at confluence, due to leakage of inhibitor from the
cell. Transformed cells, which continue to grow after a
monolayer is formed, may do so because either a) they fail to
synthesis the inhibitor or b) they do not respond to the
inhibitor. PyBHK cells, when placed in contact with a confluent
culture of normal cells, behave as normal cells. Stoker (1967a,b)
concluded that PyBHK cells may fail to synthesis the inhibitor,
but that they can still respond to the oresence of the inhibitor
(a) above). However, transformed cells, which continue to grow
when placed in contact with a confluent culture of normal cells
(b) above), may either fail to recognise the normal cell
inhibitor or may lack the junction (gap) required for transfer
of the inhibitor molecule (e.g. transformed epithelial cells do
not form low resistance junctions; Section 1.1.1.3.). The use
of the L-int$^+$ and L-int$^-$ cell lines (Section 3.5.3.) may help
to clarify some of these points, since L-int$^-$ cells begin to
grow in suspension after the monolayer is formed (e.g. do L-int$^+$
cells stop growing after a monolayer is formed). However, evidence
for an inhibitor molecule is required.

Recently, a relationship between the level of cAMP in a
cell population and the growth of the cells has been noted. Low
levels of cAMP were detected in growing cultures, but high levels
are present in confluent cultures. Transformed cells, however,
contain only low levels of cAMP, even at high cell densities
(Otten et al., 1971; Sheppard, 1972). However, since adenyl
cyclase, which catalysises the conversion of ATP to cAMP, is
located in the plasma membrane, membrane-membrane interactions
may increase the synthesis of cAMP at confluence.

4) Direct interactions between cells may be mediated by membrane-membrane interactions or by transfer of molecules from cell to cell (see 3) above). Interactions between cells in tissue culture, known to require direct contact (Martz & Steinberg, 1973), are:-

a) contact inhibition of overlapping, which gives one-cell-deep layer (monolayer) on the culture substratum.

b) contact inhibition of colony expansion. This follows from the requirement of contact inhibition of overlapping.

c) contact inhibition of speed of nuclear translocation.

A probable example of contact inhibition of overlapping in vivo is observed in epithelial wound healing (Lash, 1955), where cells moving over a wound stop when they meet, so that no multilayering of cells is observed.

Martz & Steinberg (1973) have suggested that differential adhesion, between cell-substratum and cell-cell, may explain a) and b) above.

Loewenstein & Penn (1967) have shown that epithelial cells migrate across a wound. Low resistance junctions can be detected between the cells of the two opposing borders of the wound within 30 min of the borders meeting. Therefore, direct transfer of molecules between cells may be required for contact inhibition of overlapping.

5) The activity of cells in an organ or tissue could be co-ordinated by transfer of a control molecule between the cells. One example of the co-ordinated activity of an organ (heart) has already been observed (Section 1.1.). This is mediated by ion transfer between the cells.

Since many hormones interact with the membrane of target cells, and some of these result in the synthesis of cAMP

(Pastan & Perlman, 1971), it is possible that intercellular transfer of cAMP could produce an co-ordinated response in a group of cells.

6) The transfer of molecules between cells has been suggested to explain observations in the development of multicellular organisms. Crick (1970) has calculated that the diffusion of a small molecule (e.g. cAMP) could account for the establishment of morphoenetic gradients in embryonic development, "provided a special mechanism which allows relatively quick passage of the morphogen from one cell to another in the tissue of interest" is available. Gap junctions provide this special mechanism.

Bonner (1970), working with the cellular slime mould D. discoideum has suggested that cAMP may induce cellular differentiation of cells in the slug to form stalk cells. This may require direct cell-to-cell transfer of cAMP. Ennis & Sussman (1958) has also shown in D. discoideum that apparent normal fruiting bodies are produced when 10% wild-type cells are mixed with 90% variant cells, which are blocked in development. Some type of direct cell-cell interaction may be necessary to explain this observation.

Differentiation of the ectoderm layer, present in early amphibian, depends on the organiser action of the underlying mesoderm. Kelley (1969), formed explants of mesoderm wrapped with ectoderm. When ectoderm was incubated for 3h with mesoderm, prelabelled with uridine-5-H^3, ectoderm was labelled in the nucleoli, nucleus and cytoplasm. This is consistent with the direct transfer of labelled nucleotides from mesoderm to ectoderm, as observed in Section 3.1. Therefore, providing there is cell-to-cell contact, the direct transfer of small molecules, such as nucleotides, could account for the "organiser effect" of

mesoderm on ectoderm.

In conclusion, the concept that an individual cell in a multicellular system acts as a discrete entity must be reconsidered. If all the cells can interact, then each cell can share at least some products synthesised in other cells; that is, each cell can share by cell-cell transfer, certain products of the expressed gene pool of all the other cells.

5. SUMMARY

5. Summary.

1.   The literature concerning two types of direct interaction between cells, the formation and function of low resistance junctions and metabolic co-operation between cells, is reviewed.

2.   Autoradiography was used to analyse transfer of radioactivity from prelabelled donor cells to unlabelled recipient cells in tissue culture.

3.   It was shown that uridine, thymidine, and purine nucleotides are transferred between BHK cells in contact in tissue culture. Such nucleotide transfer must involve the formation of junctions, permeable to nucleotides, between interacting cells.

4.   It was shown that RNA, DNA and protein are not transferred between BHK cells in contact in tissue culture.

5.   It was shown that nucleotide transfer is the basis of metabolic co-operation between cells.

6.   Nucleotide transfer between cells is not inhibited by actinomycin D or cycloheximide, showing that continuous RNA or protein synthesis are not required for the formation or function of intercellular junctions.

7.   Junction formation between cells is not inhibited by sub-toxic concentrations of concanavalin A, phytohaemagglutinin, nupercaine or p-hydroxymercuribenzoate.

8.   It was shown that BHK and 3T3 cells show extensive
nucleotide transfer, that HeLa cells show a reduced
nucleotide transfer and that L cells do not transfer
nucleotides.

9.   A technique was established for the isolation of cells,
which form junctions, from populations of mouse L cells.

10.   Analysis of somatic cell hybrids formed between human
cells, which can form junctions, and mouse L cells, which
cannot, show that this technique will permit the human
chromosome assignment of the genes for junction formation.

11.   The biological significance of direct interaction
between cells is discussed.

6. REFERENCES

Adams,R.L.P. & Lindsay,J.G.(1966) J.Biol.Chem. 242, 1314-1317

Adams,R.L.P., Abrams,R. & Lieberman,I.(1966) J.Biol.Chem. 241, 903-905

Adams, R.L.P., Berryman,S. & Thomson,A.(1971) Biochim.Biophys. Acta 240, 455-462

Allan,D. & Crumpton,M.J.(1973) Exp.Cell Res. 78, 271-278

Allan,D., Auger,J. & Crumpton,M.J.(1971) Exp.Cell Res. 66, 362-368

Azarnia,R., Michalke,W. & Loewenstein,W.R.(1972) J.Membrane Biol. 10, 247-258

Bendich,A., Vizoso,A.D. & Harris,R.G.(1967) Proc.Natl.Acad.Sci. U.S. 57, 1029-1035

Bennett,M.V.L., Pappas,G.D., Giménez,M. & Nakajima,Y.(1967a) J.Neurophysiol. 30, 236-300

Bennett,M.V.L., Dunham,P.B. & Pappas,G.D.(1967b) J.Gen.Physiol. 50, 1094.

Bonner,J.T.(1970) Proc.Natl.Acad.Sci.U.S. 65, 110-113

Borek,C., Higashino,S. & Loewenstein,W.R.(1969) J.Membrane Biol. 1, 274-293

Brightman,M.W. & Reese,T.S.(1969) J.Cell Biol. 40, 648-677

Bullivant,S. & Loewenstein,W.R.(1968) J.Cell Biol. 37, 621-632

Burdon,R.H.B. & Adams,R.L.P.(1969) Biochim.Biophys.Acta 174, 322-329

Bürk,R.R., Pitts,J.D. & Subak-Sharpe,J.H.(1968) Exp.Cell Res. 53, 297-301

Castillo,J.del & Katz,B.(1954) J.Physiol. 124, 586-604

Clason,A.(1971) Ph.D. Dissertation, The University of Glasgow.

Comings,D.E.(1965) Exp.Cell Res. 41, 677-681

Cox,R.P., Krauss,M.R., Balis,M.E. & Dancis,J.(1970) Proc.Natl.Acad. Sci.U.S. 67, 1573-1579

Cox,R.P., Krauss,M.R., Balis,M.E. & Dancis,J.(1972) Exp.Cell Res. 74, 251-268

Crick,F.(1970) Nature 225, 420-422

Dancis,J., Cox,R.P., Berman,P.H., Jansen,V. & Balis,M.E.(1969) Biochem.Genet. 3, 609-615

Ennis,H.L. & Sussman,M.(1958) J.Gen.Microbiol. 18, 433-449

Evans,W.H. & Gurd,J.W.(1972) Biochem.J. 128, 691-700

Friedmann,T., Seegmiller,J.E. & Subak-Sharpe,J.H.(1968) Nature 220, 272-274

Fujimoto,W.Y., Seegmiller,J.E., Uhlendorf,P.W. & Jacobson,C.B. (1968) Lancet 1968-II, 511-512

Fujimoto,W.Y., Subak-Sharpe,J.H. & Seegmiller,J.E.(1971) Proc. Natl.Acad.Sci.U.S. 68, 1516-1519

Furshpan,E.J. & Potter,D.D.(1959) J.Physiol. 145, 289-325

Furshpan,E.J. & Potter,D.D.(1968) Curr.Top.Develop.Biol. 3, 95-127

Gey,G.O., Coffman,W.D. & Kubicek,M.T.(1952) Cancer Res. 12, 264-265

Gilula,N.B. & Satir,P.(1971) J.Cell Biol. 51, 869-872

Gilula,N.B., Reeves,O.R. & Steinbach,A.(1972) Nature 235, 262-265

Goodenough,D.A. & Stoeckenius,W.(1972) J.Cell Biol. 54, 646-656

Hagiwara,S. & Tasaki,I.(1958) J.Physiol. 143, 114-137

Hopps,H.E., Bernheim,B.C., Nisalak,A., Tjio,J.H. & Smadel,J.E. (1963) J.Immunol. 91, 416.

Hurlbert,R.B. & Kammen,H.O.(1960) J.Biol.Chem. 235, 443-449

Ito,S. & Hori,N.(1966) J.Gen.Physiol. 49, 1019-1027

Jamakosmanovic,A. & Loewenstein,W.R.(1968a) Nature 218, 775.

Jamakosmanovic,A. & Loewenstein,W.R.(1968b) J.Cell Biol. 38, 556-561

Johnson,R.G. & Sheridan,J.D.(1971) Science 174, 717-719

Kaneko,A.(1971) J.Physiol. 213, 95-105

Kanno,Y. & Loewenstein,W.R.(1966) Nature 212, 629-630

Kanno,Y. & Matsui,Y.(1968) Nature 218, 775-776

Katz,B.(1966) "Nerve, Muscle and Synapse." McGraw-Hill, New York.

Kelley,R.O.(1969) J.Exptl.Zool. 172, 153-180

Kenney,F.T.(1967) Science 156, 525-528

Kolodny,G.M.(1971) Exp.Cell Res. 65, 313-324

Kolodny,G.M.(1972) J.Cell Physiol. 79, 147-150

Kuffler,S.W. & Potter,D.D.(1964) J.Neurophysiol. 27, 290-320

Lash,J.W.(1955) J.Exptl.Zool. 128, 13-27

Leibman,K.C. & Heidelberger,C.(1955) J.Biol.Chem. 216, 823-830

Lieberman,I.(1956) J.Biol.Chem. 222, 765-775

Lindholm,L. & Britton,S.(1967) Exp.Cell Res. 48, 660-665

Littlefield,J.W.(1966) Exp.Cell Res. 41, 190-196

Loewenstein,W.R. & Kanno,Y.(1964) J.Cell Biol. 22, 565-586

Loewenstein,W.R. & Kanno,Y.(1966) Nature 209, 1248-1249

Loewenstein,W.R. & Kanno,Y.(1967) J.Cell Biol. 33, 225-234

Loewenstein,W.R. & Penn,R.D.(1967) J.Cell Biol. 33, 235-242

Loewenstein,W.R., Socolar,S.J., Higashino,S., Kanno,Y. &
Davidson,N.(1965) Science 149, 295-298

Lucy,J.A.(1970) Nature 227, 815-817

Lyon,M.F.(1961) Nature 190, 372-373.

McCargow,J. & Pitts,J.D.(1971) Biochem.J. 124, 48p.

McNutt,N.S. & Weinstein,R.S.(1970) J.Cell Biol. 47, 666-688

Macpherson,I.A. & Stoker,M.G.P.(1962) Virology 16, 147-151

Marin,G. & Littlefield,J.W.(1968) J.Virol. 2, 69-77

Martz,E. & Steinberg,M.S.(1973) J.Cell Physiol. 81, 25-38

Michalke,W. & Loewenstein,W.R.(1971) Nature 232, 121-122

Migeon,B.R., Der Kaloustian,V.M., Nyhan,W.L., Young,W.J. & Childs,B.
(1968) Science 160, 425-427

O'Lague,P., Dalen,H. & Rubin,H. & Tobias,C.(1970) Science 170, 464-
466

Otten,J., Johnson,G.S., Pastan,I.(1971) Biochem.Biophys.Res.
Commun. 44, 1192-1198

Pappas,G.D. & Bennett,M.V.L.(1966) Ann.N.Y.Acad.Sci. 137, 495-508

Pappas,G.D., Asada,Y. & Bennett,M.V.L.(1971) J.Cell Biol. 49, 173-
188

Pastan,I. & Perlman,R.L.(1971) Nature New Biol. 229, 5-7

Payton,B.W., Bennett, M.V.L. & Pappas,G.D.(1969) Science 166, 1641-
1643

Penman,S., Vesco,C. & Penman,M.(1968) J.Mol.Biol. 34, 49-69

Penn,R.D.(1966) J.Cell Biol. 29, 171-174

Peracchia,C.(1973) J.Cell Biol. 57, 66-76

Perry,R.P.(1963) Exp.Cell Res. 29, 400-406

Pinto da Silva,P. & Gilula,N.B.(1972) Exp.Cell Res. 71, 393-401

Pitts,J.D.(1971) in Ciba Foundation Symposium on Growth Control
in Cell Cultures (Wolstenholme,G.E.W. & Knight,J.,eds.) pp.89-105,
Livingstone, London.

Pitts,J.D.(1972) in Third Lepetit Colloquium on Cell Interactions
(Silvestri,L.G.,ed.) pp.277-285, North-Holland, Amsterdam.

Politoff,A.L., Socolar,S.J. & Loewenstein,W.R.(1969) J.Gen.Physiol.
53, 498-515

Potter,D.D., Furshpan,E.J. & Lennox,E.S.(1966) Proc.Natl.Acad.Sci.U.S.
55, 328-336

Reese,T.S.M., Bennett,V.L. & Feder,W.(1971) Anat.Rec.Abract. 169, 409.

Revel,J.P. & Karnovsky,M.J.(1967) J.Cell Biol. 33, C7-C12

Revel,J.P. & Sheridan,J.D.(1968) J.Physiol. 194, 34p-35p

Revel,J.P., Yee,A.G. & Hudspeth,A.J.(1971) Proc.Natl.Acad.Sci.U.S.
68, 2924-2927

Rose,B.(1971) J.Membrane Biol. 5, 1-19

Rosenbloom,F.M., Kelley,W.N., Henderson,J.F. & Seegmiller,J.E.(1967)
Lancet, 1967-II, 305-306

Ruddle,F.H.(1973) Nature 242, 165-169

Sanford,K.K., Earle,W.R. & Likely,G.D.(1948) J.Natl.Cancer Inst.
9, 229-246

Satir,P. & Gilula,N.B.(1970) J.Cell Biol. 47, 468-487

Scarpa,A. & Lindsay,J.G.(1972) Eur.J.Biochem. 27, 401-407

Scherphof,G.L., Waite,M. & van Deenen,L.L.M.(1966) Biochim.Biophys.
Acta 125, 406-409

Sharon,N. & Lis,H.(1972) Science 177, 949-958

Sheppard,J.R.(1972) Nature New Biol. 236, 14-16

Sheridan,J.D.(1966) J.Cell Biol. 31, C1-C5

Sheridan,J.D.(1971a) J.Cell Biol. 50, 795-803

Sheridan,J.D.(1971b) Develop.Biol. 26, 627-636

Shoog,L. & Nordenskjöld,B.(1971) Eur.J.Biochem. 19, 81-89

Sisken,J.E. & Kinosita,R.(1961) Exp.Cell Res. 24, 168-170

Slack,C. & Palmer,J.F.(1969) Exp.Cell Res. 55, 416-419

Staehelin,L.A.(1972) Proc.Natl.Acad.Sci.U.S. 69, 1318-1321

Stoker,M.G.P.(1964) Exp.Cell Res. 35, 429-431

Stoker,M.G.P.(1967a) J.Cell Sci. 2, 293-304

Stoker,M.G.P.(1967b) Curr.Top.Develop.Biol. 2, 107-128

Subak-Sharpe,H.(1965) Exp.Cell Res. 38, 106-119

Subak-Sharpe,J.H.(1969) in Ciba Foundation Symposium on Homeostatic Regulators (Wolstenholme,G.E.W. & Knight,J., eds.) pp.276-288, Churchill, London.

Subak-Sharpe,H., Bürk,R.R. & Pitts,J.D.(1966) Heredity $\underline{21}$, 342-343

Subak-Sharpe,H., Bürk,R.R. & Pitts,J.D.(1969) J.Cell Sci. $\underline{4}$, 353-367

Todaro,G.J. & Green,H.(1963) J.Cell Biol. $\underline{17}$, 299-313

Tupper,J., Saunders,J.W.,Jr. & Edwards,C.(1970) J.Cell Biol. $\underline{46}$, 187-191

Van Zeeland,A.A., van Diggelen,M.C.E. & Simons,J.W.I.M.(1972) $\underline{14}$, 355-363

Vaughan,M.H., Warner,J.R. & Darnell,J.E.(1967) J.Mol.Biol. $\underline{25}$, 235-251

Waite,M. & van Deenen,L.L.M.(1967) Biochim.Biophys.Acta $\underline{137}$, 498-517

Warner,J.R., Soeiro,R., Birnboim,H.C., Girard,M. & Darnell,J.E.(1966) J.Mol.Biol. $\underline{19}$, 349-361

Watanabe,A. & Grundfest,H.(1961) J.Gen.Physiol.$\underline{45}$, 267-308

Weber,M.J. & Rubin,H.(1971) J.Cell Physiol. $\underline{77}$, 157-168

Weiss,M.C. & Green,H.(1967) Proc.Natl.Acad.Sci.U.S. $\underline{58}$, 1104-1111

Widmer-Favre,C.(1972) J.Cell Sci. $\underline{11}$, 261-274