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"Junctional Communication Between Animal Cells"

A thesis submitted to the University of Glasgow in partial fulfilment for the degree of Doctor of Philosophy in the Faculty of Medicine

by

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To my parents.

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Abbreviations.

Abbreviations used in this thesis are those recommended in the Biochemical Journal "Instructions to Authors" (1981),with the following exceptions:

ASV	-	Avian sarcoma virus
BSS	-	Balanced salts solution
cAMP	-	Cyclic AMP
cpm	-	counts per minute
DMSO	-	dimethyl sulphoxide
EDTA	-	ethylene diamine tetra-acetic acid
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
HCG	-	human chorionic gonadotrophin
IMP	-	intramembranous particle
K	-	thousand
NRK	-	normal rat kidney
PAGE	-	polyacrylamide gel electrophoresis
PPO	-	2,5 diphenyl oxazole
RA	-	retinoic acid
SDS	-	sodium dodecyl sulphate
src	-	sarcoma
TCA	-	trichloracetic acid
tigason	-	tri methyl, methoxy phenyl analogue of ethyl retinoate
ts	-	temperature sensitive
TPA	-	phorbol, 12 myristate 13 acetate

Summary.

1. The literature concerning the structure, permeability and function of gap junctions has been reviewed.

2. Aspects of the occurrence, metabolism and biological activities of the family of compounds derived from vitamin A known as retinoids, have been described.

3. A method has been developed by the modification of established procedures for the purification of gap junctions from small numbers of cultured cells ($\sim 10^9$ cells).

4. The method produces fractions which appear by electron microscopy to be rich in gap junctions. SDS PAGE of these fractions shows only one major protein component which has an apparent M_r of 16,000 (16K). The yield of the 16K component in different isolations has been examined, and is sufficiently reproducible (± 5%) to allow the method to be used for estimating junctional area by measuring junctional protein.

5. Further evidence has been obtained for the junctional origin of the 16K protein :

a) it is the major (sometimes only detectable) component in junctional preparations from a wide variety (mammalian, amphibian, piscine) of cultured cell types.

b) the recovery of the 16K component from Chinese hamster fibroblasts (V79 cells) before, during and after treatment with the tumour promoter, phorbol 12 myristate 13 acetate (TPA) parallels the changes in junctional area measured

morphometrically by Yancey et al, (1982) in freeze-fractured samples.

6. It has been shown that trans retinoic acid (10^{-4}M) inhibits junctional communication (assayed by measuring [^{3}H]-uridine nucleotide transfer between cells in culture) between a variety of cell types.

7. An estimate has been made of the rate of inhibition of junctional communication after the addition of retinoic acid to cell cultures. It has been shown to be rapid (half life of the open state = a few minutes or less) and to be fully and rapidly reversible.

8. Evidence has been obtained which indicates that the inhibition of junctional communication between rat liver (BRL) epithelial cells and between Syrian hamster fibroblasts (C13 cells) by 10^{-4}M trans retinoic acid appears to be caused by a reduction of junctional permeability and not by loss of the gap junction structures.

9. It has been shown that 10^{-5}M trans retinoic acid does not affect junctional communication between BRL cells and between C13 cells but that intermediate concentrations (8×10^{-5}M - 2×10^{-5}M) produce graded changes in permeability and that all the cells appear to be similarly affected.

10. It has been observed that some cell types (e.g. Chinese hamster (V79) cells and GCCM cells (derived from a human glioma))appear to be more sensitive to retinoic acid with inhibition of [^{3}H]-uridine nucleotide transfer between cells occurring at lower concentrations (10^{-4}M - 10^{-6}M).

Results have been obtained which suggest that in the V79 cell system, retinoic acid may be affecting the process of junction formation.

11. Trans retinoic acid has been purified by paper chromatography in two solvent systems and the activity (i.e. the inhibition of [^{3}H]uridine nucleotide transfer between C13 cells) has been shown to co-migrate with the major component. The inhibition is not caused by a separable minor component of commercial trans retinoic acid preparations.

12. It has been shown that treatment of BRL cells with 10^{-4}M trans retinoic acid results in both loss of junctional communication and in an increase (3 fold after 24h treatment) in the amount of the 16K protein which can be isolated in junction enriched pellets. This suggests there is an increase in the number of (non-functional) junctional plaques present.

13. V79 cells do not show an increase in the 16K protein when treated with 10^{-4}M retinoic acid which is consistent with retinoic acid affecting junction formation.

14. The importance and implication of these results have been discussed.

Chapter 1 - Introduction.

1.1. INTRODUCTION

The evolution of multicelled organisms must have required the development of cell-cell communication to allow co-ordination and control of the proliferation, differentiation and activities of the component cells. Two basic mechanisms of communication exist. The first involves the release of signal molecules which move through the extracellular fluids and interact with receptors on or in target cells (e.g. hormonal communication and chemical synaptic transmission). The second mechanism which has been of increasing importance over the past twenty years,is based on the intercellular movement of low molecular weight cytoplasmic solutes between neighbouring cells. The gap junction is thought to be responsible for this form of cell-cell communication.

1.1.1. Distribution of Gap Junctions

Gap junctions are ubiquitous structures being found in a wide variety of organisms from coelenterates (Hydra Hand and Gloebel, 1972) to vertebrates (Dewey and Barr, 1962) and arthropods (Flower, 1972; Quick and Johnson, 1977). They are also found between cells in tissue culture (Revel et al, 1971; Gilula et al, 1972), in early developmental stages of mammalian (Dulcibella and Anderson, 1975) and amphibian embryos (Hanna et al, 1980) and in larval stages of the insect (Lawrence and Green, 1975).

Excitable and non-excitable cells of both vertebrates and invertebrates are coupled by gap junctions (Revel and Karnovsky, 1967; Pappas et al, 1971; Rose, 1971; Quick and Johnson, 1977). Gap junctions are more numberous between cells in excitable tissues (Friend and Gilula, 1972).

Skeletal muscle cells, circulatory cells and many nerve cells are the only major cell types so far examined which appear to be never coupled by gap junctions.

Gap junctions have been found in so many tissues that it seems reasonable to believe that these junctions are formed between the majority of cell types in multicelled animals.

A form of junctional communication also occurs between cells in plants. Intercellular junctions, known as plasmodesmata are found between most plant cells and they may have similar permeability properties to the gap junction between animal cells (Goodwin, 1981).

1.2 STRUCTURE OF GAP JUNCTIONS.

1.2.1 Morphology of Gap Junctions.

Two common sample preparation techniques, freeze-fracture replication and thin-section of plastic embedded material,have been used to visualise gap junctions in tissues by electron microscopy. The two techniques have contributed different information about the structure of the junctions (Revel and Karnovsky, 1967; Goodenough and Revel, 1970).

In thin section preparations of a wide variety of animal tissues gap junctions are seen as regions of close apposition between plasma membranes of adjacent cells. They can have a five or seven layered appearance depending on the fixative and stain used (Dewey and Barr, 1962; Revel and Karnovsky, 1967). When tissue blocks are infiltrated with lanthanum hydroxide the extracellular space is filled with the electron opaque tracer and in occasional oblique sections this delineates hexagonal arrays of particles between the juxtapposed plasma membranes of the gap junction. The images obtained by conventional thin section and lanthanum infiltration are common for all phylogenetic forms of gap junctions although there are slight variations in the width of extracellular space or 'gap' which is around 2nm for the vertebrates (Revel and Karnovsky, 1967) and 4-5 nm for the arthropods (Flower, 1972).

Gap junctional structure is further defined by freeze-fracture analysis which reveals the internal arrangement of membranes. The fracture process preferentially splits membranes along the bilayer to expose two faces; an E-face adjacent to the cytoplasm and a P-face adjacent to the

extracellular space (Branton, 1966). Gap junctions appear by this technique as aggregates of particles. These aggregates are often referred to as junctional plaques. The junctional particles always fracture onto one face and leave corresponding depressions or pits in the opposing face (Gilula, 1972). The face onto which the junctional particles partition is a phylogenetic characteristic. For example, the junctional particles always partition to the P-face in vertebrates (McNutt and Weinstein, 1973) and molluscs (Flower, 1977) whereas in arthropods they partition to the E-face (Flower, 1972 and 1977).

In vertebrate junctions the particles are of uniform size being 7-8 nm in diameter on average (McNutt and Weinstein, 1973) and the particles are often regularly packed (McNutt and Weinstein, 1970). The particles in arthropod junctions are larger, more heterogeneous in size and are more often irregularly packed (Flower, 1972).

Early freeze-fracture preparations and lanthanum stained thin sections suggested that the gap junction particles were regularly packed in hexagonal arrays. The more recent development of the rapid-freeze technique at liquid helium temperatures, which replaces conventional fixation and infiltration with cryoprotective agents, indicates that the 'crystalline' arrangement of junctional particles may occur during some fixation procedure or in damaged tissues (Raviola et al, 1981).

Particle packing density in rapidly frozen tissues varies considerably both for the same cell type and between different cell types. In rabbit ciliary epithelium, gap junction particles are well separated with smooth membrane

matrix in between but in the corneal endothelium both loose and crystalline arrangements of particles are seen, and in the stomach and liver the particles are tightly packed but are not crystalline (Raviola et al, 1981). Crystallinity of particle packing can be induced by treatments such as exposure to divalent cations, H^+ or 2, 4 dinitrophenol, all of which have been shown to electrically uncouple cells. These changes in gap junction structure which are (usually) reversible may reflect conformational changes in particle subunit structure resulting in changes in packing and functional uncoupling (see section 1.2.4).

Considerable variation exists not only in particle packing but also in the configuration of the plaques. Most particle aggregates are roughly circular or elliptical. In cultured embryonic chick cardiac muscle cells, linear strings of particles are found alongside branched aggregates. Junctional plaques can either be in one large group as in human ovarian decidual cells or in numerous groups separated by particle free spaces as in cardiac muscle of neonatal rat (Larsen,1977).

Goodenough and Gilula (1974) have also found variations in particle size. Liver gap junctions are composed mainly of7.7 nm particles but have a peripheral line of particles 9.5-12 nm in diameter. Larger particles have also been observed loosely clustered in the vicinity of developing junctional particle aggregates (Decker and Friend, 1974; Johnson et al, 1974; Albertini and Anderson, 1974; Decker, 1976).

The variability of gap junction structure is as yet not fully understood but is possibly related to the formation,

growth and degradation of gap junctions or to their function in different tissues. On the other hand, the variations may be a consequence of the constraints placed upon them by cell architecture and the position they occupy between cells rather than any inherent differences in the junctional particles or in particle-particle interactions (Finbow, 1982). The development of isolation techniques and the subsequent isolation of gap junctions has allowed more detailed physical (structural) and chemical analysis as will be described in sections 1.2.2. and 1.2.3.

1.2.2. Isolation of Gap Junctions.

Gap junctions have been isolated by exploiting the resistance of the plaques to detergents such as deoxycholate (Benedetti and Emmelot, 1968) or sarkosyl NL97 (Goodenough and Stoeckenius, 1972). This has led to the isolation of gap junctions in morphologically pure form from mouse liver (Goodenough and Stoeckenius, 1972; Duguid and Revel, 1975; Culvenor and Evans, 1977; Ehrhart and Chaveau, 1977; Finbow *et al*, 1982), rat liver (Gilula, 1974; Finbow *et al*, 1980), mouse heart (Goodenough *et al*, 1978; Kensler and Goodenough, 1980) and from various cell types grown in culture (Finbow *et al*, 1982).

Junctional isolates from the various sources have been analysed using SDS polyacrylamide gel electrophoresis. The gel profiles obtained have shown considerable variation due to a number of reasons. Firstly, to the difficulty in determining purity of junction enriched pellets, secondly, to some of the procedures used in isolating the junctional protein (e.g. protease treatment), thirdly, to the aggregation

of membrane proteins in SDS solution, and fourthly, because the junctional protein is a relatively minor component of total membrane protein, to the small amounts of junctional protein obtained (e.g. ~ 1 μg/g wet weight liver; Finbow, 1982).

To obtain preparations of acceptable purity, treatments with hyaluronidase and with proteases (such as trypsin and collagenase) are included in the isolation protocol and it is possible that these cause a variable degradation of the junctional protein even though the morphological appearance of the gap junction after protease treatment is unaltered. Modified protocols have been devised in which exposure to proteases has been reduced to attempt to isolate the protein in an undegraded form. This, however, results in a decrease in sample purity, shown by the more complex SDS polyacrylamide gel profiles (Goodenough, 1976; Culvenor and Evans, 1977) making it more difficult to identify the junctional protein. Morphologically pure gap junctions have been isolated in the absence of protease treatment where 6M urea was used to remove contaminating proteins (Henderson et al, 1979; Hertzberg and Gilula, 1979).

The confusion surrounding the identity of the junctional protein is reflected in the various molecular weights attributed to the gap junctional protein:

10,000 (Goodenough, 1974, 76; Gilula, 1974; Henderson et al, 1979; Hertzberg and Gilula, 1979; Finbow et al, 1980).

16,000 (Finbow et al, 1982).

26,000 (Henderson et al, 1979; Hertzberg and Gilula, 1979; Finbow et al, 1980, 1982).

31,000 (Kensler and Goodenough, 1980).

33,500 (Kensler and Goodenough, 1980).

38,000 (Culvenor and Evans, 1977; Kensler and Goodenough, 1980).

The most recent study (Finbow et al, 1982) has shown that the gap junction is composed of a major protein component with an apparent M_r of 16,000. The protein has unusual aggregation properties when heated in SDS solutions. Boiling for a few minutes results in the appearance of higher molecular weight components which are believed to represent multimeric forms of the protein. Such aggregation may have mislead earlier investigators since solubilisation of gap junction enriched pellets in SDS by heating to 100°C can produce such highly aggregated forms of the protein that the material does not enter the separating gel, and the gel profiles observed are those of unaggregated contaminating proteins present.

The most abundant higher M_r species produced after heating at 100°C for 5 min is a component with a M_r of 26,000 which had previously been characterised as being of junctional origin (Henderson et al, 1979; Hertzberg and Gilula, 1979; Finbow et al, 1980). This component is believed to be a dimer of the 16K protein. Heat induced aggregation of the 16K component may produce this dimer which is a more compact molecule and therefore has a higher mobility on SDS PAGE. When the M_r of this component is estimated with reference to molecular weight markers it

therefore appears less than would be expected (i.e. it is not twice 16K).

The best evidence for the gap junctional origin of the 26,000 protein is based on the regenerating weanling rat liver system. The amount of this protein species in purified fractions isolated from regenerating liver (between 24 and 28 hours after partial hepatectomy) correlates very well with the measured changes in total area of junctional plaques seen in freeze-fracture preparations (Finbow et al, 1980). A similar correlation has now been made for the 16K junctional component (Finbow et al, 1982) isolated by a different protocol from that used previously. Additional correlative evidence for the junctional origin of the 16K component is based on the reduction of gap junctional area measured by morphometry of freeze fracture electron micrographs made after treatment of cultured V79 cells with the tumour promoting agent, TPA (4 - phorbol 12 - myristate 13 - acetate)(Yancey et al, 1982) and will be presented in this thesis.

1.2.3. Amino Acid Analysis and Chemical Composition.

A partial amino acid sequence of the major 26K polypeptide obtained from gap junction rich fractions isolated from rat liver in high yield and purity, has been determined (Nicholson et al, 1982). With the exception of a non-junctional component of M_r 38,000, all the polypeptides present on SDS polyacrylamide gel electrophoresis are found to have very similar or identical peptide maps and are thought to have arisen from proteolytic cleavage of the carboxyl terminus or aggregation of the 26K component.

The gel band characteristic of trypsin treated junction preparations (M_r 10,000) is found to be two distinct polypeptides both derived from the M_r 26,000 polypeptide.

The first 52 residues of the N terminus of the 26K component reveal a strongly hydrophobic region of 14 residues, starting from the N terminus as might be expected for a transmembrane protein (Nicholson et al, 1982).

As the cytoplasmic faces of the gap junctions are the most exposed regions during isolation it seems likely that proteolytic cleavage will occur at these sites. This is consistent with the finding by Nicholson et al (1982) that the NH_2-terminus sequence is conserved in the 26K and 10K components. Application of the Chou and Fasman paradigm to the sequence data strongly predicts the hydrophobic region of the gap junctional protein to be in a β-pleated sheet conformation.

Analyses of highly purified junctional protein have so far revealed no indication of associated carbohydrate (Griepp and Revel, 1977; Revel et al, 1978). This is unusual since most membrane proteins are known to be glycosylated. Gap junction fractions isolated by ultracentrifugation and detergent treatment contain about 47-52% lipid and 46% protein (Caspar et al, 1977; Revel et al 1980). The main phospholipid is phosphatidyl choline with a small amount of phosphatidyl ethanolamine (Revel et al, 1978).

1.2.4. Fine Structure.

Freeze fracture and thin section techniques have,in

occasional preparations,produced more detailed information about the structure of gap junctions. In some shadowed replicas of freeze-fractured tissues, a central dot 2.5nm in diameter is seen on each junctional particle. This is thought to indicate the presence of a water-filled pore (McNutt and Weinstein, 1970). In thin sectioned specimens, some particles are seen to be penetrated by a central core of colloidal lanthanum after fixation (Revel and Karnovsky, 1967) and a similar penetration can be seen in preparations of isolated junctions negatively stained with soluble heavy metal salts (Benedetti and Emmelot, 1968).

Further evidence for the existence of a hydrophilic pore along the central axis was provided in a study of the stain distribution within rat liver gap junction specimens, examined by thin section and negative staining,which showed that the stain molecules are found in two specific locations with respect to the junctional particles (Zampighi et al, 1980). Stain molecules surround the particles in the extracellular space between adjacent plasma membranes, and are also located along the central axis of each particle. The central axis measures 1-2 nm in diameter and 10nm in total length.

X-ray crystallography and electron microscopic image analysis of gap junction plaques isolated intact from mouse liver have also been used to correlate structure and chemical composition and have confirmed the general picture described above (Zampighi and Unwin, 1979).

These observations (coupled to the fact that the gap junction is believed to be permeable to small ions and molecules) lead to the channel hypothesis which envisages the junctional particles crossing the membranes of both cells

and each particle containing a water filled channel running its entire length and therefore joining the cytoplasms of the two cells (McNutt and Weinstein, 1970).

It is believed that there may be a correlation between centre-to-centre spacing of junctional subunits and uncoupling. Electrical uncoupling can be induced by experimental anoxia, lowered pH or exposure to divalent cations such as Ca^{2+}. Freeze-fracture replicas of gap junctions isolated from tissues exposed to any of the above treatments are characterised by a tighter aggregation of the junctional particles and a decrease in the overall width (cytoplasmic face to cytoplasmic face) of the junction and in the width of the gap. This crystallisation (i.e. decreased and more ordered particle spacing) has been observed in crayfish septate axons (Peracchia and Dulhunty, 1976), in mouse stomach and liver and in rabbit ciliary epithelium (Raviola et al, 1981). There is,however,no direct evidence that these crystalline junctions represent gap junctions in an impermeable state; and structural rearrangement of gap junction particles is not observed in uncoupled insect salivary glands (Revel, personal communication).

Two forms of junction conformation have also been seen in liver junctions after isolation which are thought to represent two different permeability states of the channels. One form of the isolated gap junction was produced using detergent extraction and methods directed at minimising the effects of proteolysis (Zampighi and Unwin, 1979). This form of the junction is the same as that seen in crude fractions before detergent treatment while the other form is found

only after dialysing the purified fraction against water over a period of several days. The two forms are interconvertible by the addition or removal of detergent.

The difference between these two alternative forms of the gap junction may represent a simple molecular mechanism of controlling channel permeability. High resolution electron microscopy data indicate that the changes in configuration may be achieved by the subunits on the cytoplasmic face sliding against each other, and rotating in a clockwise manner and resulting in channel closure (Unwin and Zampighi, 1980). The dimensional differences between the two channel conformations could account for observed permeability changes (Unwin and Zampighi, 1980). The junctions used in this study were isolated by detergent extraction and therefore would contain a mixture of protein,detergent and phospholipid. It is possible that on dialysing to remove the detergent some phospholipid is also removed. This may result in a rearrangement in the subunits with the protein molecules moving closer together resulting in the apparently 'closed' junction. The observations in this _in vitro_ system may not be physiologically relevant and their relation to the _in vivo_ form is unclear.

The differences in permeabilities of a series of fluorescent tagged amino acids and peptides of different size and charge have led to the suggestion that a charged group influences movement through the junctional channel (Flagg-Newton _et al_, 1979). The permeability was limited by the pore size and electronegativity of the injected molecules and the two factors were reciprocally related.

In respect to both these parameters,vertebrate junctional channels are more restrictive than invertebrate channels suggesting that vertebrate channels are narrower, more polar or both (Flagg-Newton et al, 1979).

The existence of a fixed negative charge within the junctional channels of giant lateral earthworm axons was also indicated in the experiments of Brink and Dewey (1980), who found that fluorescein diffusion was significantly suppressed in axons preinjected with aminofluorescein while carboxyfluorescein had no effect.

1.3. FORMATION AND TURNOVER OF GAP JUNCTIONS.

1.3.1. Morphological Analysis of Formation.

The information available on gap junction formation has come from morphological observations made on freeze-fracture specimens from cell types known to be coupled by junctions, such as developing embryos (Revel et al, 1973; Decker and Friend, 1974), ovarian follicle cells (Albertini and Anderson, 1974) and Novikoff hepatoma cells (Johnson et al, 1974).

Gap junctions are known to form rapidly after cells come in contact and the time scale (minutes) is such that it is thought to be too fast to allow the induction of synthesis of a new protein. It has been proposed that gap junction proteins are synthesised on endoplasmic reticulum associated ribosomes and are then transported to the cell membrane where they become associated with each other in 'formation plaques' (Revel et al, 1978). Formation plaques have been observed as early as five minutes after reaggregation of dissociated Novikoff hepatoma cells and are recognised as regions of flattened plasma membrane containing few intramembranous particles and loose groups of 9-11nm junctional particles (Johnson et al, 1974). The formation process in freeze fracture replicas of reaggregated Novikoff hepatoma cells is thought to consist of the following stages

1. development of formation plaques and reduction of the extracellular space between matched formation plaques in the adjacent cell membranes.

Formation begins when the extracellular component of

the 9-11nm particles in the opposing membranes link up and contribute to the initial cell attachment.

2. aggregation of the 9-11nm particles into tightly adherent groups indistinguishable from small gap junctions.

After the plaques form they appear to accumulate additional 9-11nm particles which may be recruited from membrane areas outside the plaque.

All these processes occur while the formation plaques are separated by 10-20nm. This space is reduced as a result of cooperative interactions of the progressively increasing numbers of 9-11nm particles.

3. growth of the small gap junctions by addition of individual particles and fusion of small aggregates (Gilula, 1972; Johnson et al, 1974).

Such well defined formation plaques have not been observed in many other systems and often the first structures seen are small gap junctions (Yancey et al, 1979).

In certain arachnids, gap junctions and tight junctions become assembled into mature structures at about the same time. In contrast with vertebrate tissues, the two junctions are composed of different sized intramembranous particles which fracture onto different fracture faces (Lane and Chandler, 1980). In such tissues it is clear that no one precursor could give rise to both junctional types, a theory which has been proposed for vertebrate tissues where both junctions are composed of 8-10nm particles which fracture on to the P face (Elias and Friend, 1976).

So far the many different systems studied all show the same temporal sequence of junction formation. That is, the

initial appearance of plaques of small gap junctions which grow in size by apparent recruitment of junctional particles. Such formation suggests that the junctional precursors are already present in the plasma membranes rather than being inserted from a cytoplasmic pool on cell-cell contact.

1.3.2. Morphological Evidence of Junction Breakdown.

Interpretation of ultrastructural data has suggested that,in vertebrate tissues,gap junctions may be removed from the cell surface by endocytosis leading to the presence of annular gap junctions in the cell cytoplasm (Albertini et al, 1975). Internalisation of gap junctions can be induced in mature rabbit ovarian follicles by injections of HCG (Bjersing and Cajander, 1974). Oestrogen appears to stimulate both formation and internalisation in several different tissues. Injections of oestrogen into female rabbits results in the formation and internalisation of gap junctions in granulosa cells of the ovarian follicle (Merk et al, 1972). (Oestrogen also induces the formation of gap junctions and the onset of electrical activity in uterine smooth muscle of the rat after ovariectomy (Bergman, 1968)).

Internalisation is achieved by the invagination of part of one cell into another at the gap junction, followed by constriction and pinching off of the invagination, isolating both junctional membranes as a cytoplasmic vesicle. Actin may play a role in this process as suggested by the basket of microfilaments observed surrounding cytoplasmic junctional vesicles in thin section of rabbit granulosa, human SW13 adrenal cortical adenocarcinoma and mouse B-16

melanoma cells (Larsen et al, 1979).

There is however no evidence for the existence of annular gap junctions in the regenerating weanling rat liver system (Yee and Revel, 1978). Between zero and twenty hours after partial hepatectomy, large gap junctions are usually located close to the bile caniculi and small gap junctions are emeshed within the strands of the zonulae occludentes. The total area of the gap junctions decreases by more than 95% between 24 and 28h (Yee and Revel, 1978; Yancey et al, 1979). By 36h numerous small aggregates of gap junctional particles begin to reappear (Yee and Revel, 1978; Yancey et al, 1979).

Freeze-fracture replicas of insect tissue during metamorphosis have been interpreted as showing an alternative method of junctional breakdown. In this system gap junctional breakdown is accomplished by a 'streaming out' of the junctional particles in linear arrays, and as development proceeds the particles become dispersed and fracture onto the E-face of the membrane (Lane and Swales, 1980). Control pupae in diapause suggest that the intramembranous groove along which the particles become aligned may be a cellular response to hormonal stimulation since glial cells in diapause remain associated and their gap junctions retain their macular larval configuration.

1.3.3. Biochemical Evidence.

As yet there is no information available on the formation and very little on the turnover of gap junctions. Ultrastructural and cell-cell coupling data have led to the belief that gap junctions are fairly stable structures, but this may be incorrect.

The turnover of the gap junction protein has been investigated *in vivo* using [^{35}S]-methionine labelled rat liver junctions (Yancey *et al*, 1981). Gap junctional protein was labelled *in vivo* with [^{35}S]-methionine and the junctions isolated at various times afterwards. Radioactivity in the junctional protein was followed with time in the M_r 10K component of the junction rich fraction which is produced from the 26K component when isolated plasma membranes are treated with trypsin. The apparent half-life of the junctional protein derived from these studies was 19 hours. This value is probably an overestimate due to the problems of reutilisation of the labelled amino acids.

The problem of reutilisation can be avoided by using a [^{14}C]-bicarbonate labelling technique. [^{14}C]-bicarbonate is fixed into the tricarboxylic acid and urea cycles, so labelling intracellular pools of arginine, aspartate and glutamate in the liver. When the [^{14}C] amino acids are released from proteins due to catabolism they are rapidly transaminated releasing [$^{14}CO_2$] into a large unlabelled CO_2 pool.

A study using mouse liver junctions labelled *in vivo* by this technique gave a value of 5.5h for the junctional half-life (Fallon and Goodenough, 1981). However, in this study *in vivo*, the junctional protein was taken to be a 21K component, the junctional origin of which is uncertain. A value of 5h is more consistent with observation in the regenerating weanling rat liver where morphological observations show that the disappearance of gap junctions occurs over a four to six hour period.

Rapid turnover of gap junctions could provide a mechanism for regulation of intercellular communication as well as junctional uncoupling via channel closure.

1.4. PERMEABILITY OF GAP JUNCTIONS.

The water filled pore in the centre of each junctional subunit is thought to provide the pathway by which substances are exchanged directly between cells. Molecules with an apparent M_r of up to 900 are able to pass through vertebrate gap junctions by passive diffusion. In arthropod gap junctions the cut off point is slightly higher and larger molecules (up to M_r 1500) can pass between coupled cells. It is difficult to define the correct molecular criteria by which to judge the likelihood of a particular substance passing through junctional channels. Clearly molecular shape as well as hydrated size will affect movement,and reports of a fixed charge in the junctional channel (Flagg-Newton et al, 1979; Brink and Dewey, 1980) suggest that charge will also be a determining factor. However M_r is a convenient measure to use in judging the likely permeability limits of the pore.

Electrophysiological, dye injection and metabolite exchange studies have been used to investigate junctional permeability.

1.4.1. Electrophysiological Techniques.

For electrophysiological measurements, two microelectrodes are inserted into different, often adjacent, cells. A current pulse applied in one cell causes a change in potential of both cells only if the cells are coupled by a low resistance pathway. (There is correlative evidence that the gap junction is such a low resistance pathway; see section 1.4.4.). The ratio between the potentials of the two cells V_2/V_1, is called the coupling ratio or coupling

coefficient and this reflects the partition of the injected current i.e. how much passes through the junctional channels and how much returns directly across the high resistance non-junctional membrane of the injected cell.

This method has the advantage that it can be used in organs and tissues *in vivo* or in tissue culture. It is also very sensitive and produces qualitative information about junctional competence. In terms of different molecular species, however, it only provides information about the passage of small current carrying ions (K^+, Cl^-). It gives no information about the movement of larger molecules. Impaling cells with microelectrodes has the disadvantage that the cells are punctured, although the extent of the damage this causes can be assessed by watching the stability of the membrane potential after penetration. The quantitative information about junctional permeability can be obtained by measurements of electrical conductance though this requires either simple, defined cell combinations (e.g. pairs of cells) or the application of cable analysis. This is technically more difficult than making simple electrical coupling (V_2/V_1) measurements.

Electrophysiological techniques have demonstrated the existence of electrical coupling between excitable cells and between non-excitable cells from both invertebrates and vertebrates (Furshpan and Potter, 1959; Furshpan, 1964; Loewenstein and Kanna, 1964; Kuffler and Potter, 1966; Kuffler *et al*, 1966), between embryonic cells (Potter *et al*, 1966; Sheridan, 1968), between cells in tissue culture (Furshpan and Potter, 1968), and between tumour cells and transformed cells (Seigenbeck *et al*, 1970).

1.4.2. Microinjection of Tracer Molecules.

i) Dye Injection Studies.

Junctional communication has been studied using fluorescent molecules as tracers. Iontophoresis of fluorescent dyes into cells and the spread of dye to surrounding coupled cells is followed by fluorescent microscopy. This method gives direct, qualitative information about junctional permeability for a range of molecular species. It can also be used *in vivo* as well as in tissue culture systems. The detection sensitivity of this technique is lower than that in electrical coupling techniques.

Fluorescent tracer molecules such as fluorescein, procion yellow and Lucifer yellow and fluorescent tagged amino acids, oligopeptides and oligosaccharides have all been found to pass between coupled arthropod cells and vertebrate cells (Furshpan and Potter, 1959, 1968; Loewenstein and Kanno, 1964, 1966; Johnson and Sheridan, 1971; Loewenstein, 1975; Simpson *et al*, 1977).

ii) Radioactive Tracers.

Junctional communication and permeability has also been studied by methods using microinjection of radiolabelled molecules. There are,however,many problems associated with this method. No satisfactory controls have been developed to insure against injection spillage artefacts, or diffusion of the radiolabelled molecule along extracellular spaces or of non-junctional transfer.

Permeability of electrically coupled cells of the leech central nervous system was investigated by microinjection of

radiolabelled fucose, glucosamine, glycine, leucine, orotic acid and uridine. Transfer of the molecules was observed in all cases only between electrically coupled cells (Rieske et al, 1975).

The results of Hermann et al (1975) showed that after injection of [^{3}H]-glycine or [^{3}H]-glucosamine into one lateral giant axon of crayfish, the labelled material only spread to other giant axons and not to associated glial cells although there are many glial processes infiltrating the fibrillar septa between the axons (Pappas et al, 1971).

Permeability of junctions between septate axons of crayfish has been examined by the combined injection of radiolabelled ions and fluorescein (to allow detection of successful injections). By this technique $^{22}Na^+$, $^{36}Cl^-$, $^{125}I^-$ $^{35}SO_4^{2-}$ and $^{42}K^+$ have been shown to pass to adjacent axons (Bennett et al, 1967). Following microinjection of the radiolabelled ions, intercellular movement of Co^{2+} and $AuCl_3OH^-$ has been detected between cells of early cleavage state Xenopus embryos (Turin, 1977).

1.4.3. Metabolite Exchange.

The phenomenon of metabolic cooperation (Subak-Sharpe et al, 1966; 69) has been helpful in investigations of junctional communication and permeability between cells in tissue culture. Metabolic cooperation experiments use mutant cell lines deficient in an enzyme of a purine or pyrimidine salvage pathway. When mutant cells and wild type cells are co-cultured, the mutant phenotype is eliminated

due to the passage from the wild type to the mutant cells of the small molecular weight metabolites which the mutant cell is unable to synthesise (due to the enzyme deficiency).

A second method, which also follows the movement of metabolites between cells (but which does not require mutant cells), has also been used to detect and measure junctional permeability. This method, like the first, depends on the impermeability of the cell membrane to the metabolites. Firstly, donor cells are labelled with a radioactive precursor, such as [^{3}H]-uridine, which is converted to labelled uridine nucleotides and incorporated into nucleic acids. Unincorporated precursor is removed by washing. Secondly, the donor cells are co-cultured with unlabelled recipient cells during which time, if junctions form between donor and recipient cells, labelled uridine nucleotides equilibrate between the cells and become incorporated into recipient cell RNA. Junctional transfer is detected and assayed after co-culture by autoradiography.

This technique provides a quantitative method of assessing junctional transfer which can be used with many cell types in culture and with several types of precursors. It also has the advantage that it provides information about large numbers of cells in one experiment, and the cells are not physically damaged during the experiment. The method has the disadvantage that the identity of the molecules which are actually transferred has to be determined for each different precursor. Also, the extent of labelling in recipient cells depends not just on the extent of transfer, but also on the activities of several enzymes and the rates of nucleic acid synthesis. It is therefore a less direct

way of measuring junctional communication than either electrophysiological or microinjection techniques.

The presence of DNA and chromosomes in intercellular bridges connecting two cells was thought to indicate a route for the intercellular transfer of genetic material (Bendich et al, 1967). Evidence has also been presented to show that RNA can pass between cells in culture (Kolodny, 1971). Kolodny co-cultivated a recipient population of cells with a donor population containing radiolabelled macromolecules. After varying periods of co-culture, the two populations of cells were separated and the recipient cells were examined for labelled macromolecules which have passed from the donor cells to the recipients. The two populations of cells were separable by centrifugation on a Ficoll gradient because the donor cells were made more dense by phagocytic ingestion of metallic tantalum powder. Following separation of the donors and recipients his results showed that labelled RNA was present in the recipient cells and this was interpreted as transfer of RNA. Kolodny also presented similar evidence in support of intercellular transfer of protein but could find no evidence of intercellular DNA transfer. No other studies have found any evidence for intercellular transfer of macromolecules and it is possible that the technique of separation and identification used by Kolodny,may not have been stringent enough leading to incorrect interpretation of the experimental results.

The transfer of [^{3}H]-uridine nucleotides between coupled cells has been used to examine the limits of junctional permeability (Pitts and Simms, 1977). Co-culture of donor

cells (prelabelled with [^{3}H]-uridine and then washed to remove unincorporated nucleotides) with recipient cells leads to the appearance of labelled material in recipient cells. This could be attributed to:

1. incorporation into recipient cells RNA of [^{3}H]-uridine lost to the medium from donor cells.
2. transfer of [^{3}H]-RNA between cells in contact.
3. transfer of [^{3}H]-uridine nucleotides between cells in contact and their subsequent incorporation into recipient cell RNA.

Several features of the transfer from donor to recipient cells suggested that the label in recipient cells was not derived from [^{3}H]-uridine in the medium. Recipient cells not in contact with donor cells were not labelled even though they were close to donor cells. Also, the grain count over recipient cells in direct contact with donor cells was 14.6% of that over donor cells and the small loss of [^{3}H]uridine from the small number of donor cells could not account for this level of incorporation in the much larger number of recipient cells.

Nucleotide transfer and RNA transfer were unambiguously distinguished by:

1. using donor cells where the label had been chased into the RNA and normal donor cells and comparing transfer
2. adding actinomycin D during co-culture.

Using chased donor cells resulted in very little transfer of labelled material to the recipient cells. Since the labelled material in such donor cells is in the form of RNA, this strongly suggests that RNA does not pass through

the gap junctions from the donor cells to recipient cells. The addition of actinomycin D was also found to reduce the grain count over recipient cells. If the activity in the recipient cells was due to RNA transfer, actinomycin D would be expected to have no effect unless it had a separate effect on the transfer process itself. This was also tested and found not to be the case.

These results indicate that the appearance of labelled nucleic acid in the recipient cells is due to the transfer of [^{3}H] uridine nucleotides between cells in contact and their subsequent incorporation into recipient cell RNA.

A recent study using metabolic co-operation has shown the forms of a variety of metabolites and a vitamin derived co-factor which are shared between cells coupled by gap junctions. The different molecular species passing through junctions which have been identified using this type of procedure are, 2-deoxy-glucose or 2 deoxy-glucose-6-phosphate, phosphoryl choline or CDP choline, proline or its precursors and lower glutamated forms of tetrahydrofolate (Finbow and Pitts, 1981). This series of experiments demonstrate the intercellular transfer of all the small molecular weight cell components examined and none of the macromolecules such as DNA, RNA or protein. These findings agree with the theory of junctional permeability being selective with regard to size, the cut off point occurring with molecular weight values of ~1000.

It seems likely that populations of coupled cells will become syncyctial with respect to their low molecular weight cytoplasmic components. The individuality of cells in a population will therefore be maintained by its

macromolecular components which will be retained within the cell, while the intercellular transfer of small molecular weight material will result in integration of the metabolic activities of the cell population.

1.4.4. Evidence that the Gap Junction is the Basis of Permeability Observations.

A correlative correspondence between the presence of gap junctions and the existence of cell-cell coupling has been shown in a number of systems.

An investigation of the fine structure of the electrical synapse in the Mauthner cells of the goldfish brain found a specialised intercellular contact (the gap junction) which was believed to represent the site of electrical transmission (Robertson, 1963). Evidence that ionic coupling is achieved via gap junctions is found in a study of 'metabolic cooperation' between Chinese hamster fibroblasts deficient in the ability to incorporate purines, and fully component cells of the same type (Gilula et al, 1972). Deficient cells are shown to incorporate purines when ionically coupled with fully competent cells and, in these instances, the presence of gap junctions is demonstrated by freeze-fracture and thin sectioning. Other reports also indicate a correlation between the morphological presence of gap junctions and electrical coupling, and that cells not coupled by junctions are not electrically coupled. (Azarnia and Loewenstein, 1977; Larsen et al, 1977).

Morphological data suggests that an increase in permeability between mammalian cells in culture after exposure to cAMP, caffeine,or a combination of the two, is associated with an increase in the number of gap junction particles in junctional plaques determined by freeze-fracture. (Flagg-Newton et al, 1981). The average size and the frequency of plaques increases and this effect is blocked by treatment with cycloheximide or puromycin, suggesting that the cAMP effect on permeability is due to a cAMP promoted proliferation of cell-cell channels. This effect of cAMP has also been observed with C1-1D mouse tumour cells (Azarnia et al, 1981). Establishment of electrical coupling (after cAMP treatment) is accompanied by development of junctional particles (see section 1.8.1.).

In some cases, however, gap junctions have been found where there is no detectable electrical coupling (see Finbow, 1982 for review).

A study of junctional communication in the peripheral vasculature by Sheridan et al,(1982) has found dye coupling between capillary cells in the apparent absence of gap junctions in freeze-fracture specimens. The intercellular transfer of dye occurs over many cell diameters within a few minutes and the transfer does not occur via the extracellular space since dye within the vessel lumen or outside the vessel walls is not taken up. The strong implication is therefore that some other structure beside the gap junction is responsible for the dye coupling in capillaries. Sheridan suggested that a possible candidate for dye coupling is the tight junction. It is however also suggested that unaggregated gap junction particles may form

channels connecting cells, and if these particles were well dispersed, they would probably be indistinguishable from the other non-junctional intramembranous particles in the membrane.

However, there are now over 70 cases from vertebrate and invertebrate systems where coupling is correlated with the presence of gap junctions. In many instances these are the only cell surface features present and, in some instances, changes in the level of intercellular coupling are directly correlated with changes in the area of gap junction interaction (Flagg-Newton et al, 1981; Meyer et al, 1981; Finbow, 1982).

1.5. CONTROL OF PERMEABILITY.

1.5.1. Evidence for Control.

i) Morphological

It has been suggested (Peracchia, 1974; Peracchia and Dulhunty, 1974) that the contracted form of the gap junction where the particles are more uniformly packed and the interparticle distance is small, is related to functional uncoupling measured by electrophysiology. However in only one study, on the crayfish septate axon, has electrophysiological analysis been combined directly with a morphological approach (Peracchia, 1974). All other studies have relied on the assumption that the 'uncoupling' treatments used caused loss of junctional communication. Thus, the validity of the frequently stated belief that the contracted state represents uncoupled junctions is uncertain.

In one study,the effects of anoxia on gap junction structure were studied in rabbit ciliary epithelium and rapid freezing of the specimens was used to minimise any fixation artefacts (Raviola et al, 1978). Connexons began to group into tiny clusters and short rows within five minutes of experimental anoxia and by thirty minutes, the entire junction had contracted either into a single aggregate or into multiple linear domains of hexagonally packed particles. It was suggested that the junctional particles have little mutual affinity in their low resistance state and are randomly distributed in discrete domains of the cell membrane (Raviola et al, 1978).

Crystallisation of gap junctions may be a result of the neutralisation of negative charges on the cytoplasmic surface of junctional particles by divalent cations or H^+, inducing the particles to associate tightly into crystalline arrays and triggering conformational changes in the particle protein, leading to a narrowing of intercellular channels (Peracchia and Peracchia, 1980).

Morphological data may provide a valuable insight into structural changes at the molecular level but should be interpreted with caution since, it is possible that, fixation procedures may themselves produce structural changes.

ii) Functional Evidence and Agents which Affect Permeability.

The concentration of free intracellular calcium is believed to play an important role in the control of uncoupling and coupling between cells. Microinjection of Ca^{2+} into cells in the salivary gland of Chironomus results in the inhibition of electrical communication. The conductance of the junctions is also depressed by treatments which are known to inhibit energy metabolism (Politoff et al, 1969). These treatments include prolonged cooling at 6-8^{o}C, exposure to cyanide, dinitrophenol, oligomycin and N-ethylmaleimide.

The passage of fluorescein and small inorganic ions between the cells was also inhibited by substitution of extracellular Na^+ by Li^+ (Rose and Loewenstein, 1971). The effect was specific for Na^+/Li^+ exchange and it is

believed that extracellular Li^+ leads to an accumulation of intracellular Ca^{2+} (Baker et al, 1971).

Oliveira-Castro and Loewenstein (1971) perforated membranes of the chironomus salivary glands and tested the coupling of the perforated cell with the surrounding cells in response to media with different ionic compositions. Junctional conductance was markedly reduced by medium containing Ca^{2+},Mg^{2+},Sr^{2+},Ba^{2+}, the effect being strongest with Ca^{2+} and weakest with Ba^{2+}. Fluorescein permeability was also affected by Ca^{2+} and the minimal effective concentration was reported to be $4 - 8 \times 10^{-5}$M.

By using aequorin (a molecule which fluoresces in the presence of Ca^{2+}) Rose and Loewenstein (1975; 76) have shown that treatments used to electrically uncouple cells all resulted in an increase in the intracellular concentration of Ca^{2+}. In a more detailed study (Rose et al, 1977) using aequorin to estimate Ca^{2+} concentration intracellularly, they found that when the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was less than 10^{-7}M the upper size limit of fluorescein conjugated peptides which could pass through junctions was in the molecular weight range 1200-900 (i.e. normal permeability), while at $[Ca^{2+}]_i$ above 5×10^{-5}M channel permeability was reduced and passage was restricted to free fluorescein (M_r 330) but there was little or no detectable reduction of electrical coupling. They attributed the decrease in molecular size exclusion with increasing $[Ca^{2+}]_i$ to changes in effective channel bore but they also considered the alternative hypothesis that junction might contain channels of different size and different calcium sensitivity. If this were the

case the result would be accounted for by an all-or-none closure of channels, with Ca^{2+} sensitivities directly related to channel size.

The results of the Ca^{2+} experiments have been questioned by Sheridan (1978) who argues that there is no satisfactory method of quantitating the aequorin response intracellularly and that the experimentors have failed to take into account the inhibitory effect of physiological (mM) concentrations of Mg^{2+} on the aequorin reaction.

Uncoupling after direct injection of Ca^{2+} has been observed in canine cardiac Purkinje cells (De Mello, 1975), reaggregated blastomeres of Xenopus (Loewenstein et al, 1978), between nerve cells in the buccal ganglion of Navanax (Baux et al, 1978) and between mouse and rat pancreatic acinar cells (Iwatsuki and Peterson, 1977).

Intracellular acidification has also been found to markedly reduce cell-cell coupling. This pH effect has been seen in whole Xenopus blastomeres (Turin and Warner, 1977), cleavage state blastomeres of amphibian (Ambystoma) and teleost (Fundulus) embryos (Spray et al, 1981) and in pancreatic acinar cells (Iwatsuki and Peterson, 1978). The closure of junctional channels at low intracellular pH in amphibian and teleost embryos appears to be a cooperative process involving several charged sites (Spray et al, 1981). It has also been suggested that protons act directly on the channel macromolecules and not through an intermediate in the cytoplasm (Spray et al, 1981).

The effects of Ca^{2+} and pH on uncoupling are difficult

to dissociate. Injection of Ca^{2+} causes an immediate decrease in intracellular pH due to an exchange for protons in the mitochondria (Meech and Thomas, 1977), making it difficult to separate the two effects to establish which is directly responsible for uncoupling (Turin and Warner, 1977).

Junctional permeability has been found to increase when cells in culture are exposed to cAMP, dibutyryl cAMP and caffeine or a combination of the three, as measured by electrical coupling and fluorescein transfer (Flagg-Newton et al, 1981; Azarnia et al, 1981).

One recently discovered class of modulators of junctional permeability is the family of retinoids (see section 1.9.). A recent study by Pitts et al (1981) has shown that 10^{-4}M retinoic acid reversibly inhibits intercellular communication, as assayed by uridine nucleotide transfer, between cells in culture. A similar result has been found with the embryonal carcinoma cell lines R53, PCC13 and H2T12 where 10^{-4}M retinoic acid inhibits intercellular communication completely and an intermediate effect is seen at 5×10^{-5}M (Hooper, personal communication), (see section 1.9.4.).

1.5.2. Possible Functions of Uncoupling.

Acetylcholine has been observed to uncouple pancreatic and lachrimal acinar cells (Iwatsuki and Peterson, 1977) suggesting that uncoupling may play a role in tissue and cellular responses to hormonal stimuli. Morphological, metabolic and electrical coupling data suggest that coupling between secretory cells of the islets of Langerhans is

modulated by secretagogues (Meda et al, 1982). The insulin content of B cells is inversely correlated with an increase in gap juncion area observed after glucose stimulation, suggesting that the extent of gap junctions is modulated according to the functional state of the cell.

Anoxia and ischaemia cause formation of cytoplasmic annular gap junctions (Yancey et al, 1978) and this may be involved in junctional breakdown. Uncoupling by this method or by channel closure may be important in allowing discrete events to occur in embryonic development (Spray et al, 1978). Uncoupling (and crystallisation) of gap junctions in response to an increase in calcium concentration could be important in ensuring communication is broken between dead or injured cells and their neighbours; (since Ca^{2+} influx into damaged cells would result in uncoupling).

Bicuculine and picrotoxin, antagonists of γ-aminobutyric acid have been found by Piccolino et al, (1982) to cause electrical uncoupling and to reduce junctional transfer of Lucifer yellow between one type of horizontal cell in the turtle retina. The authors therefore suggest that γ-aminobutyric acid may modulate horizontal cell activity in vivo by controlling junctional permeability. There is however no direct evidence to support this.

1.6. FUNCTIONS OF GAP JUNCTIONS.

1.6.1. Excitable Tissue.

Cell coupling acts as an intercellular pathway for the transmission of electrotonic signals in excitable tissues (Bennett, 1972) and as such it is used in the nervous system of both vertebrates and invertebrates (Bennett, 1972), in smooth muscle and in heart (Dewey and Barr, 1962). Chemical and electrotonic synapses can be distinguished by the directionality of transmission, electrotonic synapses being bidirectional while chemical synapses are unidirectional. Since there is direct continuity between coupled cells, action potentials can pass rapidly between the cells in a population, avoiding the delay of approximately half a millisecond at the chemical synapse due to the time taken for release, transmission and uptake of the neurotransmitter. This appears to be important in tissues such as the heart where coordinated contraction is essential, and cardiac muscle fibres are accordingly rich in gap junctions (Goodenough, 1978).

1.6.2. Non-Excitable Tissue.

In non-excitable tissues, where the gap junctions are larger and more prevalent, their function is less well defined. There are three possible ways of analysing function. The first involves genetic analysis in which cells lacking the ability to form junctions are selected and the effect of this deficiency on the subsequent growth and behaviour of the cell is studied. The second method of

analysing function is through the identification and characterisation of specific inhibitors of junctional communication. The third method involves the use of *in vitro* model systems.

Attempts to isolate mutants defective in junction formation have been surprisingly unsuccessful. It is possible that the junction-forming phenotype is very stable or perhaps the mutation is lethal. However, both these suggestions are hard to reconcile with the fact that cell lines are known (e.g. mouse L line; Pitts, 1971) which appear to be genetically incapable of forming junctions yet which grow and divide as well as junction-forming cell lines. Azarnia and Loewenstein, (1977) have shown concordant segregation of ability to form junctions, normal growth in culture and failure to grow in soft agar or as tumours *in vivo* (see section 1.8.1). The lack of success in generating communication incompetent mutants means that confirmatory data has not been obtained by other workers.

Two recently discovered inhibitors of junctional communication, the tumour promoter, TPA (Murray and Fitzgerald, 1980; Trosko *et al*, 1980) and retinoic acid (Pitts *et al*, 1981) may be valuable in characterising junctional function. Application of antisera raised specifically against gap junctions might inhibit communication by acting directly at the level of the junctional protein and may allow an examination of the consequences of the loss of intercellular communication. Inhibition of junctional communication is also caused by elevated intracellular concentrations of Ca^{2+}

and H^+ but since it is not clear exactly how this inhibition is achieved, (see section 1.5.1) such agents have not been used in functional studies.

In various tissue culture systems it has been observed that the metabolic activity of a mixed culture of coupled cells is a unique characteristic of the mixture and not simply the sum of the activities of the component cells whereas, in mixed cultures of cells which are not coupled by permeable junctions, the metabolic activity is simply the sum of the activities of the two cell types (Sheridan et al, 1979; Pitts and Shaw, 1980). Thus intercellular communication may act to integrate metabolic activities in cell populations (Pitts and Finbow, 1977). The first example of this was metabolic cooperation between $HGPRT^-$ and wild type cells (see section 1.4.3.).

Model systems have therefore suggested that gap junctions play a role in the intercellular control of enzyme activity, and intercellular growth. Rat ovarian granulosa cells and mouse myocardial cells respond to cell specific hormones by a cAMP dependent mechanism. In co-culture, these heterologous cells communicate by gap junctions, and if cells are exposed to a hormone specific for one cell type, then both cell types respond through a cell contact dependent mechanism (Lawrence et al, 1978). This model system therefore suggests that one of the functions of gap junctions may be the intercellular transfer of cAMP and amplification of hormonal response.

There are a number of situations where junctional

communication has been observed *in vivo* where it is possible to suggest what its functions may be.

i) Metabolic cooperation has been observed *in vivo* in cumulus-oocyte complexes from rat ovarian follicles. Gap junctional contacts between follicle cells and oocytes in ovarian follicles have been described in several mammalian species (Anderson and Albertini, 1976; Gilula *et al*, 1978; Moor *et al*, 1980; Moor and Smith,1980). Cell-cell communication between the cells in the complex has been investigated by both biochemical (Moor *et al*, 1980) and electrophysiological techniques (Gilula *et al*, 1978).

Intercellular coupling between cumulus cells and oocytes has been measured in tissue culture using intracellular markers derived from labelled choline, uridine and inositol (Moor *et al*, 1980). Both isolated oocytes and isolated cumulus cells can take up amino acids. The uptake of choline, uridine and inositol however,appears to be restricted to the cumulus cells and there is little uptake of these compounds by isolated oocytes. Their rate of uptake by oocytes in the cumulus-oocyte complex is high,and it is believed that these compounds enter the oocyte via gap junctions formed with cumulus cells. The role of gap junctions in this situation may be one of metabolic support.

The effect of gonadotropins on follicles both *in vivo* and in culture is to reduce,but not eliminate,intercellular communication. Cell-cell communication was characterised in cumulus-oocyte complexes from rat ovarian follicles before and after ovulation by thin section and freeze-fracture

and by electrophysiological measurements (Gilula et al, 1978). In the hours preceeding ovulation the frequency of gap junctions between cumulus cells and oocytes is reduced.

Examination of the extent of ionic coupling in cumulus-oocyte specimens reveals that ionic coupling between the cumulus and oocyte progressively decreases as the time of ovulation approaches.

Communication between cumulus cells and oocytes might fulfil two roles, initiating or directing events associated with oocyte maturation or allow the oocyte to obtain essential metabolic and trophic products by means of direct intercellular transfer through junctions (Moor et al, 1980). Gap junctions have been postulated to play a role in the co-ordination of oocyte and follicular cell differentiation in the oogenesis cycle of the insect Rhodnius prolixus (Huebner, 1981). A variety of ultrastructural methods reveal the presence of numerous gap junctions between oocyte microvilli and follicle cell processes during previtellogenesis and vitellogenesis. Junctions are however absent during atresia and chorion formation.

ii) Conventional and freeze-fracture electron microscopy have shown that gap junctions occur between both homologous and heterologous differentiated endocrine cells (Meda, 1982). Heterologous and homologous communication can be detected in whole islets of Langerhans after microinjection of the fluorescent dye Lucifer yellow. Significant changes in the frequency and size of B-cell gap junctions are observed after stimulation with glucose in culture and glibenclamide in vivo (see section 1.5.2.) suggesting that junctional changes generally participate in the response of B-cells

to a secretagogue (Meda, 1982).

The relative and absolute area occupied by gap junctions between B-cells shows a 50% increase after glucose stimulation and an even larger increase after glibenclamide stimulation. This increase is inversely correlated with the insulin content of the B-cells and it has been suggested that the development of gap junctions is modulated according to the functional status of the cells (Meda, 1982).

iii) Gap junctions may be involved in synchronisation of cellular behaviour in muscle contraction in the myometrium of both rats and humans. In the myometrium of the uterus gap junctions are absent during pregnancy but their incidence increases markedly prior to the end of pregnancy. Their presence at this stage is probably important in the onset of uniform uterine contractions (Garfield and Hayashi, 1980; Garfield et al, 1980).

iv) The patterns of junctional communication in the early mouse embryo are consistent with a possible role of gap junctions in the regulation of developmental processes (Lo and Gilula, 1980 a,b). Communication between the cells of the early mouse embryo, from the two cell stage to the preimplantation blastocyst stage, was examined by monitoring ionic coupling, transfer of fluorescein and the transfer of horseradish peroxidase which is transferred from cell-to-cell by cytoplasmic bridges, but not through gap junctions due to its size (M_r=40,000).

Cytoplasmic bridges between sister blastomeres are responsible for ionic coupling in the two, four and eight cell precompaction embryos. Junction mediated intercellular

communication (i.e. coupling in absence of peroxidase transfer) is detected for the first time at the 8 cell compaction stage when the first determination events are believed to occur in the mouse.

Intercellular communication patterns have also been studied in early molluscan development (De Laat et al, 1980). The formation of regional and temporal specific cell-cell coupling has been correlated with the determination of the mesentoblast (the stem cell of the mesoderm) and the establishment of dorso-ventral polarity.

There is however other evidence which implies that gap junctions may play no role whatsoever in the control of differentiation. Lawrence and Green (1975) found that cells in adjacent segments of the epidermis of larvae of Oncopeltus are electrically coupled to the same extent as to cells within the same segment.

However more recent work and morphological data suggest that junctions between cells in different developmental compartments may have reduced permeability and the gap junctions may therefore still actively participate in developmental control. This has been suggested by the pattern of communication between epidermal cells of the 5th instar larvae of the milkweed bug Oncopeltus fasciatus and those of maggots of the blowfly Calliphora erythrocephala. Communication was examined by ionic coupling and transfer of injected fluorescein and the small lead-EDTA complex (Warner and Lawrence, 1982). All epidermal cells, regardless of their position with respect to the segmental border, were

found to be ionically coupled. Lucifer yellow was transferred freely between cells lying in the same segment and therefore, in the same developmental compartment as defined by cell lineage. However at regions beside the segmental border only lead-EDTA was transferred freely between cells across the border, and not fluorescein. These data can be explained by either the existence of junctional particles with differing pore sizes or by variations in the number of particles. Thin section analysis however, suggests that the number of particles is the same and this implies that particles may exist with different permeability properties, due to difference in pore sizes. This selective change of permeability may be important in the control of development.

v) Compartments.

Gap junctions have been postulated to play a role in the generation of positional information and in determining the spatial pattern of cellular differentiation (Wolpert, 1978). The concept of positional information relies on mechanisms whereby cells in a developing system may have their position specified with respect to one or more points in the system. All the cells whose position have been specified with reference to the same set of points are known as a field, and it has been found that most embryonic fields involve between 50 and 100 cells. Positional information largely determines, along with the state of the cell genome and the developmental history of the cell, the nature of the molecular differentiation that the cell will undergo.

It is also believed that the same mechanisms that specify positional information may operate in different fields within the same organism as well as in different organisms from different genera or even phyla.

Postional information may be specified by an intercellular morphogenetic gradient formed via gap junctional channels. Crick (1970) calculated that a positional information gradient could be established within the appropriate time by passive diffusion of a low molecular weight morphogen. The formation of a permeability barrier by tight junctions would enhance differences between the environment of the cells buried inside an embryo or tissue, and those exposed on the outside. The presence of gap junctions linking them could then provide a pathway for transferring a morphogen and thus passively generating an intercellular gradient.

The discovery that retinoic acid can mimic the action of the zone of polarising activity (ZPA) (Tickle et al, 1982) is potentially very exciting in view of its known inhibitory effects on junctional communication (Pitts et al, 1981). The polarising region acts as a signalling source, specifying the pattern of structures which develop across the antero-posterior axis of the limb, and the behaviour of the signal from the ZPA is consistent with it being a diffusible morphogen. Local application of retinoic acid to the wing buds of embryos leads to the development of digits in a manner identical to the action of an implanted ZPA. This result may be explained in three ways. Retinoic acid itself may be a morphogen, it may be converted in the limb to a morphogenetic substance or it may affect the

distribution of the natural morphogen. The cells in the wing bud may have different sensitivities to retinoic acid which could result in inhibition of junctional communication between some cells and not others and this may affect the distribution of the natural morphogen. As yet there is not sufficient information to distinguish between these possibilities.

1.7. SPECIFICITY OF JUNCTION FORMATION.

Until recently it was believed that cells formed permeable junctions without any tissue or species specificity, then two independent pieces of work using cell culture systems indicated that this was not the case (Pitts and Bürk, 1976; Fentiman et al, 1976; 1977).

The first indication of specificity came from experiments with epithelial and fibroblastic cell types which would rapidly form homologous junctions but in mixed cultures heterologous junctions formed only very infrequently. (In about 5% of the cases examined, communication could be detected in heterologous cell pairs). It was suggested that the determining factor leading to such specificity of communication could be the frequency with which the two cell membranes came in close enough apposition to allow junction formation to occur (Pitts and Bürk, 1976).

This epithelial - fibroblastic specificity was independently discovered by Fentiman et al (1976) with human mammary duct epithelial cells and mammary fibroblasts in primary culture.

Cells termed 'universal couplers' will form junctions with any other cell types and cells falling into this category include lens epithelial cells (Fentiman et al, 1976), cells from certain breast tumours (Fentiman et al, 1976) and keratinocytes from guinea pig ear (Hunter and Pitts, 1981). There are also cells which do not form any detectable junctions in culture. These are all tumour cells and are probably derived from cell types which form junctions

normally (Borek *et al*, 1969; Fentiman *et al*, 1977).

As yet the phenomenon of communication specificity as mediated by specificity in gap junction formation is not understood, and its possible function *in vivo* is unclear.

1.8. JUNCTIONAL COMMUNICATION AND CANCER.

1.8.1. The Role of Junctional Communication in Cancer.

Many studies have now shown that whereas some types of malignant cells are totally incapable of forming permeable junctions (Loewenstein and Kanno, 1966, 67; Kanno and Matsui, 1968; Azarnia and Loewenstein, 1971; Fentiman et al, 1976; Corsaro and Migeon, 1977) other malignant cells still have the ability to form these junctions (Furshpan and Potter, 1968; Sheridan, 1970; Johnson and Sheridan, 1971; Pitts, 1972; Azarnia and Loewenstein, 1976; Fentiman et al, 1976; Corsaro and Migeon, 1977).

Morphological data suggest that cancer cells which do form junctions, possess fewer gap junctions than their normal counterparts (Prutkin, 1975; Schenk, 1980; Kocher et al, 1981).

A genetic correlation has been established between coupling and tumourigenicity in experiments where communication - incompetent cancer cells were hybridised with communication - competent normal cells. The ability of the hybrid cells to make junctions and their tumourigenicity was then investigated (Azarnia and Loewenstein, 1973, 77).

The resumption of normal growth properties of the hybrids was accompanied by the ability to communicate. The analysis was carried further using the mouse cancer cell line Cl-1D. In this case the communication - competent cell line was a human skin fibroblast and the hybrid system was genetically less stable. The hybrid generations progressively lost human chromosomes in culture, resulting in the production of segregants. Some of these segregants

reverted to the malignant state, and these cells were tested for communication incompetence to determine if reversion to the malignant state was accompanied by communication incompetence. This was the pattern which was found. The normal growth behaviour in the segregants was found to be related to communication competence. These results although very suggestive, do not,however,prove that loss of junctional communication has a primary or causal role in cancer.

It is interesting to note that the level of cyclic AMP is abnormally low in a number of diverse tumour cells (Burk, 1968; Anderson et al, 1973 a; b). Several tumour cell lines in culture have been temporarily induced to assume normal growth patterns and/or shapes when cyclic AMP or dibutyryl cAMP were added to the medium (Hsie and Puck, 1971; Otten et al, 1971; Johnson and Pastan, 1972) and in vivo intraperitoneal injection of dibutyryl cyclic AMP has been reported to arrest the growth of mammary tumours in the rat (Choo-Chung and Guillino, 1974). This however remains the only such report. Junctional permeability has recently been found to be increased when cells are exposed to dibutyryl cyclic AMP (Flagg-Newton et al, 1981). The effect was found to be due to both an increase in junctional transit rate and in the number of transferring cell interfaces. Azarnia et al (1981) additionally found that exposure of the junction - incompetent cell line, derived from mouse tumours (C1 - 1D), to cyclic AMP lead. to the acquisition of permeable junctions. Confirmation of this rather surprising result awaits further experimental data.

The cellular location of the src gene product of a strain of avian sarcoma virus has been identified in transformed chick fibroblasts and has been found to be principally located at the inner surface of the plasma membrane and, in particular, at the gap junctions (Willingham et al, 1979). Altered junctional capacities may be fundamental to the expression of the ASV induced transformed phenotype (Atkinson et al, 1981). The extent and rate of dye transfer between ts (src) virus transformed NRK cells has been found to be dependent on the temperature at which the cells are grown. Dye transfer is decreased by approximately 70% when the cells are grown at the permissive temperature (i.e. when cells are transformed) compared to cells grown at the non-permissive temperature. The src gene product is believed (Kreuger et al, 1980, a;b) to have tyrosine phosphotransferase activity and it is possible that phosphorylation of the gap junction protein could result in a conformational change switching the junctional channel from the open to a closed state.

There are several reports in the literature that tumour promoters such as TPA, inhibit or reduce junctional communication between animal cells in tissue culture (Yotti et al, 1979; Murray and Fitzgerald, 1979; Trosko et al, 1980; Newbold and Amos, 1981; Enomoto et al, 1981; Newbold, 1982; and Pitts, unpublished results). This has lead to the formulation of a new hypothesis for tumour promotion (Newbold, 1982). It is proposed that promoters might permit the expression of a premalignant phenotype which had previously been masked by communication with surrounding

normal cells, perhaps by inhibiting passage of growth regulatory factors from normal cells to their 'initiated' neighbour. Initiating doses of potent carcinogens do not result, in tumours, in a reduction of intercellular communication. Communication, is however, inhibited by the application of a promoter and the initiated cell would become effectively isolated from its neighbours. Along with induced local hyperplasia, the promoter would therefore allow the expansion of a clone of initiated cells, some of which (perhaps those on the inside of the clone) would be protected from the growth controlling influences of normal cells. These cells would then divide further and progress towards full malignancy by repeated selection of fitter variants.

One attractive feature of this hypothesis is that it does not rely on permanent loss of junctions in cancer cells. Similar transient losses of gap junctions have been observed in other situations such as regenerating liver where gap junctions are known to be greatly reduced 24 - 28h after partial hepatectomy (Yee and Revel, 1978).

1.8.2. Growth Control.

Models have been proposed based on an intimate relationship between junctional communication and the control of cell proliferation (Loewenstein, 1968; Burton, 1971; Socolar, 1973). It is proposed that every cell is capable of synthesising a growth control molecule (which is able to pass through gap junctions) either at specific times in the cell cycle (Loewenstein, 1968) or in

a sinusoidal fashion throughout all the phases of growth (Burton, 1971). The growth control substances are rapidly broken down and should their concentration within a cell fall below a certain threshold value, then the cell will cease to grow. In a dense population of cells, junctional transfer of the growth control molecule from synthesising to non-synthesising cells will serve to dilute the concentration and thus growth will be inhibited. If cells are uncoupled, no dilution of the growth control substance will occur and this will give rise to uncontrolled or cancerous growth.

Despite the attractiveness of these models, there is no direct experimental evidence to support them. Indeed, since they were proposed a number of studies have shown that many types of malignant cells have the ability to form junctions (see section 1.8.1.). It would appear that there is no direct link between loss of the ability to form permeable junctions and the onset of cancerous growth. This may reflect that there are many causes of cancer and loss of the ability to form permeable junctions is one of them.

1.9. RETINOIDS.

1.9.1. Occurrence, Inter-relationships and Metabolism of Retinoids.

Vitamin A is a term which covers a group of related compounds, including the naturally occurring retinoids (see below), and was first discovered in eggs, milk, butter and fish liver oils (Stepp, 1909; 1911; McCollum and Kennedy, 1916; Drummond, 1920). Plants contain carotenoids which provide the only source of vitamin A in animals.

The parent retinoid is the all-trans vitamin A alcohol, trans retinol its naturally occurring oxidation products are trans retinal (vitamin A aldehyde) and trans retinoic acid (vitamin A acid) (see Fig. 1). The common structural features of these compounds include a six membered ring, and a dimethyl substituted all trans tetraene chain (see section 2.1.10). The different regions of the retinoid molecules can be chemically modified in various ways giving rise to the group of compounds known as the synthetic retinoids.

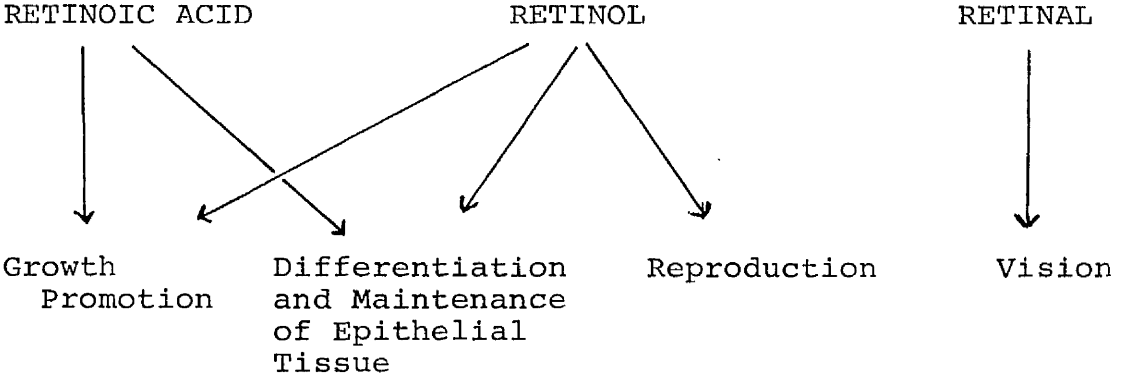


Fig. 1.

Dietary vitamin A is provided either as retinyl esters or as β-carotene. Retinyl esters are hydrolysed by specific esterases and absorbed by intestinal mucosal cells. β-carotene is absorbed then cleaved to give two molecules of retinal which are reduced to retinol. Retinol is esterified before leaving the intestinal mucosa and the retinyl esters pass into the lymphatics where they are transported as chylomicrons to the blood from which they are taken up by the liver and stored (Huang and Goodman, 1965; Goodman, et al, 1966).

Mobilisation and transport of retinol from liver storage requires hydrolysis of retinyl esters followed by conjugation of the free retinol with a specific transport protein known as retinol binding protein (RBP). This binding protein has a molecular weight close to 20,000 (Stubbs et al, 1979; Rao et al, 1979) and has a single binding site for one molecule of retinol. The holoprotein moves into circulation where it binds to prealbumin and the resulting 1:1 molar RBP - prealbumin complex transports retinol to target organs. Uptake by target organs is mediated via membrane receptors (Krishna and Cama 1979).

Unlike retinol and retinal, retinoic acid can not be stored and is rapidly metabolised in many tissues such as kidney and intestine, to 5, 6 epoxyretinoic acid and retinoyl β-glucuronide,before excretion (Dunagin et al, 1965; Ito et al, 1974). Relatively little is known about the plasma transport of retinoic acid except that it is bound to serum albumin. Cellular uptake is believed to be mediated via receptors on cell membranes.

Intracellular binding proteins for retinol and retinoic

acid have been identified in several tissues. Both cytosol retinol binding protein (CRBP) and cytosol retinoic acid binding protein (CRABP) have molecular weights close to 14,600 and have a single binding site for one molecule of retinoid ligand. In general, the affinity of synthetic analogues of both retinol and retinoic acid for their binding proteins, parallels a variety of measurable biological activities suggesting that, in these cases at least, the binding proteins mediate the mechanism of action of the retinoids. (see section 1.9.3.).

1.9.2. Effects of Retinoids.

Both retinol and retinoic acid can participate in various metabolic reactions. Some of these reactions occur in many cell types while others may be limited to specific cells containing special enzymes or proteins.

i) Effects on Glycoproteins

Retinoids are known to induce two different types of changes in cell surface glycoproteins. A non-specific effect resulting from release of lysosomal enzymes (Borek et al, 1973; Brandes et al, 1974; Hogan-Ryan and Fennelly, 1978) and a specific effect caused by enhanced glycosylation of discrete glycoproteins (Jetten et al, 1979 a;b).

Retinol treatment of L1210 leukaemia cells in culture causes changes in cell surface glycoproteins attributable to the release of lysosomal neuraminidase (Brandes et al, 1974) while a more specific effect is seen in retinol stimulation of cell surface galactosyl transferase activity in 3T3 cells (Dorsey and Roth, 1974). Cells from mouse

epidermis (a keratinising tissue), when cultured in the presence of retinyl acetate, display the phenotype of a secreting epithelium with markedly altered glycoprotein synthesis (Adamo et al, 1979).

In contrast to the glycosylation catalysed by dolichol (Lenarz, 1975),retinol catalyses transfer of monosaccharides, rather than oligosaccharides. Since retinol and dolichol coexist in many cell types (De Luca, 1977) either or both may participate in the in vivo glycosylation of any given glycoprotein.

Retinoids also effect glycosaminoglycan and proteoglycan production, with different cell types in culture responding differently. In corneal cell cultures, a stepwise increase in retinoic acid concentration in the medium, from 10^{-9}M to 10^{-6}M leads to a gradual decrease in the incorporation of labelled glucosamine (Dahl and Axelsson, 1980). Undifferentiated mesenchymal cells and mature chondrocytes display a decrease in glycosaminoglycan synthesis (Soiursh and Meier, 1973; Pennypacker et al, 1978), whereas dermal fibroblasts, and epidermal cells show enhanced glycosaminoglycan synthesis (Yuspa and Harris, 1974; Shapiro and Poon, 1976; Jetten et al, 1979).

ii) Effect on Differentiation.

Retinoids are known to play a role in maintaining and inducing differentiative functions of epithelial tissues. Vitamin A deficiency causes alterations in epithelial tissues with a decrease in the number of mucus secreting cells and development of squamous metaplasia, a process similar to that induced by chemical carcinogens. Excess vitamin A causes keratinising tissues to become mucus

secreting and to produce typical goblet cells (De Luca et al, 1972).

Low concentrations of retinoic acid (10^{-9} - 2 x 10^{-5}M in different investigations) stimulate several murine embryonal carcinoma cell lines, (even those previously considered incapable of differentiation) to give rise to a variety of cell types indistinguishable by morphological criteria and cell surface features from normal differentiated tissue cells (Jetten, et al, 1979).

There are a number of reports which suggest that retinoic acid can induce abnormalities in pattern formation in developing tissues and organs.

Injection of retinoic acid (4 x 10^{-5}M) in the chick embryo causes formation of feathers on the foot scales (Dhouailly et al, 1980; Hardy et al, 1981). The foot skin regions affected by retinoic acid treatment are believed to be those in which scale morphogenesis was starting,or was about to start,at the time of exposure to retinoic acid (Dhouailly et al, 1980).

Newall and Edwards (1981 a,b) have found that application of retinoic acid, retinol and retinyl paimitate at concentrations between 5 x 10^{-7}M and 5 x 10^{-6}M cause the development of cleft palate in mouse embryos. Retinoic acid is more active in vitro than either retinol or retinyl palmitate. They suggest that this effect is due to elevated retinoid levels interfering with normal biosynthesis of glycoprotein in cells of the embryo. Congenital limb defects are also produced in mouse fetuses following maternal administration of retinoic acid (3 x 10^{-4}M) (Kwasigroch and Kochhar, 1980) and similar malformation patterns can also

be induced by excess retinoic acid (4×10^{-4}M) in rat fetuses (Kistler, 1981).

A recent study has indicated that retinoids alter pattern formation specifically in the proximo-distal axis of regenerating axolotl limbs (Maden, 1982). Forelimbs were amputated through the mid-radius and ulna, and the animals were immediately placed in solutions of either retinoic acid (1.2×10^{-4}M) retinol (1.2×10^{-4}M), retinol acetate (1.2×10^{-4}M and 6×10^{-4}M) or retinol palmitate (1.2×10^{-4}M and 6×10^{-4}M), for 12 days and then transferred to normal water. After one month, the elements removed by amputation had regenerated in control animals whereas animals treated with a retinoid showed abnormalities in the proximo-distal axis of the regenerated elements.

Retinoic acid, at concentrations of 2×10^{-2}M - 3×10^{-2}M, has been shown to mimic the action of a small group of cells at the posterior margin of the limb bud which act as a signalling region to specify the pattern of structures which develop across the antero-posterior axis of embryonic limb (Tickle et al, 1982). Indirect evidence suggests that the signal from the polarising region is a diffusible morphogen. As yet there is no evidence to clarify whether retinoic acid itself is a morphogenetic substance or alternatively, whether it affects the distribution of the natural morphogen. (see section 1.6.2. (v)).

iii) Anti-carcinogenic Activity.

The anti-carcinogenic activity of retinoids was first discovered in epithelial tissues grown in organ culture. Retinol was found to suppress the induction of hyperplasia and metaplasia by methylcholanthrene and reverse already

established pre-cancerous changes (Lasnitski, 1955). To date,retinoids have been shown to be capable of preventing development of tumours of the skin (Bollag, 1972; Prutkin, 1973), respiratory tract (Cone and Nettesheim, 1973), urinary tract (Bollag and Ott, 1970; Evard and Bollag, 1972) and mammary gland (Chytil and Ong, 1976).

iv) Effect on Growth.

Retinoids can modulate the growth and influence specific cellular processes of various cells in culture. Vitamin A has been found to stimulate mitosis and enhance cell proliferation in newly explanted chick heart fibroblasts (Lasnitski, 1955) and growth of 3T3 and NIL8 cells is stimulated when grown in low concentrations of serum in the presence of retinoids (Dicker and Rozengurt, 1979).

Growth inhibitory effects on a wide number of cells in culture are also elicited by retinoids and these effects can be divided into two classes, with effects on growth rate and effects on density dependent growth. Kochhar et al, (1968) reported retinol treatment inhibited the growth rate of 3T6 fibroblasts before the cultures became confluent. An apparently different type of growth inhibition is seen in cultures of transformed L929 cells exposed to retinoic acid (Dion et al, 1977). Treated cultures reach confluence at a 2 to 4 times lower cell density than do untreated cultures. Retinoic acid may restore normal density dependent growth control to transformed cells.

1.9.3. Mechanism of Action.

The mechanism of retinoid action is unknown but two

possibilities have been proposed to explain growth inhibitory effects in culture. Binding of the retinoid to the specific cellular retinoid binding proteins and the translocation of this complex to the nucleus may result in changes in gene expression (Chytil and Ong, 1979; Jetten and Jetten, 1979). Alternatively, since retinoids have been demonstrated to be involved in glycosyl transfer reactions (De Luca, 1977), inhibition of growth may be due to both qualitative and quantitative change in cell surface membrane glycoconjugates. These mechanisms may operate in concert in some cells and individually in others, so that even cells where no cellular binding protein is found, may be responsive

Retinoic acid binding protein (CRABP) has been postulated to play a critical role in retinoid stimulation of embryonal carcinoma cells differentiation (Jetten and Jetten, 1979). The ability of various natural and synthetic retinoids to stimulate differentiation of PCC4 AzaR1 cells has been compared with their capacity to compete with [^{3}H]-retinoic acid for binding sites on the cytosol retinoic acid binding protein. The two activities were found to correlate well. Two cell lines derived from the PCC4 AzaR1 cells by treatment with the mutagen N-methyl-N^1-nitro-N-nitrosoguanidine, fail to differentiate during exposure to retinoic acid *in vivo* or *in vitro* (Schindler *et al*, 1981). The rate of retinoic acid uptake in cells from the two mutant lines does not appear to be reduced compared with the rate in cells from the parental line but the specific cytoplasmic retinoic acid binding protein activity is virtually absent in both mutants. These results strengthen the view that differentiation of embryonal carcinoma cells in response

to retinoic acid requires formation of retinoic acid - CRABP complexes.

Some mechanisms have been postulated to explain the anti-carcinogenic activity of retinoids such as labilisation of cell surface membranes resulting in leakage of intracellular content and necrosis (Matter and Bollag, 1977; Mayer et al, 1978). Retinoid induced labilisation of lysosomal membranes leading to increased lysosomal enzyme activities and cell destruction is also thought to be a possible mechanism (Shamberger, 1971). Inhibitory effects of retinoids on cell growth may act to decrease cell proliferation and the increased DNA synthesis induced by exposure to carcinogens (Lasnitski, 1976; Chopra and Wilkoff, 1977 a;b).

1.9.4. Effects of Retinoids on Communication.

The effects of retinoids on junctional communication have been studied on cells in culture using the technique of metabolic cooperation. Communication is detected by measuring the extent of transfer of [^{3}H]-uridine nucleotides from pre-labelled donor cells to recipient cells in contact. Junction formation and/or junctional transfer is blocked by 10^{-4}M retinoic acid (Pitts et al, 1981; Hamilton et al, 1982). Retinoic acid (10^{-4}M) also blocks communication through established junctions between cultured cells and the inhibition is fully and rapidly reversible (Pitts et al, 1981; Hamilton et al, 1982).

Morphological studies on the application of retinoic acid to tumours show retinoid induced proliferation of gap junctions (Prutkin, 1975; Elias and Friend, 1976; Elias

et al, 1981).

Topical application of retinoic acid to keratocanthomas leads to an increase in the number of gap junctions between the tumour cells (Prutkin, 1975). This increase may facilitate and mediate mucus metaplasia and this view is supported by the observation that the gap junctions, abundant in the mucus producing tumours, are sparse when the tumour reverts back to the dry keratonic condition upon cessation of retinoic acid treatment (Prutkin, 1975).

Exposure of chick embryo shank skin to retinoic acid in organ culture has also been observed to induce mucus metaplasia accompanied by tight junction formation and an increase in gap junctional area (Elias and Friend, 1976). In addition topical retinoic acid treatment induces a greater than two fold increase in gap junctions in human basal cell carcinomas (Elias et al, 1981).

There is insufficient experimental data to relate the observed effects of retinoic acid on gap junctional area and its apparently contradictory effects on junctional communication measured by functional tests, or to relate either of these to the observed effects on growth and differentiation. This project was designed to investigate the relationship between the morphological and functional observations, in part by the development of procedures for the isolation and biochemical characterisation of gap junctions from cultured cells, and to further characterise the mechanism of inhibition of retinoic acid on intercellular communication.

Chapter 2 - Materials and Methods.

2.1. MATERIALS.

2.1.1. Chemicals.

Protease Type X (Thermophilic bacterial protease : thermolysin E.C. 3.4.24.4), trypsin (E.C. 3.4.21.4), glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12, from rabbit muscle). N,2,4 DNP L-aspartic acid, N,N di 2,4 DNP L-lysine, all trans retinoic acid and tetradecanoyl phorbol 13-acetate (TPA) were supplied by Sigma Chemical Co. Ltd., Dorset.

Thin layer silica gel chromatography plates were obtained from Camlab, Cambridge and DMSO from Koch Light Laboratories Ltd., Colnbrook, Bucks.

D19 developer, Photo Flo, AR10 fine grain autoradiographic stripping film and Kodak X-Omat S X-ray film were supplied by Kodak Ltd., England.

Amfix was supplied by May and Baker Ltd., Dagenham. Depex and Giemsa stain were obtained from Hopkin and Williams, Essex.

Urea'acrylamide and N,N methylene bis acrylamide were supplied by British Drug House Ltd., London.

Triton-X-100 was obtained from Rohm and Haas, Croydon.

Sarkosyl NL97 detergent was supplied by ICN Pharmaceuticals, Irvine, California.

Phosphotungstic acid was supplied by EMscope, London.

Ninhydrin was supplied by Cambrian Chemicals Ltd., Croydon.

Low molecular weight standards for SDS PAGE,and Coomassie Brilliant Blue R250 were obtained from Bio-Rad, Irvine, California.

13 cis retinoic acid and Tigason (see 2.1.11) were the kind gift of Prof. R. Mackie, Dermatology Department, Glasgow University.

All other reagents used were BDH "Analar" grade.

2.1.2. Radiochemicals.

[5 - ^{3}H] uridine (specific activity 30 Ci . $mmol^{-1}$). [Methyl - ^{3}H] thymidine (25 Ci $mmol^{-1}$), [G - ^{3}H] hypoxanthine (1 Ci $mmol^{-1}$) and [L - ^{35}S] methionine (<1000 Ci . $mmol^{-1}$) were supplied by Amersham International Ltd.

Na ^{125}I (1000 Ci/ml in Na OH) carrier free was supplied by the Radiodispensary, Western Infirmary.

2.1.3. Trypsin solution.

Trypsin	2.5g
NaCl	6g
sodium citrate	2.96g
Phenol Red	0.015g

Distilled water to 1 litre.

pH 7.8.

2.1.4. Versene in Phosphate Buffered Saline.

NaCl	8g
K Cl	0.2g
NaH_2PO_4	1.15g
KH_2PO_4	0.2g
versene	0.2g
1% Phenol Red	1.5 ml

Distilled water to 1 litre.

pH 7.2.

2.1.5. Balanced Salts Solution (BSS).

Na Cl	6.8g
K Cl	0.4g
$Ca\ Cl_2\ .6H_2O$	0.393g
$Mg\ So_4\ .7H_2O$	0.2g
$Na\ H_2\ PO_4.2H\ O$	0.14g
1% Phenol Red	1.5 ml

Distilled water to 1 litre

pH 7.2.

2.1.6. Triton Extraction Buffer.

Triton-X-100	10ml
NaCl	8.77g
$Na_2\ H\ PO_4$ An.	0.575g
$Na\ H_2\ PO_4\ 2H_2O$	0.148g

Distilled water to 1 litre.

2.1.7. Formal Saline

100ml 40% (W/V) formaldehyde plus 900ml 0.08M NaCl, 0.2M $Na_2\ SO_4$.

2.1.8. Cell Growth Medium

Cells were grown in Glasgow Modification of Eagle's medium supplemented with 10% calf serum, or 10% foetal calf serum (RTG_2 cells). Glioma cells were cultured in Hams F10 supplemented with 10% foetal calf serum.

See Flow Laboratories Manual for formulations.

2.1.9. Cultured Cell Lines.

The following established cell lines were used:

a) BHK 21/C13 : Syrian hamster kidney fibroblast line (MacPherson and Stoker 1962).

b) TG_2 : stable mutant of the cell line BHK 21/C13 lacking the enzyme hypoxanthine : guanine phosphoribosyl transferase (Marin and Littlefield 1968).

c) MDCK : Madin - Darby canine kidney epithelial line (Rindler et al 1979).

d) BRL : Buffalo rat liver epithelial line (Coon 1968).

e) V79 : Chinese hamster lung fibroblast line (Ford and Yerganian 1958).

f) L929/A9 : stable mutant of the L mouse fibroblast line lacking the enzyme hypoxanthine:guanine phosphoribosyl transferase (Littlefield, 1966).

g) RTG_2 : rainbow trout gonadal tissue fibroblast line (Wolf and Quimby 1962).

h) 3T3 : Swiss mouse fibroblast line (Todaro and Green 1963).

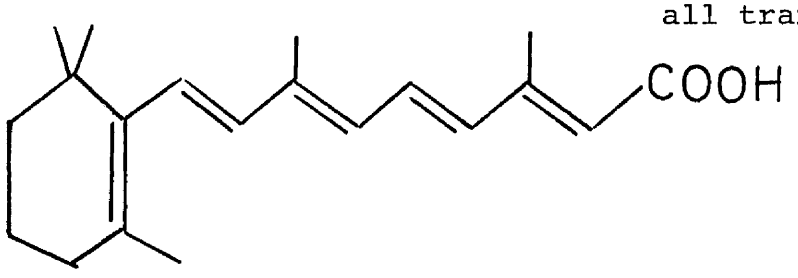
i) Xen : Xenopus laevis kidney epithelial line.

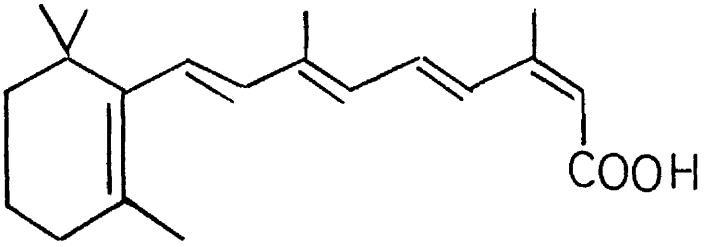
j) G-CCM : cell line derived from human glioma

k) NOR-T : normal adult glial cell line.

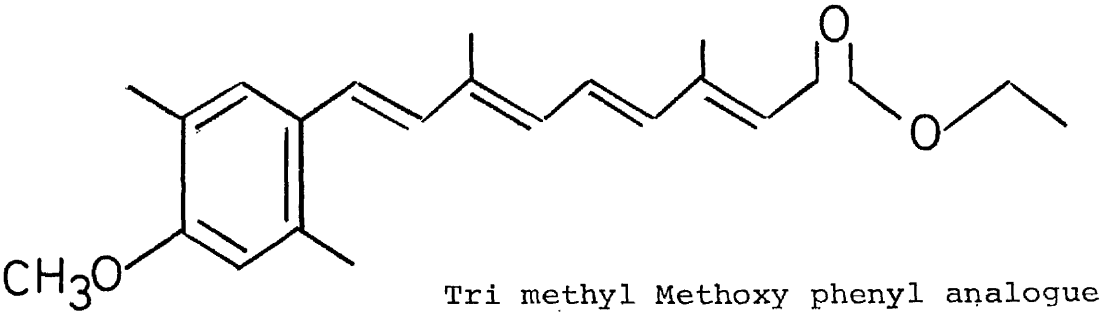
2.1.10 Structural Formulae.

all trans retinoic acid





13 cis retinoic acid



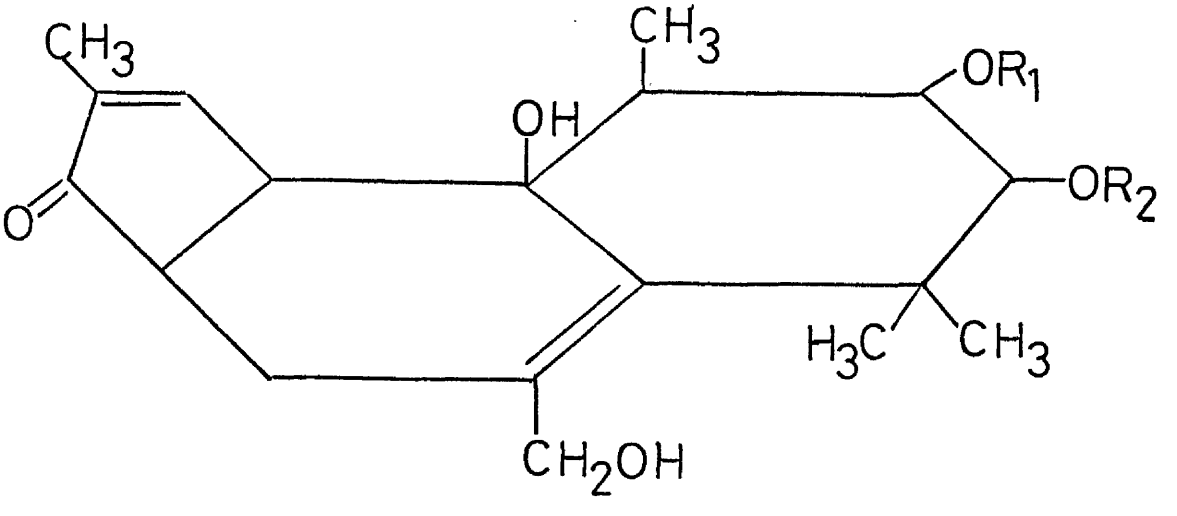
Tri methyl Methoxy phenyl analogue
of ethyl retinoate
(TIGASON)

R_1 = tetradecanoate

R_2 = acetate

tetradecanoyl phorbol 13-acetate

(TPA)



2.2. METHODS.

2.2.1. Maintenance of Cell Lines.

Cell lines were grown at 37°C as monolayers in Roux bottles or 20 oz roller bottles in an atmosphere of 5% CO_2, 95% air using Glasgow Modification of Eagle's Medium (EC_{10}) supplemented with 10% calf serum and containing 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin.

Cells were subcultured 1:3 every 3 days by rinsing the cell sheets twice with a 1:1 mixture of trypsin (see section 2.1.3.) and versene (see section 2.1.4.) and then suspending the trypsinised cells in growth medium. Single cell suspensions were obtained by pipetting gently up and down several times. Cell numbers were obtained by counting on a haemocytometer.

Cells for experiments involving uridine nucleotide transfer (see section 2.2.8.), measurement of incorporation of labelled precursors (see section 2.2.11) and cell growth (see section 2.2.12.) were grown in EC_{10} on plastic petri dishes (Nunc, Gibco Biocult, Paisley, Scotland) in a humidified incubator at 37°C containing an atmosphere of 5% CO_2, 95% air.

The isolation of gap junctions from cultures requires larger cell numbers and consequently cells were grown as monolayers in 20 oz roller bottles in 150 ml EC_{10}.

2.2.2. Isolation of Gap Junctions From Cells in Culture: Protocol 1.

Gap junctions were isolated from 18 just confluent 20 oz bottles of BRL cells (total 3 x 10^9 cells). The

medium was removed and the cells washed with ice-cold BSS (see section 2.1.5.).

Triton extraction buffer (see section 2.1.6.) (12ml) was added to each bottle and the bottles left to rotate at 37°C for 5 min. The triton extracts were decanted, combined and centrifuged at 3000g for 10 min at 0°C. The supernatant was removed and centrifuged again at 3000g for 10 min at 0°C.

These two low speed centrifugations remove triton insoluble material (e.g. nuclei, cytoskeletal and matrix proteins). The supernatants contain triton solubilised material (e.g. membranes, cytosol and residual triton insoluble material).

The final low speed supernatant was centrifuged at 40,000g for 45 min at 0°C. The resulting pellets were resuspended in small volumes of 1mM $NaHCO_3$ (pH 7.4) at 0°C, the total volume made up to 50ml with 1mM $NaHCO_3$ (pH 7.4) at 0°C and recentrifuged as before. The pellets were resuspended in a total of 9ml 1mM $NaHCO_3$ (pH 7.4), 3ml trypsin (0.025%) added and the solution incubated at 37°C for 20min. Sarkosyl NL97 detergent (12ml 0.001% in 1mM $NaHCO_3$ (pH 7.4)) was added, the solution mixed and immediately centrifuged at 40,000g for 45 min at 0°C.

Since gap junctions are resistant to detergents such as sarkosyl NL97, the high speed centrifugation pellets the junctions along with membrane vesicular material. The pellet was further purified using discontinuous sucrose density gradients.

The pellet was resuspended in 1ml 1mM $NaHCO_3$ (pH 7.4) at 0°C and loaded onto discontinuous sucrose gradients of

3.5ml 54% sucrose ($^{w}/v$ in distilled water) and 4.5ml 32% sucrose ($^{w}/v$ in distilled water) and centrifuged at 209,000g for 90 min at $0^{o}C$. The material at the 32%/54% sucrose interface was collected with a pasteur pipette, diluted to 14ml with 1mM $NaHCO_3$ (pH 7.4) at $0^{o}C$ and centrifuged at 209,000g for 60 min at $0^{o}C$, to yield a pellet enriched in gap junctions.

2.2.3. Isolation of Gap Junctions from Cell Cultures: Protocol 2.

Gap junctions were isolated from 3 confluent 20 oz roller bottles of various cell types (total 4.5 x 10^8 cells).

The medium was removed and triton extraction buffer (12ml) added immediately to each bottle and the bottles left to rotate for 5 min at $37^{o}C$. The triton extracts were decanted combined and then centrifuged at 3000g for 10 min at $0^{o}C$. The supernatants were removed and centrifuged at 209,000g for 30 min at $0^{o}C$. The pellets were resuspended in a small volume of 1mM $NaHCO_3$ (pH 7.4) at $0^{o}C$, the combined volume made up to 12.5ml and centrifuged at 250,000g for 30 min at $0^{o}C$. The supernatant was discarded and the pellet resuspended in 1.5ml 6M urea (in distilled water), 0.5ml trypsin (0.1% w/v in 6M urea) added and the solution incubated at $37^{o}C$ for 20 min. Sarkosyl NL97 was added to a final concentration of 0.5%, the solution mixed then layered onto 9ml 32% sucrose (w/v in distilled water) and centrifuged at250,000g for 1h at $0^{o}C$, to yield a junction enriched pellet.

2.2.4. SDS Polyacrylamide Gel Electrophoresis.

Microslab (6 cm x 0.5 cm running gel, stacking gel, and 0.75 mm thick gels) polyacrylamide gel electrophoresis was carried out as described by Amos (1976) with the modifications of Laemmli (1976).

Running gels were 13.75% acrylamide, 0.3% N,N methylene bis-acrylamide (w/v) in 0.375M tris HCl buffer (pH 8.8). Stacking gels were 6% acrylamide, 0.15% N,N methylene bis acrylamide (w/v) in 0.125M tris HCl buffer (pH 6.8).

Samples were solubilised at room temperature in 1% SDS (containing 0.001% bromophenol blue (w/v) tracker dye). 5-15µl samples were applied per gel slot and the gels run at 15mA for 1.5h.

Gels were stained in 0.125% Coomassie Brilliant Blue in 5% acetic acid, 12.5% isopropanol in water (v/v) for 8h and destained in several changes of 5% acetic acid, 16% methanol in water (v/v).

The M.Wt marker mixture contained lysozyme (M.Wt 14,300), soybean trypsin inhibitor (M.Wt 21,000), carbonic anhydrase (M.Wt 30,000), ovalbumin (M.Wt 45,000) bovine serum albumin (M.Wt 68,000) and phosphorylase B (M.Wt 94,000).

2.2.5. Gel Scanning.

Slab gels were scanned using a Joyce-Loebel microdensitometer. The relative amounts of proteins were obtained by cutting out the appropriate peaks and weighing on a fine balance.

2.2.6. Standardisation of Gel Scans.

Gels were run with different concentrations of M.Wt standard proteins and the gels scanned using a Joyce-Loebel microdensitometer, which measures the absorption due to Coomassie stain. Lysozyme and carbonic anhydrase peaks were cut out and weighed and there was found to be a linear response of area under the peak to protein concentration.

Several scans of one gel showed that the scans differed by less than 2%.

2.2.7. Electron Microscopy of Isolated Gap Junctions.

Gap junction pellets were isolated as described (see section 2.2.2. and section 2.2.3.). The pellet was taken up in a small volume (10µl) of 1mM $NaHCO_3$ (pH 7.4). An aliquot (2µl) of the resuspended pellet was placed in a small weighing boat and 2µl of 2% phosphotungstic acid (pH 7.0) added. This solution was then placed on a carbon coated copper electron microscope grid. The liquid was carefully absorbed with filter paper, and the grid blotted carefully on the underside. The grid was air dried and viewed in a Phillips 301 electron microscope.

2.2.8. Uridine Nucleotide Transfer.

The passage of labelled molecules derived from [5-^{3}H] uridine through intercellular junctions from prelabelled donor to recipient cells was used as a measure of the extent of junctional communication between cells in culture.

Recipient cell cultures were prepared by plating 10^5 cells in 2.0ml EC_{10} in 3cm diameter plastic petri dishes each containing three sterile glass coverslips (13.0mm

diameter) and incubating at 37°C overnight.

Donor cell cultures were prepared by plating 2 x 10^5 cells in 2.0ml EC_{10} in 3cm diameter plastic petri dishes (without coverslips) and incubating at 37°C overnight. The donor cells were then labelled for 4h by replacing the medium with 2.0ml EC_{10} containing 20µCi [5-^{3}H] uridine (specific activity 30 Ci mmole^{-1}). Residual [5-^{3}H] uridine was removed by washing the donor cell cultures three times with 2.0ml sterile BSS at room temperature. The donor cells were then detached by washing with 2 changes of trypsin : versene (1:1) and suspended in 2.0ml EC_{10} at room temperature. 100µl aliquots of the labelled donor cell suspension were added to the dishes of unlabelled recipient cells and the mixtures co-cultured for 3h.

Following co-culture the medium was removed, the cells were washed twice with 2.0ml BSS at 0°C and fixed with 2.0ml formal saline for 30 min at 20°C.

The coverslips were removed, placed in racks and the acid soluble nucleotides removed by washing twice with 5% TCA at 0°C for 5 min and twice with water at 0°C for 4 min. The coverslips were finally rinsed in ethanol and dried.

Coverslips were mounted (cells uppermost) with Depex on microscope slides and processed for autoradiography (see section 2.2.9.).

2.2.9. Preparation of Slides for Autoradiography.

Slides were dipped in gelatin/chrome alum and dried at room temperature. They were then covered with AR10 fine grain autoradiographic stripping film, dried under a

stream of cold air and placed in a light tight box. Autoradiographs were stored at room temperature in the dark. The period of exposure varied depending whether the autoradiographs were used for grain counting studies (short exposures) or for photography (long exposures).

After the exposure period, slides were developed with D19 developer at 20°C for 5 min, washed in water for 2 min and fixed for 5 min with a 1:5 dilution (v/v) of Amfix. After washing for 4 min with water, slides were stained using freshly diluted Giemsa (1:20 in water) for 1 min. The stained, washed autoradiographs were air dried and second coverslips mounted on top with Depex.

Photographs were taken with a Leitz Orthomat photomicroscope using Ilford Pan F 35mm film.

2.2.10. Quantitation of Autoradiography.

Developed autoradiographs show heavily labelled donor cells in which the distribution of autoradiographic grains is mainly cytoplasmic due to the conversion of nuclear RNA into longer lived cytoplasmic forms during the co-culture period. If cell-cell communication has occurred, recipient cells in contact with donors are lightly labelled over the cytoplasm with heavier labelling over the nucleus. Recipient cells not in contact with donors have only the autoradiographic background level of grains.

Autoradiographic grains were counted over 50 recipient cells in contact with donor cells and 50 isolated recipient cells. The means and standard deviations of the grain counts for the two populations were used to calculate

t-values using the students t-test, and the values of P were derived from statistical tables. A P value of <0.05 was defined as indicating positive nucleotide transfer, and a P value of >0.05 was defined as showing no significant difference between the two populations of grain counts.

2.2.11. Incorporation of Labelled Precursors.

Cell cultures growing in 3cm diameter plastic petri dishes were labelled with the appropriate [^{3}H]-precursor : details of the labelling procedures are given in the figure legends.

After incubating at 37^{o}C for the times shown in the legends, the radioactive medium was removed and cell monolayers washed four times with 2ml BSS at 20^{o}C. The incorporation of radioactivity into acid soluble and acid insoluble fractions was measured as follows. The acid soluble pool was extracted with 2 x 1.5ml 5% TCA at 0^{o}C and the extracts combined. The cell layer was further washed with 1.5ml 5% TCA and 1.5ml distilled water, and then the acid insoluble pool extracted with 2ml 0.1M NaOH for 1h at 20^{o}C.

Acid insoluble (after acidification with 0.3ml 1M HC1) and acid soluble extracts were added to 10ml triton-toluene (1:2 v/v) scintillation fluid containing 0.5% (w/v) PPO scintillant.

Samples were then counted in a liquid scintillation counter. The efficiency of counting calculated from the channels ratio varied by no more than 5% between different samples using the same counting system.

Replica cultures varied by no more than 10% from their respective mean.

2.2.12. Measurement of Rate of Cell Growth.

4×10^5 cells were plated in 5ml EC_{10}, or 5ml EC_{10} + 25µl DMSO or 5ml EC_{10} + 25µl DMSO containing 2×10^{-2}M trans retinoic acid (final concentration 10^{-4}M) and allowed to spread for 2h. Cell number was estimated by trypsinising the cells (see section 2.2.1.) suspending in growth medium and counting using an improved Neubauer haemocytometer. This was called Time Oh, and the cell number was then estimated at 24h intervals. Two dishes of cells were counted for each treatment and the average cell number calculated.

2.2.13. Chromatography of Retinoic Acid.

13 cis retinoic acid, all trans retinoic acid and Tigason (see section 2.1.10) were applied (10µl 20mM solution in EtoH) to silica gel thin layer chromatography plates (19cm x 20cm). Chromatography solvent systems used to separate the retinoids were chloroform:petroleum ether (4:1), chloroform : acetone (4:1) and chloroform : acetone (99:1).

Spots were detected by viewing under ultra violet light of wavelength 254nM and 350nM.

2.2.14. Elution of Chromatographically Purified All Trans Retinoic Acid.

All trans retinoic acid, 13 cis retinoic acid and Tigason were chromatographically separated in the solvent system chloroform : acetone (99:1) as described in section 2.2.13.

The all trans retinoic acid was eluted by cutting out the spot, clamping it between two clean glass slides and dipping one end in alcohol. A microcapiliary tube was attached to the other end of the thin layer piece to facilitate the solvent flow. The trans retinoic acid was eluted in a small volume of EtOH (50µl) in an EtOH saturated atmosphere and then diluted with EC_{10} to give a final concentration of 10^{-4}M. The effect of this on uridine nucleotide transfer was then tested (see section 2.2.8.).

2.2.15. Iodination of Gap Junctions.

The most successful method of iodinating gap junctions was found to be with the chloramine T catalysed reaction.

Gap junctions were isolated from 18 roller bottles of BRL cells as described (see section 2.2.2.). The junction rich pellet was suspended in 25µl 50mM phosphate buffer (pH7.4). $Na^{125}I$ (400µCi in water) was added and the reaction started by the addition of 25µl chloramine T (3.5 mg/ml)in 50mM phosphate buffer (pH7.4). The reaction was allowed to proceed for 1min at 20°C before the addition of 100µl Na metabisulphite (2.4mg/ml in 50mM phosphate buffer (pH7.4)). The sample was loaded onto 9ml 32% sucrose (w/v in distilled water) and the reaction vessel washed out with 200µl KI (20mg/ml in 50mM phosphate buffer (pH7.4)). The washings were added to the sample and the tube centrifuged at 209,000g for 90min. This centrifugation further purified the junction enriched pellet and removed contaminating, unbound iodide.

2.2.16. Proteolytic Digestion and Peptide Mapping of Iodinated Gap Junctions.

i) Gap junction enriched pellets were isolated from 18 roller bottles of BRL cells, (see section 2.2.2.), iodinated (see section 2.2.15) and analysed by SDS PAGE (see section 2.2.4.).

The labelled junctional protein band, (M_r 16K) was cut from the Coomassie stained gel with a sharp razor blade, rinsed with 1ml H_2O and dried under vacuum in a dessicator to remove traces of destaining solvents. The protein was eluted with 0.5ml SDS extraction buffer in 1% SDS (w/v in 50mM TEA HCl (pH 8.0) containing 5% (v/v) 2 mercaptoethanol, 5mM, EDTA and 0.5mg GAPDH (Unlabelled GAPDH is added as a carrier to facilitate recovery of the iodinated gap junction protein which is present in small amounts). The mixture was incubated at 20°C for 1h during which time the gel piece swells and absorbs the extraction buffer. The gel piece was then crushed with a glass rod and the mixture further incubated at 65°C for 15min. After standing at room temperature until no colour was visible in the gel pieces, the sample was filtered through glass wool and the protein precipitated (see section 2.2.16(ii)).

ii) Recovery of Eluted Protein.

3m KOAc (70µl) was added to the eluted protein, mixed thoroughly and the mixture left on ice for 30min. A flocculent precipitate appeared and this was pelleted by centrifugation at 1000g at room temperature for 5min. The pellet was resuspended in 2ml acetone : HCl (0.85 : 100

v/v), a further 40µg GAPDH added and the tube centrifuged again at 1000g at room temperature for 5min. The supernatant was discarded and the previous washing step repeated. The pellet was then resuspended in 1ml 10% TCA and incubated at $0^{o}C$ for 30min. The precipitated material was centrifuged as before and finally washed with acetone. The precipitated protein was dried overnight in a vacuum dessicator containing solid NaOH to adsorb residual acid.

iii) Alkylation of Iodinated Protein.

The dry protein (labelled junctional protein and carrier GAPDH) was dissolved in 0.5ml 8M urea in 100mM Tris HCl (pH 8.5) which had been degassed and left under N_2 at reduced pressure in a dessicator, for 30min.

Fresh 10mM DTT (in water) was added to a final concentration of 2mM and the solution replaced in an atmosphere of N_2 in the dessicator under reduced pressure for 60min at room temperature. Iodoacetamide (40mM in water) was then added to a final concentration of 10mM and the solution again placed under N_2 at room temperature in a dessicator at reduced pressure for 60min.

The protein solution was finally dialysed against 0.5% NH_4HCO_3 (five changes of 1 litre) before proteolytic digestion (see section 2.2.16(iv)).

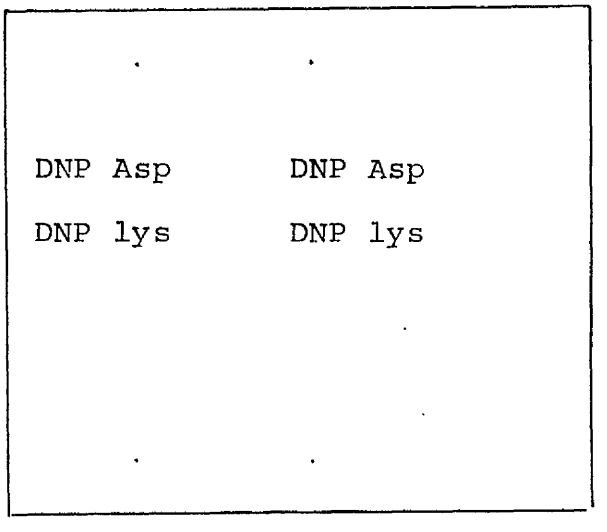
iv) Thermolytic Digestion.

Thermolysin (200µg/ml in 1mM HCl) was added (30ml) (to give a ratio of thermolysin : protein of 1µg:100µg)

and the mixture incubated at 55°C for 2h. A further 30µl thermolysin (200µg/ml in 1mM HCl) was added and the mixture again incubated at 55°C for 2h. The digested protein was then freeze dried, and the peptide products mapped (see section 2.2.16 (v)).

v) Peptide Mapping.

The freeze dried digested protein was suspended in a small volume (5µl) BAWP (Butanol : acetic acid : water : pyridine 15:3:12:10). This was applied to the origin of a silica gel thin layer chromatography plate (10cm x 10cm). For maps at pH 6.5 the origin was placed in the middle of one side of the plate, and for those at pH 3.5, the origin was put nearer the anode (since most proteins are positively charged at this pH).



DNP Asp and DNP Lys at 1mg/ml (in 1mM HC^{1}) were applied (2µl) opposite the digested protein spots to monitor the electrophoresis.

Using two pieces of Whatman 3MM chromatography paper, buffer was applied evenly to the TLC plate.

The pH 6.5 buffer was 10% pyridine, 0.5% acetic acid (v/v in water), and the pH 3.5 buffer was 0.5% pyridine, 5% acetic acid (v/v in water).

The plate was electrophoresed at 400v for 15min and dried overnight.

Chromatography was carried out in the second dimension using BAWP (Butanol : acetic acid : water : pyridine 15:3:12:10).

The plate was dried thoroughly and sprayed lightly with 0.25% ninhydrin ($^{w}/v$) in acetone.

The digested peptide fragments of GAPDH were visible as discrete purple spots when left for 8-16h at room temperature.

Thermolytically digested iodinated gap junctional protein was identified by autoradiography using Kodak X-omat S X-ray film and exposing for 1 week with an intensifying screen (Ilford Ltd., England).

Chapter 3 - Isolation and Identification of the Junctional Protein From Cultured Cells.

3.1. INTRODUCTION

The chemical structure of the gap junction has been investigated by isolating morphologically intact plaques and examining the protein component using SDS polyacrylamide gel electrophoresis. This has however failed to unequivocably identify the junctional protein(s) due to the problems associated with isolating pure fractions of gap junctions. There is no definitive criteria of purity except for morphological appearance of fractions in electron micrographs, gap junctions are a relatively minor membrane species, and the use of proteases to remove contaminants has introduced problems. By morphological criteria gap junctions are not affected by protease treatment but proteins may,however,be specifically or generally cleaved resulting in degradation products.

To date all published work on the identification of the gap junctional protein has been based on junctional isolates from whole tissues such as liver. A new method has been developed by which gap junctions can be isolated from cells grown as monolayers in cell culture (Finbow et al, 1982). Since cells in culture are more amenable to direct experimentation (e.g. cloning, mutant selection and pulse labelling), it is now possible to expose cells to different treatments and examine the effect on the amount of the recoverable gap junctional protein and hence indirectly on the extent of gap junctional plaques at the cell surface. The method of isolation involves differential ultracentrifugation and discontinuous sucrose density gradients and therefore will isolate only junctional plaques and not individual isolated junctional particles

(see section 6.3.).

The first section of the results describes the development of a modified extraction procedure which allows analysis of quantities of cultured cells small enough for easy experimental manipulation and for a series of comparative experiments to be undertaken which examine the effect of retinoic acid on the isolation of junctional protein and hence on the area of gap junctions between cells (see section 5).

The second section of the results describes evidence which shows a correlation between junctional area measured by freeze-fracture morphometry and the recovery of the protein component believed to be of junctional origin, from ^{V79} cells treated with TPA.

3.2.1. Isolation of Gap Junctions From Cultured Cells.

The method which has been devised for the isolation of gap junctions from cultured cells by Finbow et al, (1982) yields junction enriched fractions from BRL cells in culture which, on SDS polyacrylamide gel electrophoresis, show the presence of only one major protein with an apparent M_r, 16,000 (16K) (Fig. 2).

Morphological examination of such junction enriched pellets shows the major visible component has the characteristic appearance of a gap junction (Fig. 3). This technique however requires relatively large cell numbers using 18 roller bottles (2.7×10^9 cells) for each isolation, which makes investigations based on a series of comparative experiments impractical, and as a first step towards looking at the effect of retinoic acid on the isolation of junctions, it was important to scale down the isolation procedure.

After several preliminary attempts to modify the procedure which resulted either in yields too small for analysis or in poor separation (e.g. Fig 4) where it was not possible to associate any one band with the junctional fraction, a method was developed (see section 2.2.3), for the isolation of gap junctions from 3 roller bottles of cells (4.5×10^8) using a combination of 6M urea and trypsin to remove contaminating non-junctional proteins from a crude triton extract of the cell monolayer. SDS polyacrylamide gel electrophoresis of the junction enriched pellets isolated by the improved method show the presence of one major protein, M_r, 16,000, which can be seen after staining with Coomassie (fig. 5). The final junction

enriched pellets produced by this method are variably contaminated with a fibrous component which appears to be insoluble in SDS and,when applied to the gels,fails to enter the running gel but remains in and on the stacking gel (e.g. see Fig. 5). This can be removed without affecting the recovery of the 16K band or the gap junctions seen in electron micrographs,by a short low speed centrifugation before running samples on gels.

Electron micrographs of gap junction fractions isolated by this procedure from BRL cells show them (Fig. 6) to be morphologically more complex than those isolated using the method of Finbow et al, 1982. The biochemical purity is however sufficient (e.g. see Fig 5) to allow unambiguous identification and quantitative measurement of the 16K protein and it is therefore practical to use this method to examine the effect of retinoic acid on the isolation of the junctional protein and hence, on the area of gap junctions present between cells (see section 1.9.4.).

3.2.2. The Junctional Protein

The 16K protein was found to be the only constituent in junction fractions isolated using the modified protocol from the cell lines BRL, V79, C13 and RTG_2 (Fig. 7) all of which form functionally detectable junctions. The protein was however also found to be present in similar fractions from A9 cells (Fig. 7) which have been shown previously not to communicate either with cells of the same type or with any other cell type tested (Pitts, 1971). Since the isolation procedure isolates junctional plaques

and not individual junctional particles, it is possible that A9 cells may have junctional plaques present at the cell surface which are defective in some way, preventing effective intercellular communication.

This protein is the same as the 16K component isolated by Finbow et al, (1982) from mouse liver. Both proteins are the only major protein present in junction enriched fractions and both have the same mobility on SDS gels. They also show identical heat induced aggregation in SDS buffers. Fig. 8 shows that solubilising the 16K protein by heating to 100°C in 1% SDS prior to SDS polyacrylamide gel electrophoresis results in aggregation of the 16K protein to higher molecular weights of 26,000 (and in some experiments also 39,000 and 56,000) thought to represent multimeric forms (dimer, trimer, tetramer) of the protein. Prolonged boiling of the junction fraction isolated from BRL cells results in such extensive aggregation that a large proportion of the material is so highly aggregated that it fails to enter the running gel. This aggregated material can be shown to originate from the 16K protein by eluting the 16K protein from the gel, and heating to 100°C in 1% SDS. When the sample is rerun, the aggregated material is again visible (results not shown). Heat induced aggregation is concentration dependent, and is not seen in isolations using fewer cells. This aggregation is a sufficiently unusual property (most proteins dissolve more readily in SDS at 100°C) to be used as a criterion for the identity of the 16K protein from cultured cells and from mouse liver.

Aggregation of the 26K junctional protein has been

reported by Henderson et al (1979) and heat treatment in SDS solutions has also been observed to cause aggregation of some other membrane proteins such as myelin proteins (Morrell et al, 1975) and proteins from lens fibre membrane (Broekhuyse and Kuhlmann, 1979).

To use the isolation method for quantitative measurements of the junctional protein (and hence junctional area) after treating cells in different ways (e.g. with retinoic acid), the yield of the 16K protein must be reproducible.

The yield of the 16K protein isolated in parallel experiments was investigated to assess reproducibility by adding equal amounts of triton extracts of cells labelled with [^{35}S] methionine to different triton extracts made from the same numbers of cells (Figs. 9, 10). Table 1 shows that the amount of labelled material recovered in each of the three separate isolations was within 3-5% of the mean value. This means that the reproducibility is sufficiently high to allow a series of comparative experiments.

Two systems one in vivo (Finbow et al, 1982) and one in culture, have been used to show that the 16K protein is of junctional origin. In regenerating rat liver gap junctions are virtually absent 24-28h after partial hepatectomy (Yee 1978). Finbow et al, (1982) have shown that the amount of recoverable 16K protein from 30h post-operative regenerating liver is greatly reduced when compared to control amounts. Peroxisomal bound urate oxidase crystals which co-migrate with rat liver gap junctions

provided an excellent internal control for variation in recovery during the purification procedure. The recovery of urate oxidase crystals was the same in both normal and regenerating liver. The results of this study (Finbow et al, 1982) show a strong morphological and biochemical correlation as to the junctional origin of the 16K protein.

Treatment of V79 cells with 2×10^{-7}M TPA causes inhibition of nucleotide transfer (Yotti et al, 1979). Freeze-fracture analysis of TPA treated V79 cells shows this is due to a TPA induced removal of junctional plaques from the cell surface, (Yancey et al, 1982), where treatment with TPA causes a 95% reduction in the area of gap junctions. This morphological data was correlated with a biochemical study of the effect of TPA on the isolation of the 16K protein. Gap junction fractions from TPA treated V79 cells were isolated and analysed by SDS polyacrylamide gel electrophoresis (Fig. 11). The amount of the 16K protein in TPA treated V79 cells fell to 13% of that in control cells (Fig 12 and Table 2). V79 cells which had been exposed to TPA for 18h and then left in normal medium for a further 27h showed almost control levels of the 16K protein (Fig 12 and Table 2) with only a 5% decrease. The TPA containing medium, control medium and medium from those cells previously exposed to TPA, was tested in a uridine nucleotide transfer experiment. The medium containing TPA inhibited junctional communication between V79 cells (Table 3) as has been reported previously (Yotti et al, 1979). The % decrease in the amount of

the 16K protein isolated in separate experiments from TPA treated V79 cells compared to control V79 cells varied from 60-87%.

The results correlate with morphological data and offer further evidence as to the junctional origin of the 16K protein.

TPA has no detectable effect on junctional communication between BRL cells as measured byuridine nucleotide transfer. BRL cells were treated with TPA for 18h before junction isolation and the junction enriched fraction analysed by SDS PAGE (Fig. 13). TPA was found to have only a slight effect on the recovery of the 16K protein, causing a reduction of 11% in the amount of the 16K protein compared to that isolated from control BRL cells. (Fig. 14 and Table 4). The TPA containing medium was found to have no effect on uridine nucleotide transfer between BRL cells (Table 5).

The 16K junctional protein was iodinated, digested with thermolysin and the peptide products mapped (Fig. 15). Glyceraldehyde -3- phosphate dehydrogenase was added to facilitate the recovery of the junctional protein which is present in very small quantities. The peptide products of GAPDH were mapped and visualised after spraying with ninhydrin (Fig. 16). The map of GAPDH can be used as an internal control to check the reproducibility of the mapping procedure.

The results show that it is possible to digest iodinated junctional protein into a small,useful number of peptides separable by two dimensional mapping. The value of this approach will be discussed in section 6.1.

Figure 2 SDS PAGE of Gap Junction Enriched Fractions Isolated from Cultured BRL Cells.

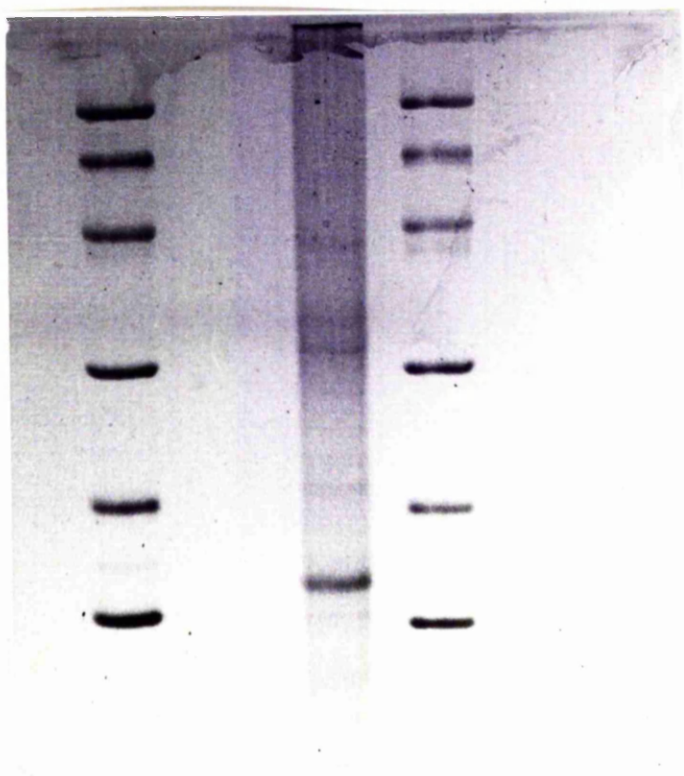
Gap junctions were isolated from 18 confluent roller bottles of BRL cells by the method of Finbow et al, (1982) as described in section 2.2.2.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature, and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 Bio-Rad M.Wt marker proteins

Lane 2 junction enriched pellet from BRL cells

Lane 3 Bio-Rad M.Wt marker proteins.



LANE 1 2 3

Figure 3 Electron Micrograph of Junction Enriched Pellet Isolated from Cultured BRL Cells and from Mouse Liver.

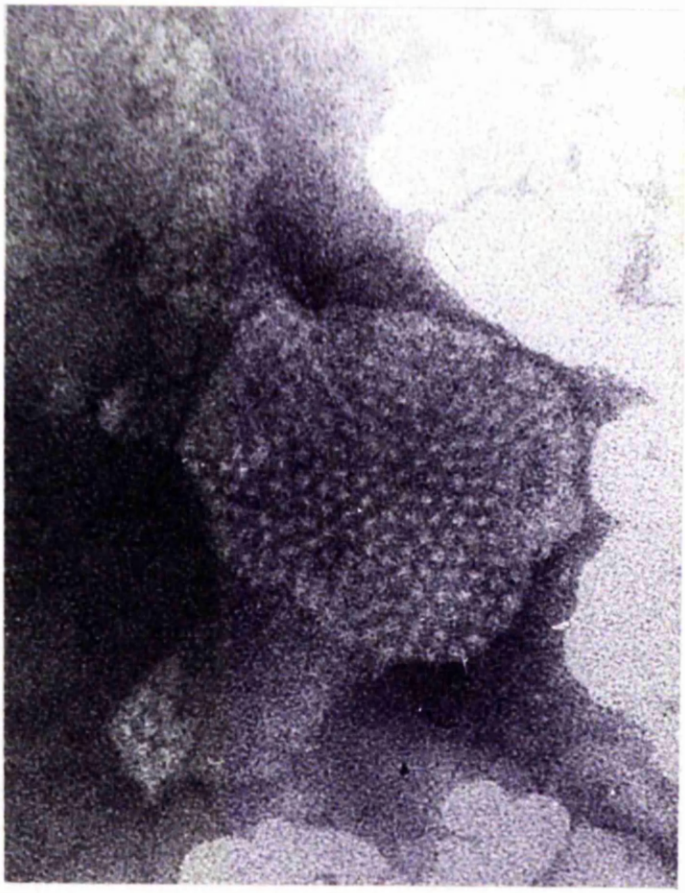
Gap junctions were isolated from 18 confluent roller bottles of BRL cells and from 25 mouse livers by the method of Finbow et al, (1982) as described in section 2.2.2.

The final junction enriched pellet was resuspended in a small volume (10µl) 1mM $NaHCO_3$ (pH 7.4). A fraction of the suspended pellet (2µl) was placed in a small plastic weighing boat and 2µl 2% phosphotungstic acid (w/v in water) (pH 7.0) added. The mixture was then dropped onto a carbon coated copper electron microscope grid. Excess liquid was removed by blotting the underside of the grid with filter paper. The grid was allowed to dry and examined with a Phillips 301 electron microscope.

a) BRL cells (Magnification 200,000)

b) mouse liver (Magnification 108,000)

a)



b)

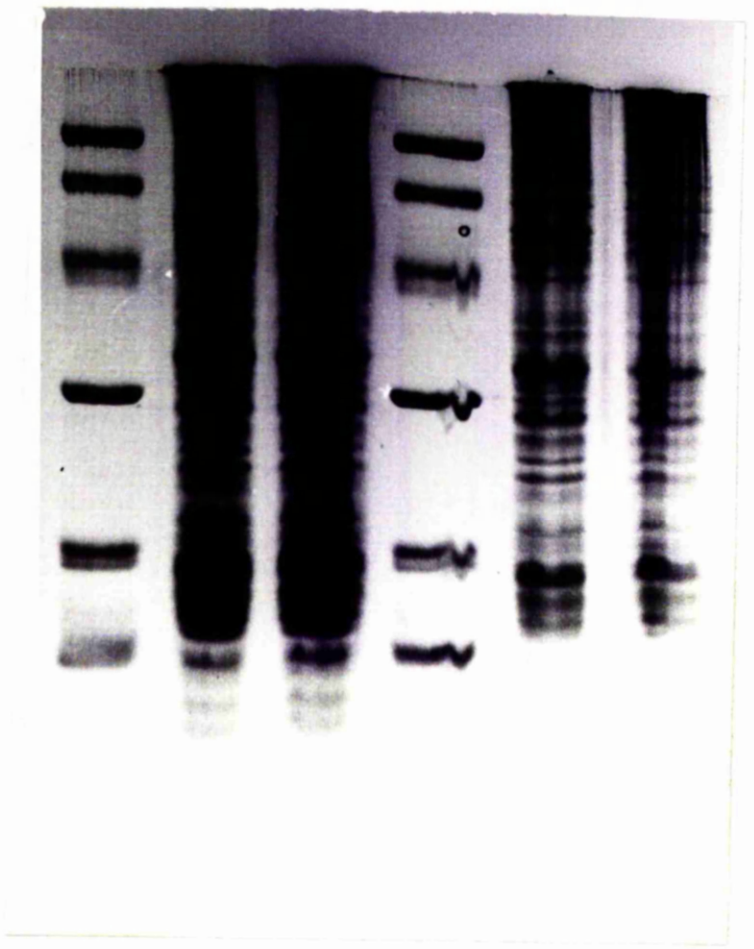
Figure 4 Isolation of Gap Junctions From Cultured BRL and CK Cells by a Scaled Down Procedure.

Gap junctions were extracted from 3 confluent roller bottles of BRL cells or canine kidney (CK) cells in 12ml 1% triton, 0.15m NaCl, 5mM phosphate buffer (pH 7.4) per bottle.

Contaminating nuclei and cytoskeletal fragments were pelleted by centrifugation at 3000g at $0^{o}C$ for 10 min. The supernatant was centrifuged at 209,000g for 30 min and the resulting pellet resuspended in 1.5ml 6M urea, 0.5ml 2% sarkosyl NL97 (w/v in 6M urea) was added to give a final concentration of 0.5%. This was layered onto 32% sucrose and centrifuged at 209,000g for 2h.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature, and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio-Rad M.Wt. marker proteins
Lane 2 : junction enriched pellet from BRL cells (10µl sample)
Lane 3 : junction enriched pellet from CK cells (10µl sample)
Lane 4 : Bio-Rad M.Wt. marker proteins
Lane 5 : junction enriched pellet from BRL cells (5µl sample)
Lane 6 : junction enriched pellet from CK cells (5µl sample)



LANE 1 2 3 4 5 6

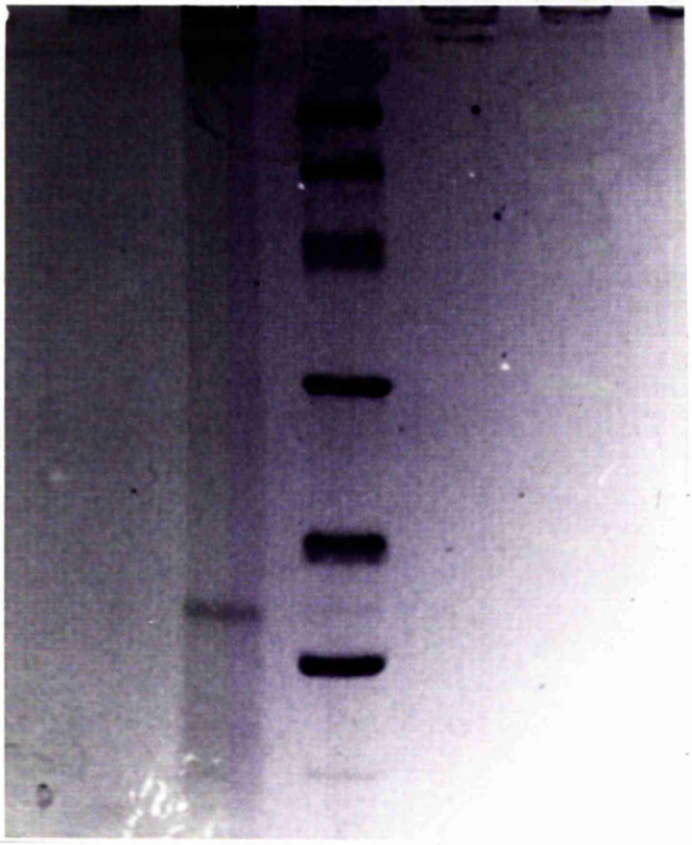
Figure 5 Isolation of Gap Junctions From Cultured BRL Cells By Modified Method. (Protocol II)

Gap junctions were isolated from 3 confluent roller bottles of BRL cells in 12ml 1% triton, 0.15m NaCl, 5mM phosphate buffer (pH 7.4) per bottle. The triton extracts were combined and centrifuged at 3000g for 15min. The pellet was discarded and the supernatant centrifuged at 109,000g for 30min. The resulting pellet was resuspended in 2ml 1mM $NaHCO_3$ (pH 7.4) at $0^{o}C$, diluted to a total volume of 30ml with the same buffer and centrifuged at 109,000g for 30min. The pellet was resuspended in 1.5ml 6M urea, 0.5ml trypsin (1mg1ml w/v in 6M urea) added and the solution incubated at $37^{o}C$ for 20min. Sarkosyl NL97 detergent was then added to a final concentration of 0.5% (100µl of 10% sarkosyl w/v in 6M urea). The solution was gently mixed, layered onto 32% sucrose and centrifuged at 150,000g for 90min.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : junction enriched pellet from BRL cells

Lane 2 : Bio-Rad M.Wt. marker proteins



LANE 1 2

Figure 6 Electron Microscopy of Gap Junction Enriched Pellets Isolated by Improved Isolation Method (Protocol II).

Gap junctions were isolated from 4 confluent roller bottles of BRL cells as described in section 2.2.3.

The final junction enriched pellet was prepared for electron microscopy as described in section 2.2.7.

a) BRL cells

Magnification x 110,000

a)

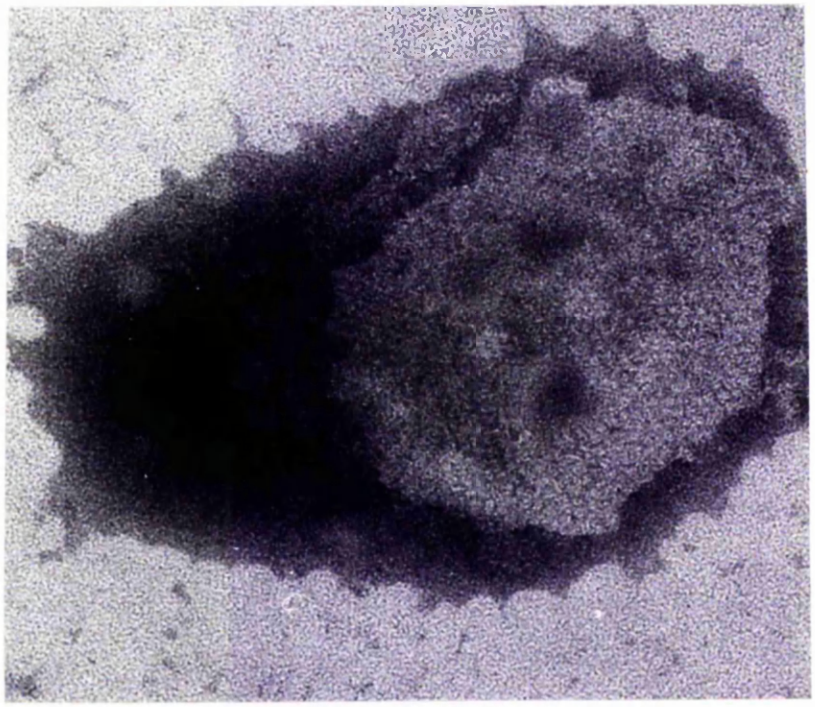


Figure 7 Isolation of Gap Junctions From Different Cell Types in Culture.

Gap junctions were isolated from three confluent roller bottles of BRL, C13, V79 cells and from 9 roller bottles of A9 cells using isolation Protocol II as described in section 2.2.3.

The RTG_2 cells did not grow well in roller bottles and were grown instead as monolayers in 30 75cm^2 plastic flasks. Gap junctions were extracted in 10ml 1% triton, 0.15m Nacl, 5mM phosphate buffer (pH 7.4) per flask. The rest of the isolation procedure was as described in section 2.2.3.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

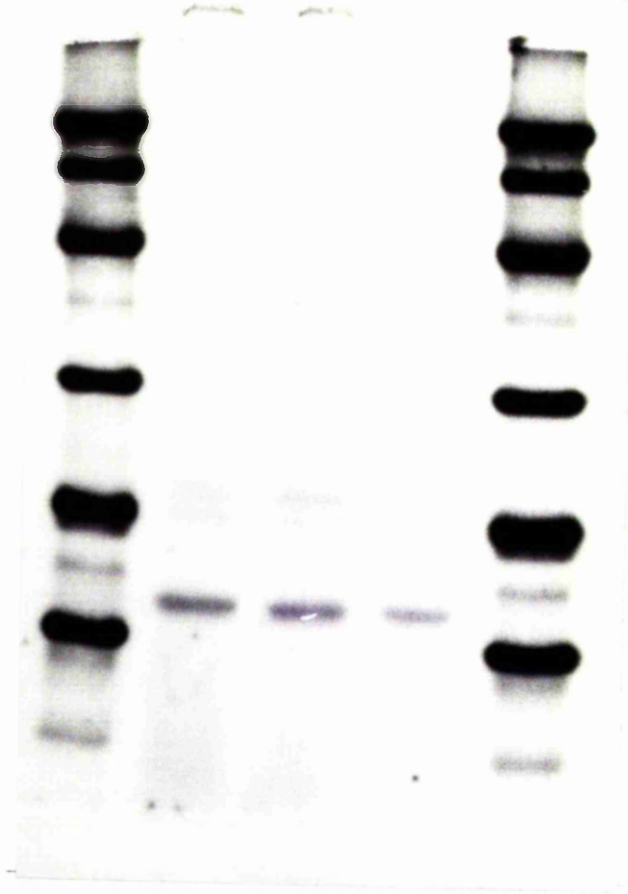
A) Lane 1 : Bio-Rad M.Wt marker proteins
Lane 2 : junction enriched pellet BRL cells
Lane 3 : junction enriched pellet V79 cells
Lane 4 : junction enriched pellet C13 cells
Lane 5 : Bio-Rad M.Wt. marker proteins

B) Lane 1 : Bio-Rad M.Wt. marker proteins
Lane 2 : junction enriched pellet A9 cells

C) Lane 1 : junction enriched pellet RTG_2 cells
Lane 2 : Bio-Rad M.Wt. marker proteins

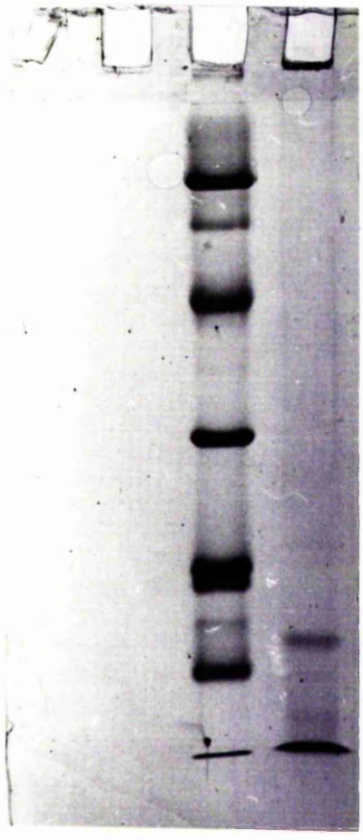
(This gel was stained using the modified silver stain (Oakley et al, 1980)).

A)



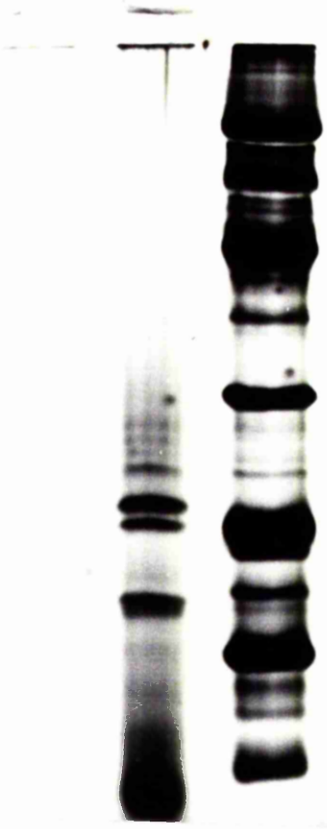
LANE 1 2 3 4 5

B)



LANE 1 2

c)



LANE 1 2

Figure 8 Heat Induced Aggregation of the 16K Protein Isolated From BRL Cells.

Gap junctions were isolated from 18 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

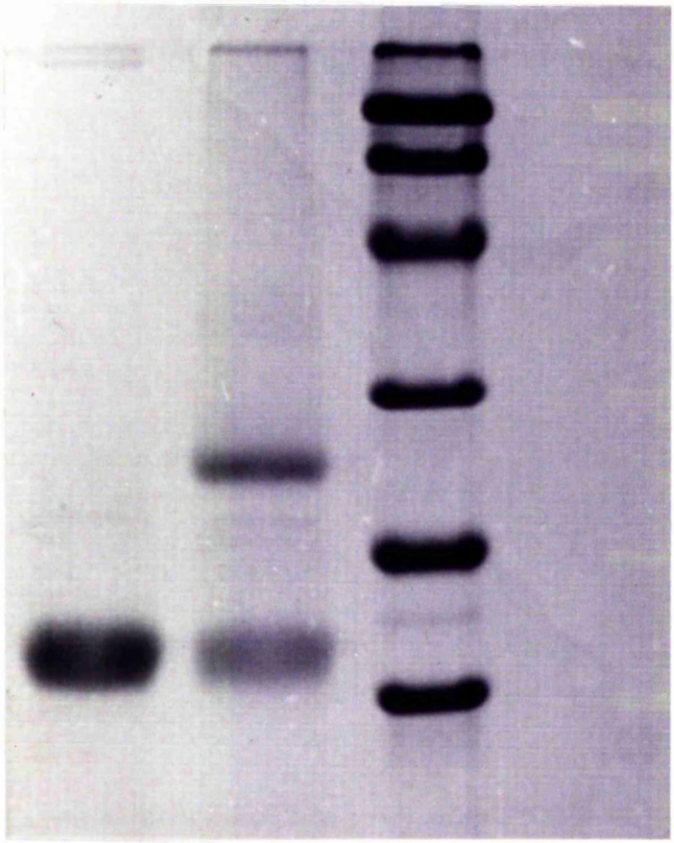
After dissolving the final junction enriched pellet in 40µl 1% SDS, the sample was split into two equal aliquots, 20µl were left at room temperature for 10min and the other 20µl heated to 100°C for 5min.

The samples were then analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : junction enriched pellet from BRL cells solubilised at room temperature.

Lane 2 : junction enriched pellet from BRL cells solubilised in SDS at 100°C.

Lane 3 : Bio-Rad M.Wt. marker proteins.



LANE 1 2 3

Figure 9 Quantitation of Isolation Protocol II.

Three $75cm^2$ flasks of sub-confluent BRL cells were labelled overnight with 200µCi [35_S] methionine in 30ml methionine free medium per flask. The labelled medium was removed and the cell layer washed twice with 25ml neutralised BSS at 0^oC to remove any unincorporated methionine. Gap junctions were extracted in 12ml 1% triton, 0.15m NaCl 5mM phosphate buffer (pH 7.4) per flask, and the triton extracts pooled. The pooled extract was then divided into three parts and equal aliquots added to triton extracts from three separate, but parallel, isolations of gap junctions from 4 confluent roller bottles of BRL cells (see section 2.2.3.). The remainder of the isolation protocol was as described in section 2.2.3.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Unlabelled gap junction protein was visualised by staining with Coomassie Brilliant Blue as described in section 2.2.4.

Lane 1 : Bio-Rad M.Wt. marker proteins

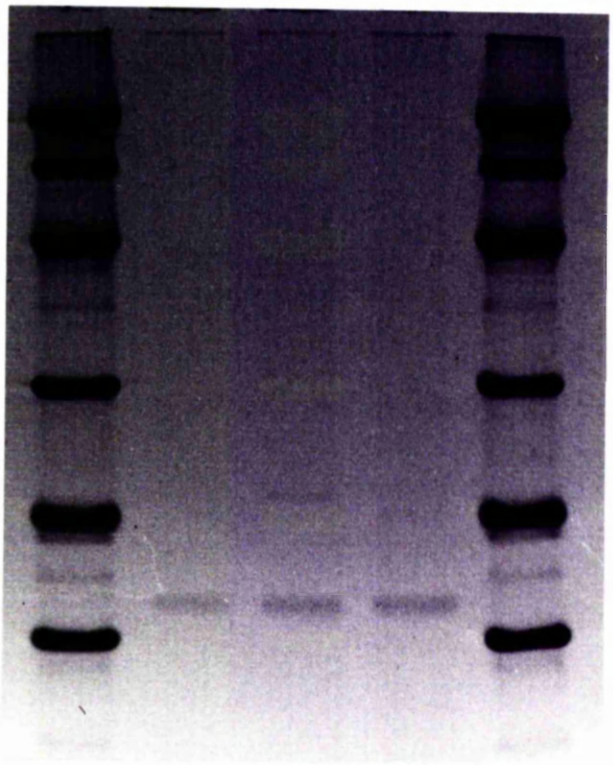
Lane 2 : junction enriched pellet from 4 roller bottles of BRL cells

Lane 3 : junction enriched pellet from 4 roller bottles of BRL cells

Lane 4 : junction enriched pellet from 4 roller bottles of BRL cells

Lane 5 : Bio-Rad M.Wt. marker proteins

Quantitation of Radioactivity - see Fig 10 and Table 7.



LANE 1 2 3 4 5

Figure 10 Recovery of Radioactivity - Microdensitometer Traces of Autoradiographs.

For experimental details see legend to Fig 9.

The wet Coomassie stained gel was placed on filter paper and dried under vacuum. The gel was then placed against Kodak Orthomat X-ray film and left at -70^{o}C for 49 days.

The resulting autoradiograph was scanned using a Joyce-Loebel microdensitometer (see section 2.2.5.).

Scan of autoradiographs of labelled material associated with the gap junction enriched pellet from

a) 4 bottles of BRL cells

b) 4 bottles of BRL cells

c) 4 bottles of BRL cells

a)

b)

c)

Table 1 Recovery of Radioactivity - Quantitation of Microdensitometer Traces of Autoradiographs.

For experimental details see legend to Fig 9

Gel scans were quantitated as described in section 2.2.5.

[^{35}S] Methionine Incorporated

Sample	Area under 16K peak	Variation from Mean
Junction prep from 4 bottles BRL cells	0.032	5%
Junction prep from 4 bottles BRL cells	0.034	0%
Junction prep from 4 bottles BRL cells	0.035	3%
	Mean Value = 0.034.	

Figure 11 The Effect of TPA on the Isolation of Junctional Protein from V79 Cells.

Gap junctions were isolated from 45 confluent roller bottles of V79 cells using Isolation Protocol II as described in section 2.2.3.

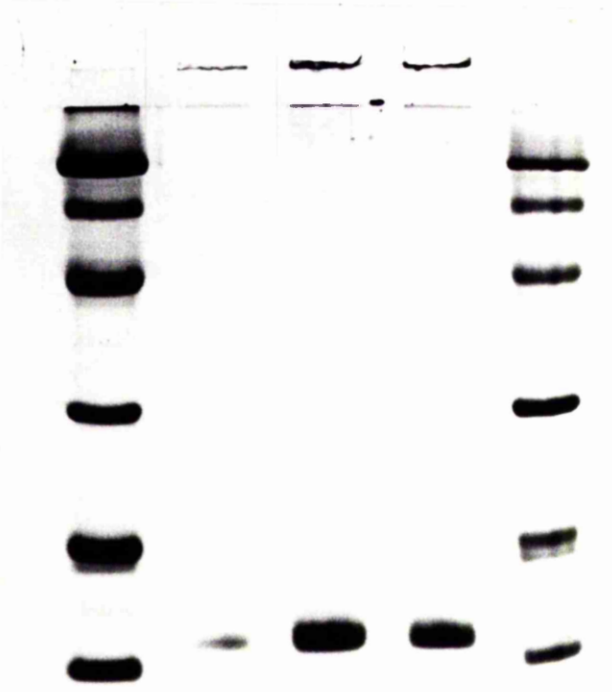
45h before junction isolation, the medium was removed from 15 roller bottles and replaced with 150ml EFC_{10} + 120µl 2 x 10^{-4}m TPA (final concentration 2 x 10^{-7}m). 18h later the medium + TPA was removed, and the cells washed four times with 100ml neutralised BSS at 37°C.150ml EFC_{10} was added and the cells incubated at 37°C for 27h before junction isolation.

The medium was removed from the remaining 30 roller bottles of V79 cells 18h before junction isolation and replaced with 150ml EFC_{10} (control) or with 150ml EFC_{10} + 120µl 2 x 10^{-4}M TPA (final concentration 2 x 10^{-7}M).

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio-Rad M.Wt. marker proteins
Lane 2 : junction enriched pellet V79 + 2 x 10^{-7}M TPA
Lane 3 : junction enriched pellet V79 control
Lane 4 : junction enriched pellet V79 TPA reversal
Lane 5 : Bio-Rad M.Wt. marker proteins.

For quantitation of protein see Fig 12 and Table 3.



LANE 1 2 3 4 5

Figure 12 Treatment of V79 Cells with TPA - Quantitation of Relative Amount of 16K Protein.

For experimental details see legend to Fig 11.

The relative amount of the 16K protein in control, TPA treated and TPA reversal V79 cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

a) control V79 cells
b) 2×10^{-7}M TPA reversal V79 cells
c) 2×10^{-7}M TPA treated V79 cells

For relative amounts see Table 2.

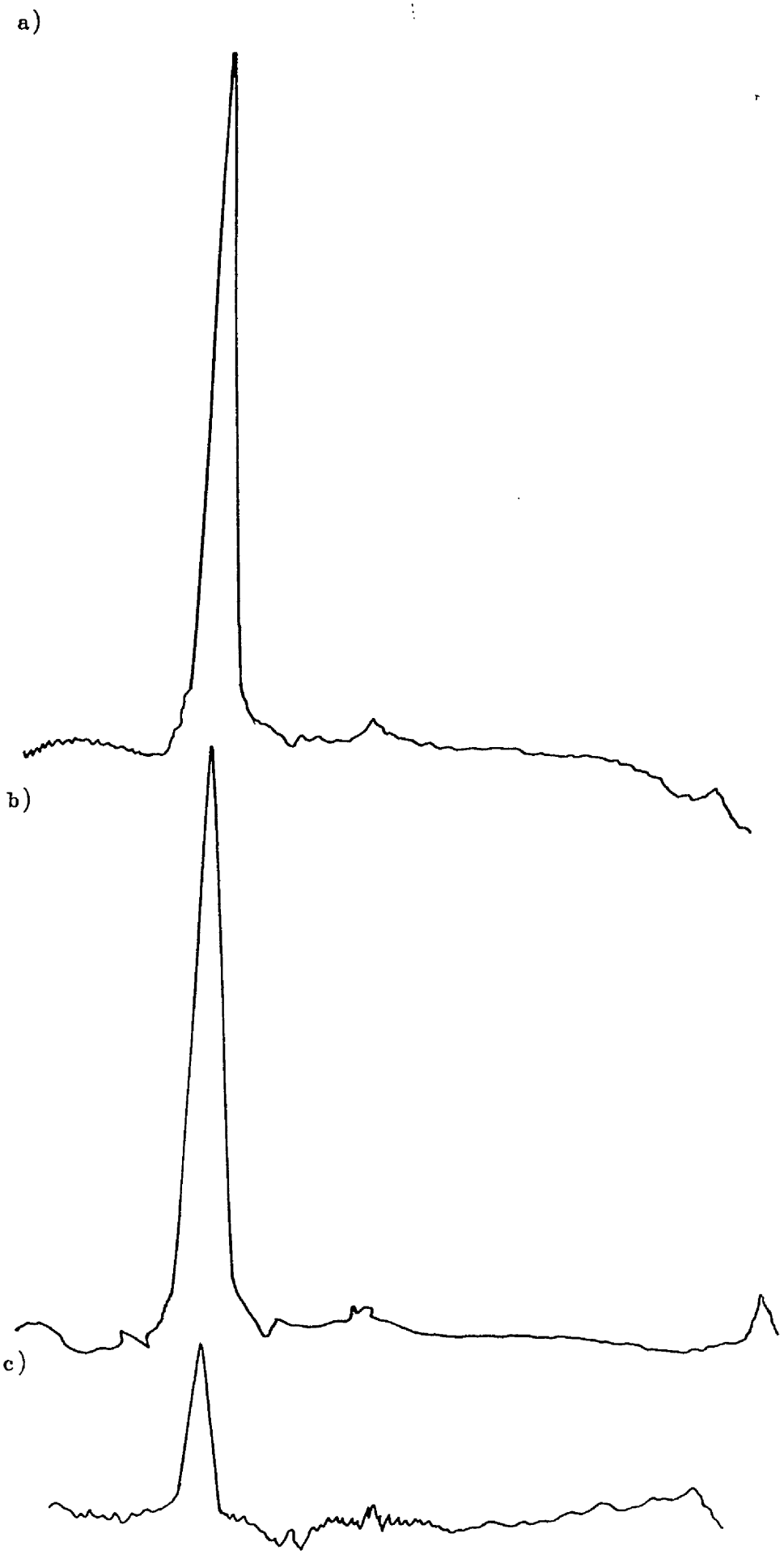

a)
b)
c)

Table 2 Treatment of V79 Cells with TPA - Quantitation of Relative Amounts of 16K Protein.

For experimental details see legend to Fig 11.

The relative amount of the 16K protein in control, TPA treated and TPA reversed V79 cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

Results are shown for 5 separate experiments.

*Quantitation of relative amount of 16K protein treated and isolated as described in Fig 11.

	Area under 16K Peak	% decrease
Control	0.0493	-
TPA	0.0066	87
$TPA_{reversal}$	0.0469	5
Control	0.0101	-
TPA	0.0025	76
$TPA_{reversal}$	0.0087	13
Control	0.0042	-
TPA	0.0017	60
Control	0.0057	-
TPA	0.0035	62
Control	0.0084	
TPA	0.0017	80

Table 3 Effect of TPA on Uridine Nucleotide Transfer Between V79 Cells.

V79 cells were treated with 2 x 10^{-7}M TPA for 18h, or with 2 x 10^{-7} TPA for 18h followed by a 27h reversal, before gap junction isolation (as described in legend to Fig 11).

The TPA containing medium, control medium and TPA reversal medium were removed and their effect tested on uridine nucleotide transfer between V79 cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml control medium, 2.0ml medium + 2 x 10^{-7}M TPA, or with 2.0ml medium from TPA reversed V79 cells.

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.).

	Recipients in Contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f	P
control	55.5 (6.9)	3.5 (2.3)	49.5	98	<0.001
2×10^{-7}M TPA	4.6 (2.6)	4.2 (2.2)	0.9	98	N.S.
TPA reversal	55.0 (6.8)	4.0 (2.1)	50.1	98	<0.001

Figure 13 Effect of TPA on the Isolation of the 16K Protein From BRL Cells.

Gap junctions were isolated from 15 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation the medium was removed from 5 roller bottles and replaced with 150ml EFC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans retinoic acid to give a final concentration of 10^{-4}M trans retinoic acid.

The medium was removed from the remaining 10 bottles 18h before junction isolation and replaced with 150ml EFC_{10} (control) or with 150ml EFC_{10} + 120µl 2 x 10^{-4}M TPA to give a final concentration 2 x 10^{-7}M.

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio-Rad marker proteins

Lane 2 : RNase and cyt C markers

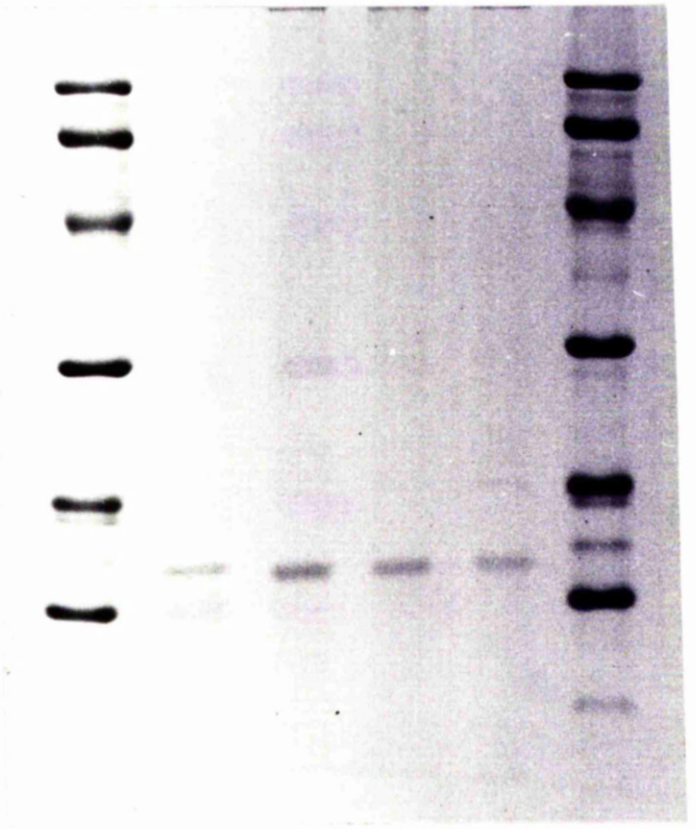
Lane 3 : junction enriched pellet BRL cells + 10^{-4}M trans RA

Lane 4 : junction enriched pellet BRL cells control

Lane 5 : junction enriched pellet BRL cells + 2 x 10^{-7}M TPA

Lane 6 : Bio-Rad M.Wt. marker proteins.

For quantitation of protein see Fig 14 and Table 4.



LANE 1 2 3 4 5 6

Figure 14 Treatment of BRL Cells with TPA - Quantitation of Relative Amount of 16K Protein.

For experimental details see legend to Fig 13. The relative amount of the 16K protein in control, TPA treated and trans RA treated BRL cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

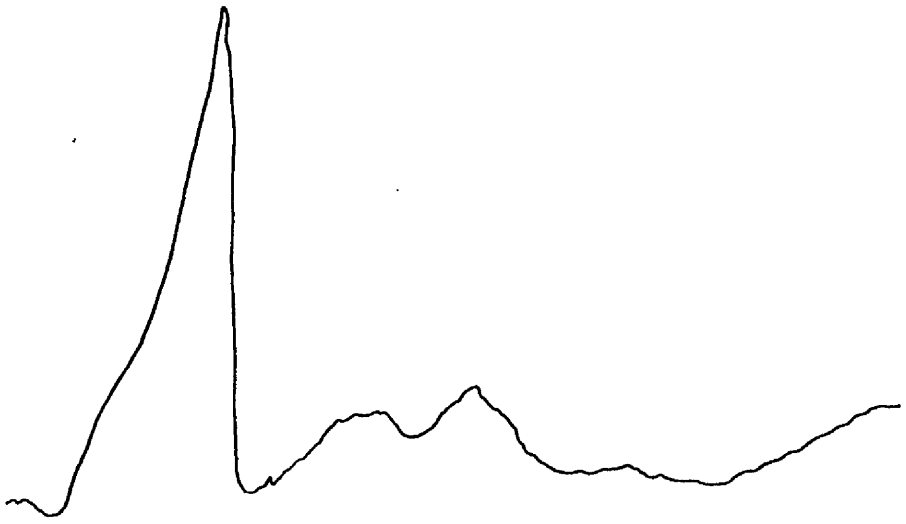
a) BRL cells + 10^{-4}M trans retinoic acid

b) BRL cells + 2 x 10^{-7}M TPA

c) control BRL cells.

For relative amounts of protein see Table 4.

a)



b)

c)

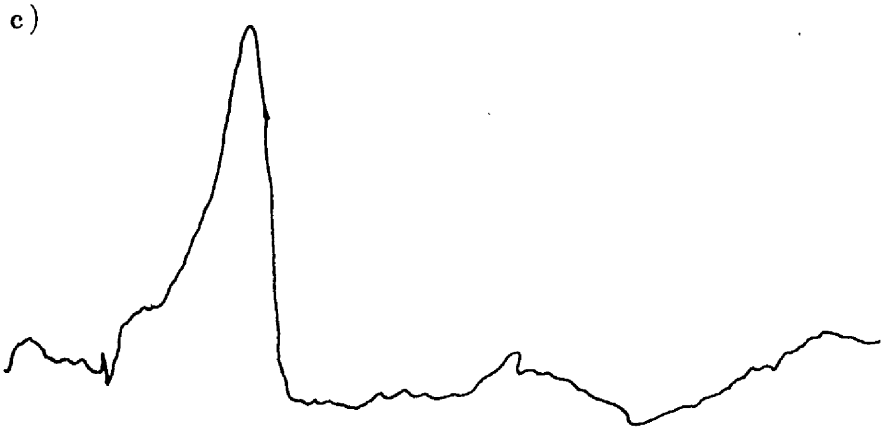


Table 4 Treatment of BRL cells with TPA - Quantitation of Relative Amount of 16K Protein.

For experimental details see legend to Fig 13. The relative amount of the 16K protein in control, TPA treated and trans retinoic acid treated BRL cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

	Area Under 16K Peak	% difference with respect to control
control	0.040	-
2×10^{-7}M TPA	0.036	-10
10^{-4}M RA	0.048	+20

Table 5 Effect of TPA and Trans Retinoic Acid on Uridine Nucleotide Transfer Between BRL Cells.

BRL cells were treated with 10^{-4}M trans retinoic acid for 24h, or with 2 x 10^{-7}M TPA for 18h before gap junction isolation (as described in legend to Fig 13).

The TPA containing medium, control medium and trans retinoic acid containing medium were removed and their effect tested on uridine nucleotide transfer between BRL cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml control medium, 2.0ml medium + 2 x 10^{-7}M TPA, or with 2.0ml medium + 10^{-4}M trans retinoic acid.

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.).

	Recipients in contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f.	P
control	36.7(5.1)	4.5(1.6)	42.0	98	<0.001
10^{-4}M trans retinoic acid	4.9(1.3)	4.3(1.8)	1.9	98	N.S.
2 x 10^{-7}M TPA	39.4(6.3)	5.5(1.3)	32.7	98	<0.001

Figure 15 Thermolytic Map of Iodinated 16K Protein Isolated From BRL Cells.

Gap junctions were isolated from 18 confluent roller bottles of BRL cells using the method of Finbow et al (1982) (Isolation Protocol I : see section 2.2.2.).

The final junction enriched pellet was iodinated (see section 2.2.15) and analysed by SDS polyacrylamide gel electrophoresis. The labelled 16K protein was eluted from the gel as described in section 2.2.16(i). The eluted protein was recovered by acetone precipitation (see section 2.2.16(ii)) and alkylated (see section 2.2.16(iii)) prior to thermolytic digestion (see section 2.2.16(iv)).

Thermolysin (200µg/ml in 1mM HC1) was added (30µl) to give a ratio of thermolysin : protein 1µg:100µg, and the mixture incubated at 55^{o}C for 2h. A further 30µl thermolysin (200µg/ml in 1mM HC1) was added and the mixture again incubated at 55^{o}C for 2h. The digested protein was freeze dried and the peptide products mapped (see section 2.2.16(v)).

Peptide mapping was carried out at pH 3.5 and pH 6.5 (see section 2.2.16(v)). The digested peptide fragments of the labelled 16K protein were identified by autoradiography using Kodak X-omat S X-ray film and exposing for 1 week with an intensifying screen.

a) pH 3.5

b) pH 6.5

a)

b)

Figure 16 Thermolytic Map of Glyceraldehyde -3- Phosphate Dehydrogenase.

Glyceraldehyde -3- phosphate dehydrogenase (540µg) was alkylated (see section 2.2.16(iii)) prior to thermolytic digestion (see section 2.2.16(iv)).

Thermolysin (200µg/ml in 1mM HC1) was added (30µl) to give a ratio of thermolysin : protein 1µg : 100µg, and the mixture incubated at 55°C for 2h. A further 30µl thermolysin (200µg/ml in 1mM HC1) was added and the mixture again incubated at 55°C for 2h. The digested protein was freeze-dried and the peptide products mapped (see section 2.2.16(v)).

Peptide mapping was carried out at pH 3.5 and pH 6.5 (see section 2.2.16(v)). The digested peptide fragments were visualised by spraying the TLC plate with 0.25% ninhydrin (w/v) in acetone, and leaving at room temperature for 8-16h.

a) pH 3.5

b) pH 6.5

a)

b)

3.2.3. Conclusions.

A new method has been developed for the isolation of gap junctions from cultured cells using a relatively small number ($\sim 10^9$) of cells. SDS polyacrylamide gel electrophoresis of junction enriched fractions isolated from cultured cells using the new method, reveals the presence of one major protein M_r 16,000 (16K). This 16K protein isolated from cultured cells is the same as the 16K protein isolated from mouse liver as determined by mobility on SDS gels, and aggregation in SDS buffers at 100°C. Further evidence is presented to support the belief that this 16K protein is of junctional origin. Experiments show that the yield of the 16K junctional protein in separate isolations is reproducible.

Chapter 4 - The Effect of Retinoic Acid on Junctional Communication Between Cells in Culture.

4.1. INTRODUCTION.

Vitamin A derived retinoids such as retinoic acid play an important role in the control of growth and differentiation in cells and tissues of higher organisms. Retinoids induce changes in cell surface glycoproteins (Borek et al, 1973; Brandes et al, 1974; Hogan-Ryan and Fennelly, 1978; Jetten et al, 1979; a, b) and can effect glycosaminoglycan and proteoglycan production (Dahl and Axelsson, 1980). They are also involved in maintaining and inducing differentiated functions of epithelial tissues (De Luca et al, 1972). Retinoids show anti-carcinogenic activity (Elias et al, 1980; 1981), and have recently been shown to be capable of altering pattern formation in a regenerating system (Maden, 1982) and in a developmental system (Tickle et al, 1982).

Retinoic acid has been found to affect junctional communication between cells in culture (Pitts et al, 1981) but as yet the relationship between this and the other effects of retinoic acid is unknown. If retinoic acid is inhibiting junctional communication by acting specifically on junction formation or on junctional permeability as opposed to causing cellular changes which result indirectly in changes in junctional communication, then it may prove to be a valuable tool for elucidating the function(s) of gap junctions in culture and in vivo.

4.2. THE EFFECT OF RETINOIC ACID ON JUNCTIONAL COMMUNICATION BETWEEN CELLS IN CULTURE.

The earlier observations of Pitts et al, (1981) have been confirmed and extended. The effect of retinoic acid on junctional communication between BRL, C13, RTG_2 Xen and V79 cells was examined using uridine nucleotide transfer. When cells (donor cells) are pre-labelled with [5-^{3}H]uridine for 3h, then washed and cocultured with unlabelled cells (recipient cells), recipient cells in contact with donors are labelled only if the two cell types form junctions. Treatment of BRL and C13 cells with 10^{-4}M trans retinoic acid during coculture of donor and recipient cells gives a unimodal distribution of grain counts over all recipient cells, with the level of grains over recipients in contact with donor cells being indistinguishable from those over cells not in contact with donors, i.e. being reduced to background levels (Table 6). A similar effect has also been seen by others using 3T3 cells and TG_2 cells (Hamilton et al, 1982). The mean grain count over Xenopus and RTG_2 recipients in contact with donors is also reduced in the presence of 10^{-4}M trans retinoic acid, but not all recipients in contact with donors have background grain counts (Table 7). This can be seen more clearly by presenting the grain counts over recipient cells as histograms (Fig 17). Transfer of [^{3}H]-uridine nucleotides between primary and secondary Xenopus and RTG_2 recipient cells is inhibited in the presence of 10^{-4}M retinoic acid in the same way as transfer between donors and primary recipient cells (Table 8). This will be discussed later in this section. It would appear therefore that amphibian (Xen) and fish (RTG_2)

cells are less sensitive to retinoic acid under these conditions.

The background level of grain counts over the recipient cells in contact with donor cells could be due to retinoic acid causing (i) a general toxic effect, inhibiting the metabolic activities of cells, (ii) inhibition of RNA synthesis, preventing incorporation of [^{3}H]-uridine nucleotides into recipient cell RNA, (iii) inhibition of uptake of [^{3}H]-uridine or of the conversion of [^{3}H]-uridine to [^{3}H]-uridine nucleotides or (iv) inhibition of transfer of [^{3}H]-uridine nucleotides from donor cells to recipient cells.

The concentration of retinoic acid used to inhibit uridine nucleotide transfer is high and could be toxic. Cell growth is a good general measure for toxicity so the effect of 10^{-4}M retinoic acid on the growth of those cell types where retinoic acid appears to inhibit uridine nucleotide transfer was examined. BRL, C13, 3T3, V79 and RTG_2 cells were plated in control medium and in medium containing retinoic acid and allowed to spread for 2h. The cell number was then estimated over 24h intervals (Figs 18-22). The fold increase in cell number after 72h was the same in control and retinoic acid treated BRL (Fig 18), V79 (Fig 19) and RTG_2 (Fig 20)cells. There was a reduction of 5% in the fold increase in retinoic acid treated C13 cells after 72h (Fig 22) and 16% in 3T3 cells exposed to retinoic acid for 72h (Fig 21). In all cases the cultures continued to grow and the cells remained attached to the surface of the petri dish without any detectable changes in morphology. The morphology of donor

cells which attached and spread in the presence of 10^{-4}M retinoic acid in the previous experiments was also indistinguishable in appearance from donors plated in the absence of retinoic acid (see Fig 27).

To further distinguish between the possible explanations, the effect of retinoic acid on RNA and DNA synthesis was studied by measuring the incorporation of [5-^{3}H] uridine, [methyl-^{3}H]-thymidine and [G-^{3}H] hypoxanthine into both acid soluble (nucleotide) and acid insoluble (nucleic acid) fractions. The medium was removed from V79, BRL, C13, 3T3 and RTG_2 cells which had been grown overnight, and replaced with medium containing 10^{-4}M retinoic acid, control medium or medium + DMSO. The cells were either pulsed for 1h with the labelled precursor immediately, 3h or 24h later, and the incorporation into acid soluble and acid insoluble fractions measured (Tables 9,10,11).

Trans retinoic acid (10^{-4}M) reduced incorporation of the labelled precursors into both acid soluble and acid insoluble fractions. The extent of inhibition increases with length of exposure to 10^{-4}M trans retinoic acid, and after 24h, in some instances it is as high as 70% (incorporation of [G-^{3}H] hypoxanthine and [methyl -^{3}H]-thymidine into acid insoluble fractions in V79 cells). Results from replicate dishes in this experiment varied by less than 10%. There were however larger variations from experiment to experiment, ranging from 20-30%. This may be due to cells being more sensitive to retinoic acid at different stages of growth.

In the experiments involving uridine nucleotide transfer,

cells are exposed to retinoic acid for 3h only, after which time all the cell types are affected less. Inhibition of [5-^{3}H]uridine into acid insoluble pools after 3h exposure to retinoic acid ranges between 9% (BRL cells) and 47% (V79 cells). In all cases the inhibition of incorporation of [5-^{3}H]uridine into acid insoluble fractions is insufficient to explain the large decrease in grains over recipient cells in contact with donor cells which is observed in uridine nucleotide transfer experiments. This ranges from V79-99.5%, C13-99.2%, BRL-98% to RTG_2-70.5%. Likewise, the reduction of incorporation of labelled precursors into acid soluble fractions in the pre-labelled donors is not high enough to account for the reduction in grain counts over the recipient cells in contact with donor cells. This reduction is most likely therefore due to, at least in a large part, inhibition of nucleotide transfer.

These results showing the effect of retinoic acid on the incorporation of [methyl - ^{3}H]-thymidine into acid insoluble fractions where inhibition observed after 24h treatment was between 50-70% appear to contradict the earlier studies on cell growth. In the cell growth experiments, cells were plated in the presence of 10^{-4}M retinoic acid whereas in the experiments on incorporation of precursors, junctions have been established prior to the addition of retinoic acid. It is conceivable that a sudden transition from the coupled to uncoupled state leads to a transient disturbance of DNA synthesis (at ~24h) but from which the cells recover.

Retinoic acid (10^{-4}M) has however, been reported to decrease the growth rate of C13 cells by 0-34% (Murphy,

personal communication). This variability, like that observed between the different (but apparently similar) incorporation experiments mentioned earlier, suggests either that small differences in concentration of retinoic acid in this concentration range, or that undefined differences in cellular physiology or growth conditions make large differences in the observed effects. These variations contrast with the very consistent effects of 10^{-4}M retinoic acid in uridine nucleotide transfer.

The conclusion that retinoic acid has a prime effect on the transfer process and not on the incorporation of transferred nucleotides is supported by two further observations: (i) the pattern of labelling in donor cells in uridine nucleotide transfer experiments shows the usual change in the donors from predominantly nucleolar and nuclear labelling to cytoplasmic labelling (i.e. RNA turnover and the synthesis of stable cytoplasmic forms is continuing as normal) and

(ii) in experiments described by Hamilton et al, (1982) and below (Table 13) wild type C13 cells in mixed cultures with HGPRT$^-$ TG_2 cells continue to incorporate [G-^{3}H]-hypoxanthine after the addition of 10^{-4}M retinoic acid while incorporation into the mutant cells is completly inhibited (through an effect on junctional communication).

Further experiments designed to understand the variations in incorporation of nucleic acid precursors and growth of cells in retinoic acid were not pursued.

These results therefore suggest that retinoic acid inhibits the transfer of [^{3}H]-uridine nucleotides from donor cells to recipient cells. This inhibition of cell

communication could be due to an effect on 1) junctional formation, 2) junction breakdown, 3) junctional permeability, or 4) to retinoic acid acting as a detergent and causing removal of active junctional plaques from the cell surface.

The results in Table 12 show that inhibition of junctional communication between cells by retinoic acid is rapid i.e. within minutes. By comparing the grain counts above the background level in control and retinoic acid treated cells in overexposed autoradiographs, it can be calculated that there is a 98% reduction in the grain count.

$$\frac{\text{Recipient cells in contact + Retinoic Acid (360h exposure)} - \text{Isolated Recipient Cells + Retinoic Acid (360h exposure)}}{\text{Recipient cells in contact control (360h exposure)} - \text{Isolated Recipient Cells Control (360h exposure)}} \times 100$$

$$= \frac{15.5 - 12.3}{174.0 - 18.2} \times 100 = 2\%$$

Therefore in the retinoic acid treated cells, either transfer continues at the normal rate for less than 2% of the time or falls immediately to 2% of the rate which then remains constant for the full 3h. The estimates are maximum values as no significant incorporation above background can be detected even after prolonged autoradiographic exposure. It is most likely that the rate of transfer decreases (exponentially) during the first few minutes (or less) of the 3h coculture period to some undetectable (perhaps zero) level.

Rapid inhibition of nucleotide transfer has also been

reported by Pitts et al, (1981), who found that retinoic acid inhibited transfer of [G-^{3}H] hypoxanthine between already coupled C13 and TG_2 cells within minutes.

To confirm that retinoic acid affects established junctions, and to obtain an estimate for the rate of reversal of inhibition of junctional communication, C13 and TG_2 cells were grown overnight to allow the formation of junctions. The cells were then treated with 10^{-4}M retinoic acid for 1h and the cells pulsed with [G-^{3}H] hypoxanthine for the last 15min (45-60 min) after addition of retinoic acid. The retinoic acid was removed and the cells fixed and prepared for autoradiography either immediately or at various times afterwards. Even 5min after the removal of retinoic acid, transfer of labelled material from C13 cells to TG_2 cells could be detected (Table 13 and Fig 23). It appears that communication is restored very rapidly after removal of retinoic acid since it would take a measurable time for transfer and incorporation of labelled material from C13 cells to TG_2 cells.

This result also shows (c.f. Figs 18-22) that retinoic acid (10^{-4}M) does not have a lasting toxic effect on cells since the inhibition is fully and rapidly reversible.

The results which show that inhibition and reversal of junctional communication by retinoic acid occur rapidly suggest indirectly that retinoic acid is affecting junctional permeability. For these results to be explained by an effect on formation and turnover of gap junctions, the turnover time must be fast leading to rapid breakdown. Estimates for the half-life of the junctional protein however vary from 5.5h (Fallon and Goodenough, 1981) to 19h

(Yancey et al, 1981) (see section 1.3.3.), and it seems unlikely that this could account for the rate of inhibition of intercellular communication which is observed.

Preliminary experiments showed that concentrations of 10^{-4}M retinoic acid fully inhibit uridine nucleotide transfer between BRL cells and between C13 cells. This concentration of retinoic acid is high and the effect of lower (more physiological) concentrations on uridine nucleotide transfer between homologous cultures of BRL, C13 and V79 cells was examined to determine if there was any intermediate effect. BRL and C13 cells are known to form stable communication in homologous culture and it is possible that these cells form junctions in excess of functional requirements and that retinoic acid may have an effect at lower concentrations which is not seen due to the excess of gap junctions. V79 cells, on the other hand, are very mobile cells which make and break junctions easily and it may therefore be possible to detect effects of lower concentrations of retinoic acid.

Medium was removed from recipient V79, BRL and C13 cells, prior to the addition of donor cells prelabelled with [5-^{3}H]-uridine, and replaced with control medium or medium containing various concentrations of retinoic acid (10^{-4}M - 10^{-7}M). Cells were cocultured for 3h, fixed and prepared for autoradiography and the effect of retinoic acid on uridine nucleotide transfer examined by grain counting. Inhibition of uridine nucleotide transfer between BRL cells and between C13 cells was seen only with 10^{-4}M retinoic acid (Table 14). V79 cells appeared to be more sensitive with an intermediate effect being seen with 10^{-5}M retinoic acid. This was examined in more detail in later experiments.

The effect of concentrations of retinoic acid between 10^{-4}M and 10^{-5}M on uridine nucleotide transfer between BRL and between C13 cells was investigated to determine whether there was a gradual loss of communication between 10^{-4}M-10^{-5}M or whether 10^{-4}M was the only concentration at which inhibition was observed. Tables 15, 16 and Fig 24 show that as the concentration of retinoic acid rises over this concentration range, all the recipient cells show fewer grains i.e. all cell junctions have decreased permeability. This could be due to 1) increasing retinoic acid concentrations decreasing the number of fully open channels or 2) a retinoic acid induced decrease in effective diameter in all channels or 3) to a decrease in the number of channels present due to removal of junctional plaques from the cell surface. It would be possible to distinguish between 1) and 2) using probes of different molecular weight such as those used by Simpson et al, (1977). If 1) was correct, then all the molecules would pass through the channels but at a slower rate, while if 2) was correct there would be a reduced cut off point where only probes below a certain size would pass through. The third possibility will be discussed in section 5.2. which examines the effect of retinoic acid on junctional plaques.

Since the uridine nucleotide transfer assay is dependent on junction formation as well as junctional permeability it could be argued that retinoic acid affects formation and not permeability. It is however possible to distinguish the two effects within the same assay by comparing transfer from donors to primary recipient cells with transfer from primary recipient cells to secondary

recipient cells. A primary recipient cell is a cell which is in direct contact with the pre-labelled donor cells while a secondary recipient is a cell which is in direct contact with a primary recipient but not with donor cells. Since recipient cells are plated out at the beginning of the experiment, 21h before the addition of retinoic acid, junctions between primary and secondary recipient cells are already established before donors are added. Examination of primary to secondary recipient transfer at concentrations of retinoic acid between 10^{-4}M and 10^{-5}M (Fig 25) shows a similar pattern of inhibition compared to that between donors and primary recipients. This supports the earlier conclusion that the mechanism of trans retinoic acid inhibition of junctional communication is via an effect on junctional permeability. As mentioned previously, the effect of retinoic acid (10^{-4}M) on uridine nucleotide transfer between primary and secondary recipient cells in Xenopus and RTG_2 cultures also shows the same pattern of inhibition as that observed between donor cells and primary recipients (i.e. some transfer fully inhibited and some unaffected) which again suggests that retinoic acid is affecting junctional permeability as opposed to formation.

V79 cells were found earlier to be more sensitive to retinoic acid i.e. junctional communication appeared to be inhibited at lower concentrations of retinoic acid. Because intermediate effects could be seen making this system more sensitive than BRL and C13 cell systems where an all or nothing response to retinoic acid is seen (i.e. either all cells are unaffected or all are inhibited), this was taken as an opportunity to compare the effects of 13 cis and trans

retinoic acid on junctional communication. Their effect on uridine nucleotide transfer between homologous V79 and C13 cells cocultured for different times was examined.

Donor cells were prelabelled with [5-^{3}H]-uridine and cocultured with unlabelled recipient cells for 30min, 60min and 180min in the presence and absence of 10^{-7}M - 10^{-4}M cis and trans retinoic acid. Cells were fixed and prepared for autoradiography and grains counted over recipient cells. There was complete inhibition of nucleotide transfer between C13 cells with only 10^{-4}M cis or trans retinoic acid. The inhibition was complete at all times (Table 17). An effect on nucleotide transfer between V79 cells on the other hand, was seen at concentrations as low as 10^{-6}M cis or trans retinoic acid after 30min coculture (Table 18). As the length of coculture increased, the inhibitory effect of retinoic acid on uridine nucleotide transfer decreased. At 60min and 180min there was inhibition with only 10^{-4}M, 10^{-5}M trans retinoic acid and 10^{-4}M cis retinoic acid. The effect of cis and trans retinoic acid on uridine nucleotide transfer between V79 cells and C13 cells can be seen by plotting the grain counts over recipient cells as histograms (Fig 26). There was a unimodal distribution of grain counts over C13 primary recipient cells with either all cells having a background level of grains (in presence of 10^{-4}M trans or cis retinoic acid) or having grain counts indistinguishable from those in control cultures i.e. all heavily labelled. As the concentration of retinoic acid increased, the number of V79 primary recipient cells with background levels of grains increased, while there were also cells which

communicated at control levels. This suggests that in the V79 cell system retinoic acid may inhibit communication by inhibiting junction formation. This may be achieved by inhibiting cell movement such that either cell contact or the process of formation of gap junctions occurred with reduced frequency. It therefore appears that some cell types are more sensitive to the effects of retinoic acid than others.

Another cell type which has recently been shown (Scott and Pitts, unpublished observations) to have junction communication characteristics similar to V79 cells is the cell line GCCM which was derived from a human glioma. Earlier observations have shown that after a 1h coculture, GCCM cells show a very low level of communication with less than 5% of the recipient cells in contact with donors being labelled (Scott and Pitts, unpublished observations). By increasing the coculture period to 3h it is possible to increase the number of communicating cells to 50% of the cells in contact with donor cells (Morgan, personal communication). Heterologous cultures of GCCM donor cells and NORT (normal human glial cell line) recipient cells show the more usual higher levels of communication with all recipient cells in contact with donors being labelled (i.e. the same level of communication as between BRL cells and C13 cells, Scott and Pitts, unpublished observations).

By using the above combinations of cells (GCCM->GCCM and GCCM->NORT) and the two coculture times (1h and 3h), it was possible to look at the effect of retinoic acid on communication between three different cell systems which showed various levels of communication and to see if

retinoic acid inhibited at low concentrations only those cells which showed low levels of communication in untreated cultures.

Prior to the addition of pre-labelled donor cells, the medium was removed from recipient cells and replaced with medium containing concentrations of cis and trans retinoic acid at concentrations from 10^{-7}M to 10^{-4}M. Donor and recipient cells were cocultured for 1h (GCCM->GCCM) and 3h (GCCM->GCCM, and GCCM->NORT). The GCCM->GCCM 3h coculture was fully inhibited by concentrations down to 10^{-6}M trans retinoic acid and by 10^{-4}M cis retinoic acid, but only partially inhibited by 10^{-6}M - 10^{-5}M cis retinoic acid (Table 19). Inhibition of communication between GCCM and NORT cells was seen only with 10^{-4}M cis and trans retinoic acid (Table 19) (c.f. BRL and C13 cells). Communication between GCCM cells after a 1h coculture is low and this was unaffected by any concentration of retinoic acid (cis or trans) (Table 19). The inhibition of communication between GCCM cells in a 3h coculture is similar to that observed between V79 cells. This again suggests that cells with intermediate levels of communication may be more sensitive to cis and trans retinoic acid at lower concentrations. To see if this generalisation would hold in other systems, junctional communication between different cell types was tested in an attempt to find another system comparable to V79 and GCCM cells. Other systems in which below average levels of junctional communication between donor and recipient cells occurs, are in heterologous cultures of BRL, C13, and CK cells (Pitts and Bürk, 1977; Hunter and Pitts, 1981). The data in Table 20 shows that BRL, C13 and CK cells

communicate only in homologous cell combinations, and no detectable communication is observed in heterologous cell combinations (A9 cells do not form functionally detectable junctions and were included as a negative control). These systems are therefore suitable for examination but time did not permit an investigation of the effect of different concentrations of cis and trans retinoic acid on uridine nucleotide transfer between these cells

Since a high concentration (10^{-4}M) of the commercially available retinoic acid is required to cause complete inhibition of junctional communication between all cell types tested, it is possible that the trans retinoic acid may be contaminated with another substance (such as a structural isomer) which is more potent (i.e. effective at lower concentrations). Trans retinoic acid was chromatographed in two systems (Table 21) which separated it from two other retinoids, 13 cis retinoic acid and the aromatic retinoid,tigason (see section 2.1.10. for formulae). The eluted trans retinoic acid was added to recipient cells prior to the addition of labelled donor cells, and the effect on uridine nucleotide transfer analysed as described previously. The activity (i.e. ability to inhibit uridine nucleotide transfer) was found to run with the trans retinoic acid (Table 22 and Fig 27) showing that trans retinoic acid is most likely to be the compound which must be added to cells in culture to cause inhibition of junctional communication. It is also possible however that trans retinoic acid could be converted in cells to an active derivative, and it may be that cells like V79 cells are able to make more of this derivative and so junctional

communication between these cells is inhibited at lower concentrations of retinoic acid.

Table 6 The Effect of Retinoic Acid on Uridine Nucleotide Transfer between BRL cells and between C13 cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium (control), 2.0ml medium + 10µl DMSO (DMSO) or with 2.0ml medium + 10µl DMSO containing 2×10^{-2}M trans retinoic acid (final concentration in medium 10^{-4}M).

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.). The procedure used to analyse the data is described in section 2.2.10.

	Donor	Recipient	Recipients in contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f.	P.
control	BRL	BRL	36.4(3.9)	4.3(1.9)	51.8	98	<0.001
	C13	C13	42.5(3.9)	4.0(2.3)	58.5	98	<0.001
DMSO	BRL	BRL	36.1(5.4)	3.5(1.7)	72.9	98	<0.001
	C13	C13	43.5(3.1)	4.0(1.9)	76.5	98	<0.001
10^{-4}M Retinoic Acid	BRL	BRL	2.1(1.7)	1.6(1.4)	1.52	98	N.S.
	C13	C13	3.7(1.3)	3.4(1.5)	0.96	98	N.S.

Table 7 The Effect of 10^{-4}M Retinoic Acid on Uridine Nucleotide Transfer Between RTG_2 cells and between Xenopus Cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing 2 x 10^{-4}M trans retinoic acid.

After 3h co-culture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.). The procedure used to analyse the data is described in section 2.2.10.

In these experiments, Xenopus cells were cultured at 28°C and RTG_2 cells at 18°C.

	Donor	Recipient	Recipients in contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f.	P
control	RTG_2	RTG_2	54.2(17.9)	4.8(3.8)	19.2	98	<0.001
	Xen	Xen	56.3(22.9)	2.2(2.0)	16.5	98	<0.001
10^{-4}M trans retinoic acid	RTG_2	RTG_2	15.9(7.3)	1.3(1.2)	13.7	98	<0.001
	Xen	Xen	24.2(13.9)	2.4(1.6)	10.9	98	<0.001

Figure 17 Distribution of Grain Counts Over Recipient Cells in Contact with Donor Cells.

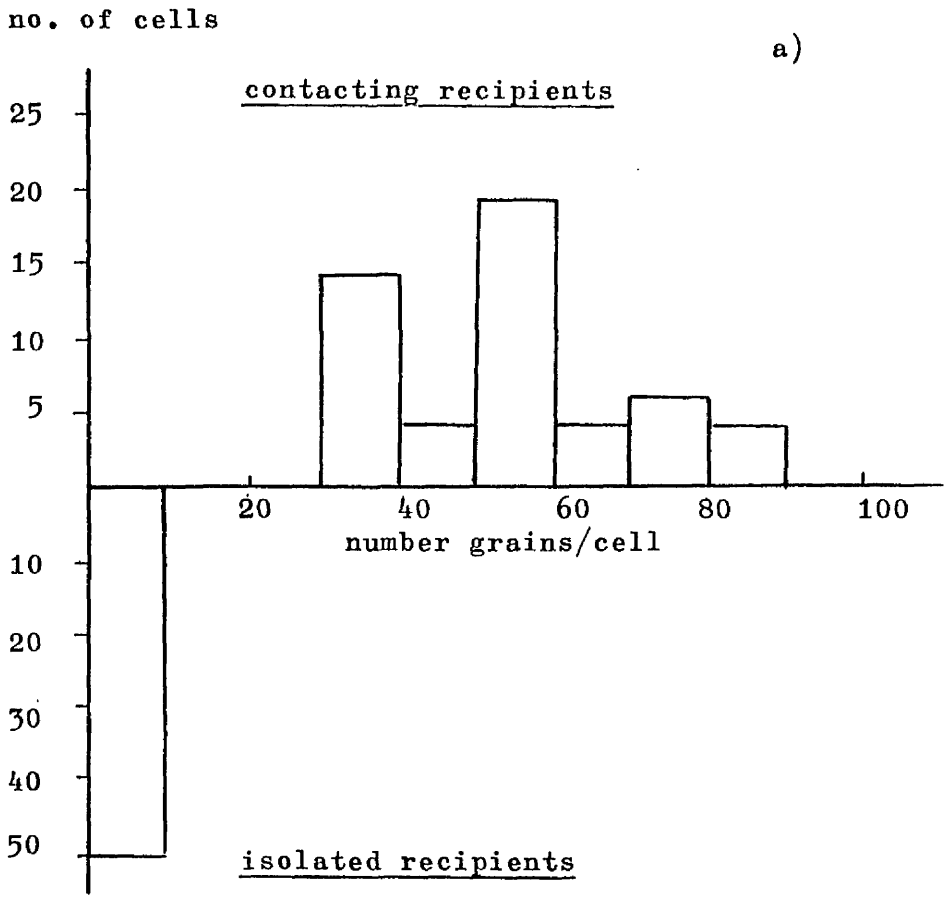
For experimental details see section 2.2.8. and legend to Table 7.

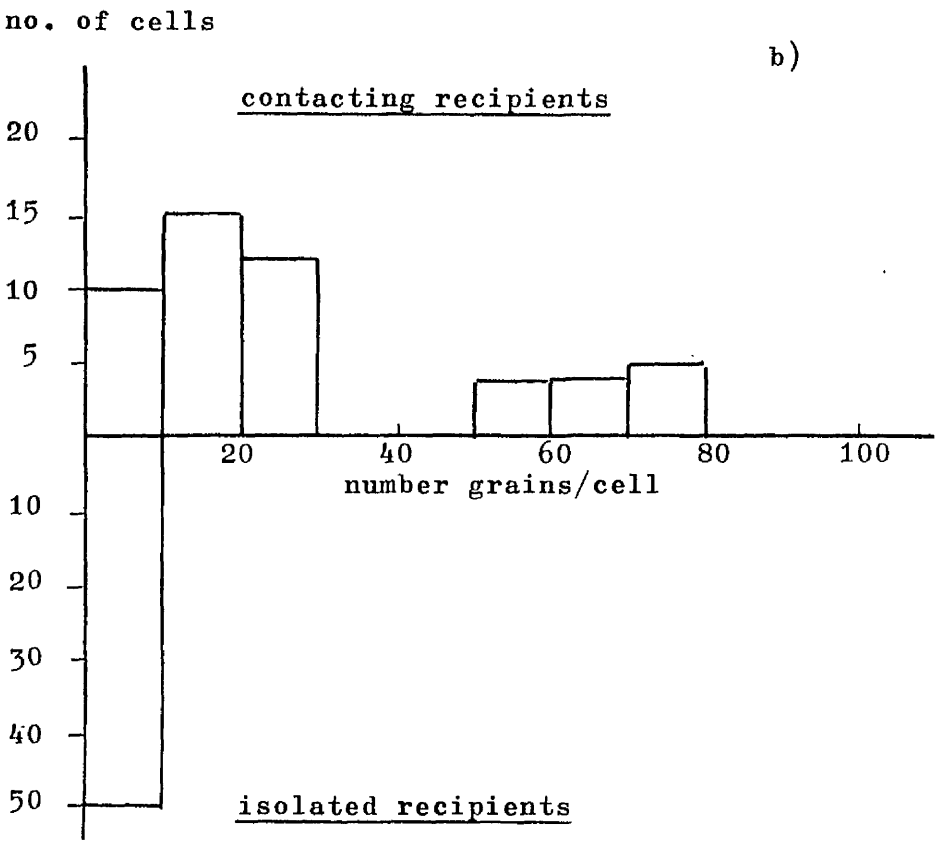
a) RTG_2 control

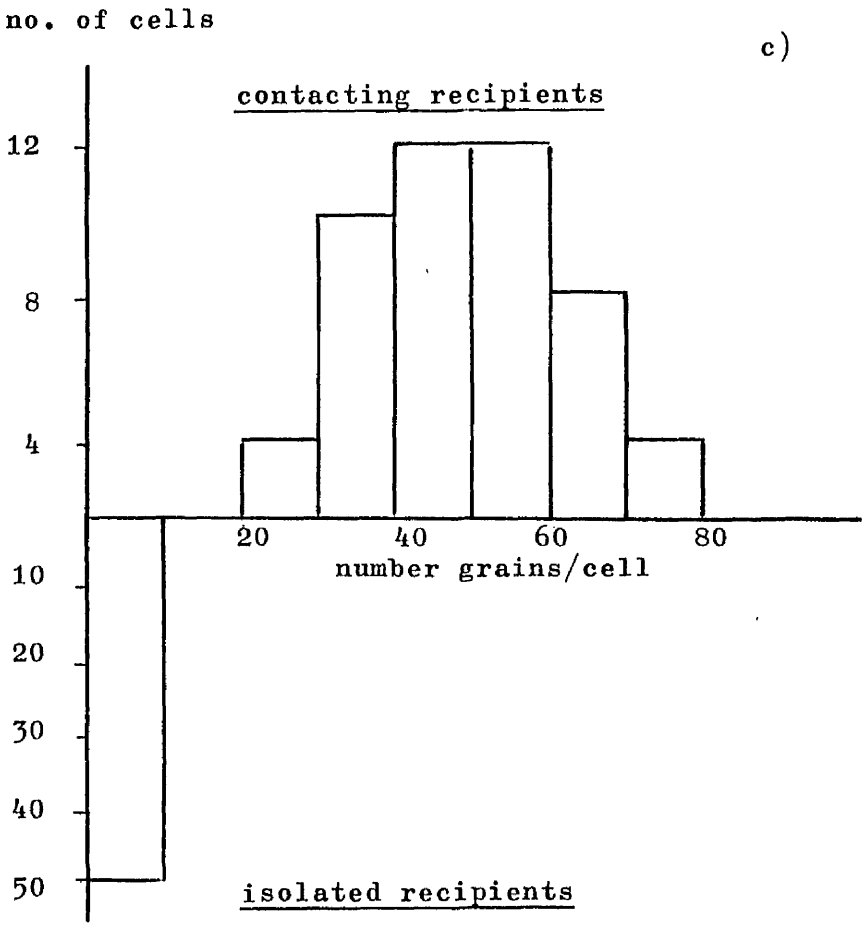
b) RTG_2 + 10^{-4}M trans retinoic acid

c) Xenopus control

d) Xenopus + 10^{-4}M trans retinoic acid


no. of cells
a)
contacting recipients
25
20
15
10
5
20
40
60
80
100
number grains/cell
10
20
30
40
50
isolated recipients


no. of cells
b)
contacting recipients
20
15
10
5
20
40
60
80
100
number grains/cell
10
20
30
40
50
isolated recipients


no. of cells
c)
contacting recipients
12
8
4
20
40
60
80
number grains/cell
10
20
30
40
50
isolated recipients

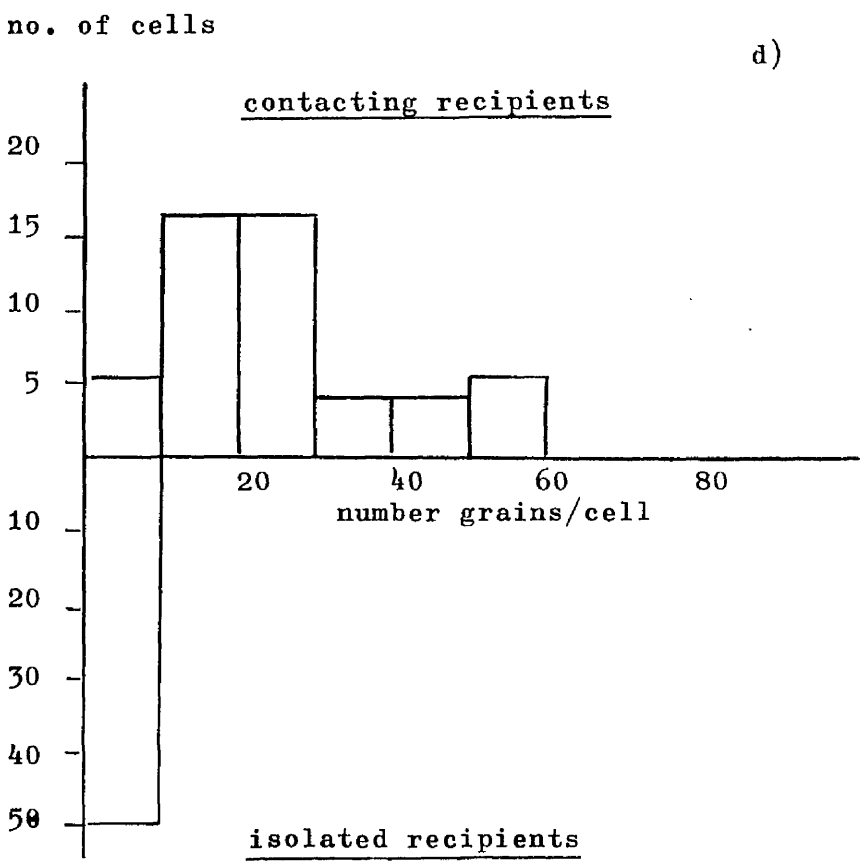

no. of cells
d)
contacting recipients
20
15
10
5
20
40
60
80
number grains/cell
10
20
30
40
50
isolated recipients

Table 8 The Effect of 10^{-4}M Retinoic Acid on Uridine Nucleotide Transfer Between Primary and Secondary Recipient Cells.

For experimental details see legend to Table 7.

Grains were counted over 20 secondary recipient cells in contact with primary recipient cells (see section 2.2.10). Primary recipients are recipient cells in contact with donor cells and secondary recipients are recipient cells in contact with primary recipient cells (but not in contact directly with donor cells).

	1° Recipient	2° Recipient	Recipients in contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f.	P
control	RTG_2	RTG_2	23.1(7.9)	4.8(3.8)	9.1	38	<0.001
	Xen	Xen	22.6(13.0)	2.2(2.0)	7.4	38	<0.001
10^{-4}M trans retinoic acid	RTG_2	RTG_2	8.5(4.1)	1.3(1.2)	7.4	38	<0.001
	Xen	Xen	15.6(11.9)	2.4(1.6)	4.8	38	<0.001

Figure 18 Effect of 10^{-4}M Trans Retinoic Acid on Growth of BRL Cells.

For experimental details see section 2.2.12.

Each point represents the average value of duplicate estimations of cell number.

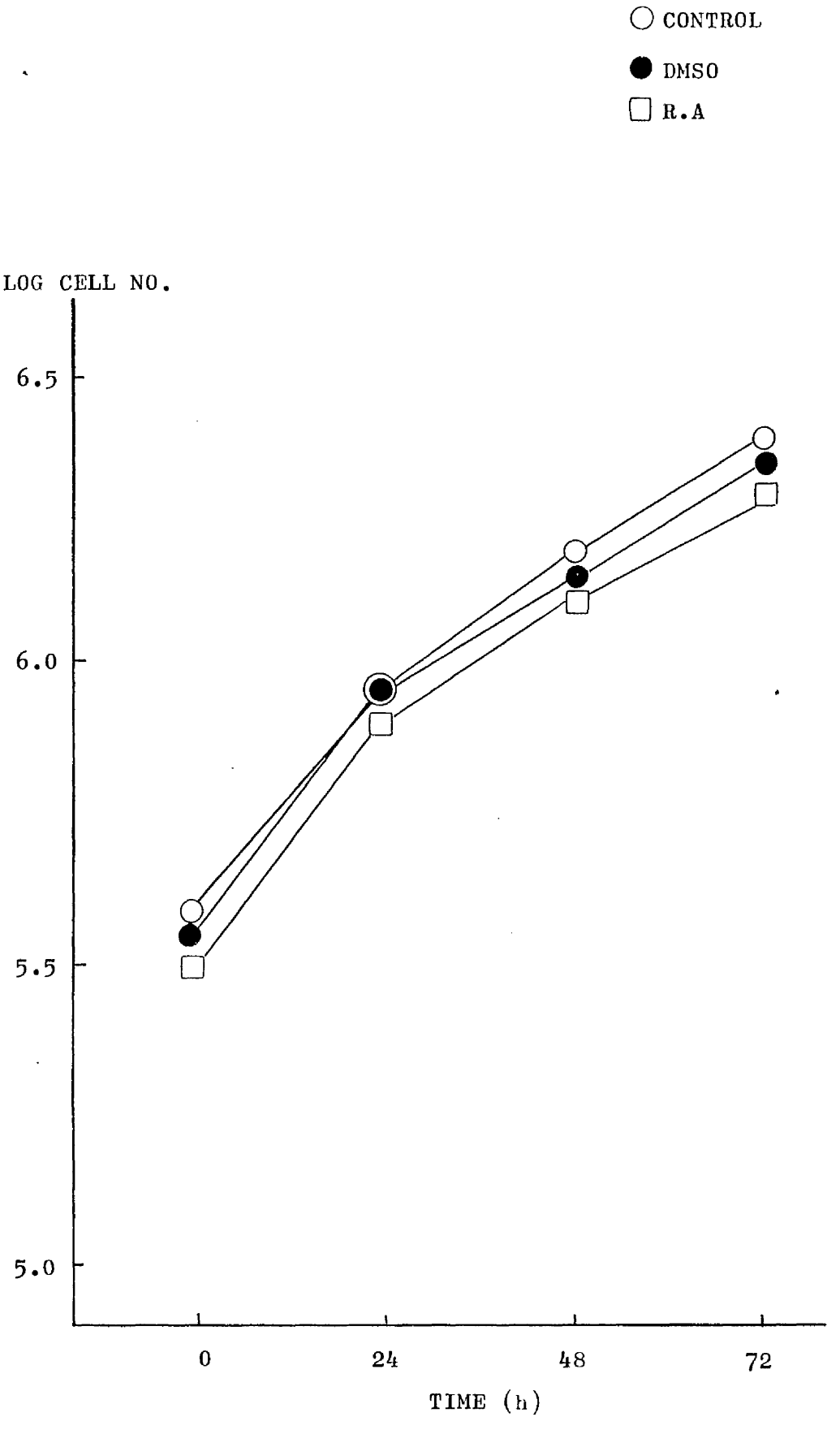

○ CONTROL
● DMSO
□ R.A
LOG CELL NO.
6.5
6.0
5.5
5.0
0
24
48
72
TIME (h)

Figure 19 Effect of 10^{-4}M Trans Retinoic Acid on Growth of V79 Cells.

For experimental details see section 2.2.12.

Each point represents the average value of duplicate estimations of cell number.

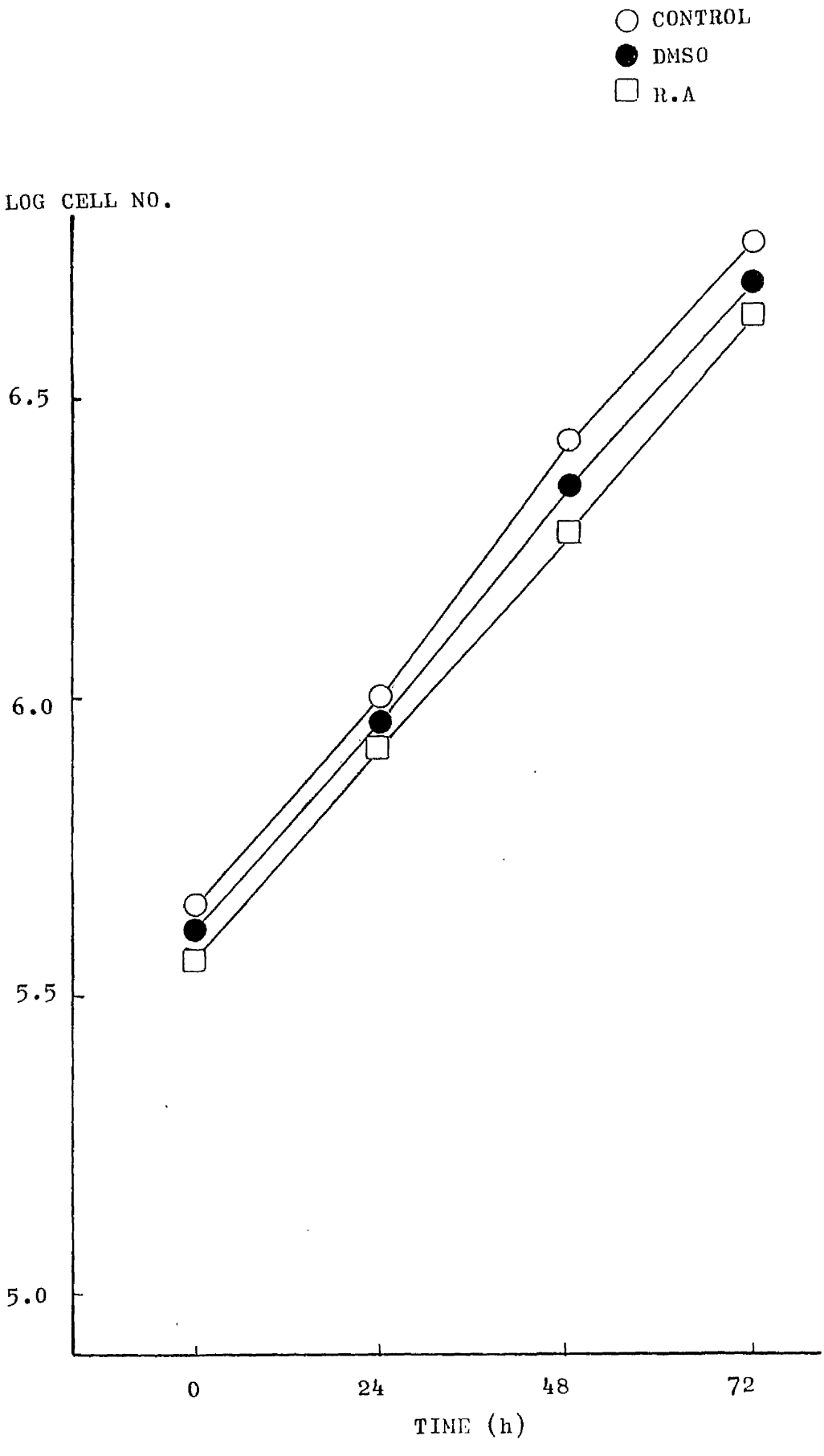

CONTROL
DMSO
R.A
LOG CELL NO.
6.5
6.0
5.5
5.0
0
24
48
72
TIME (h)

Figure 20 Effect of 10^{-4}M Trans Retinoic Acid on Growth of RTG_2 Cells.

For experimental details see section 2.2.12.

Each point represents the average value of duplicate estimations of cell number.

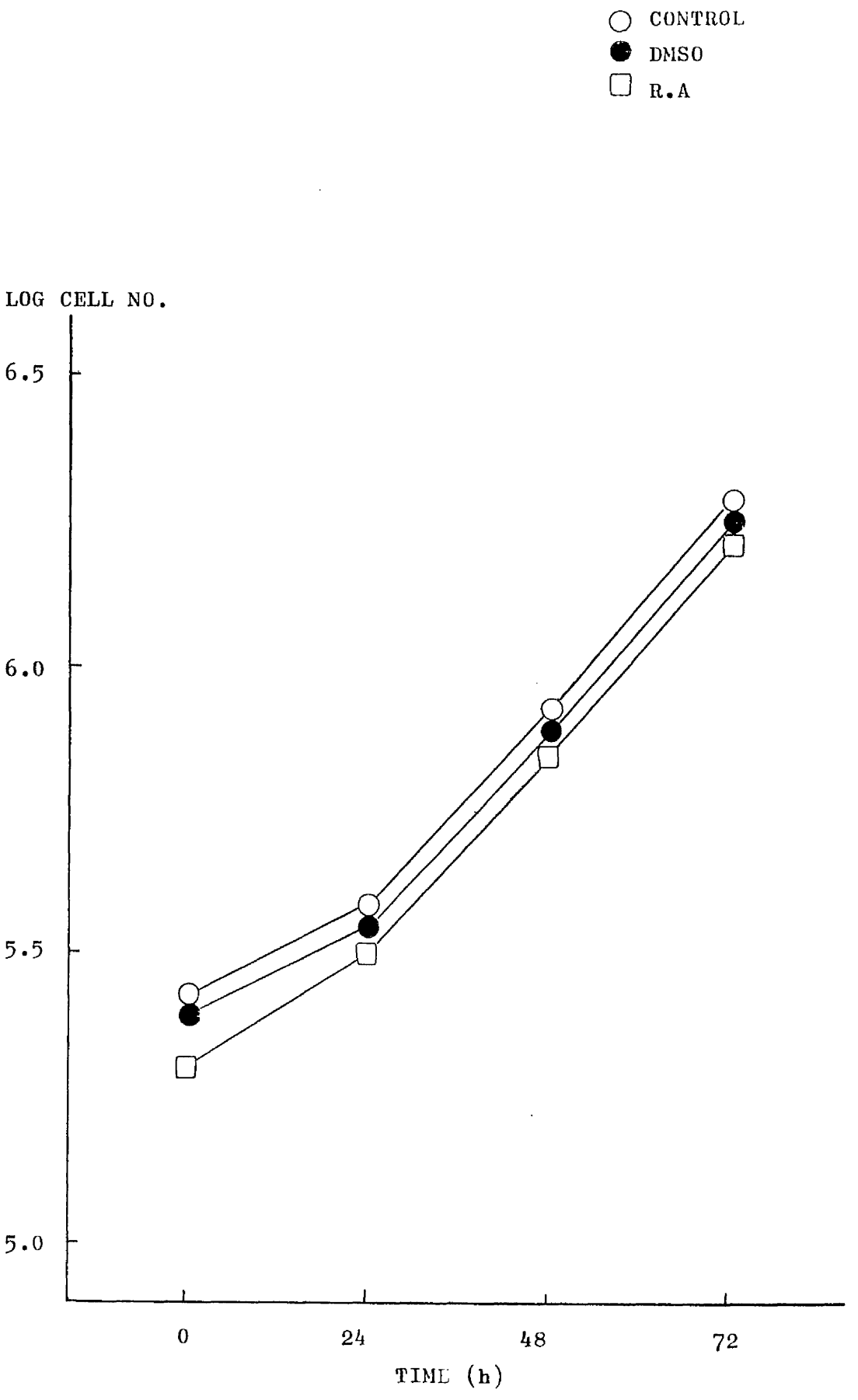

CONTROL
DMSO
R.A
LOG CELL NO.
6.5
6.0
5.5
5.0
0
24
48
72
TIME (h)

Figure 21 Effect of 10^{-4}M Trans Retinoic Acid on Growth of 3T3 Cells.

For experimental details see section 2.2.12.

Each point represents the average value of duplicate estimations.

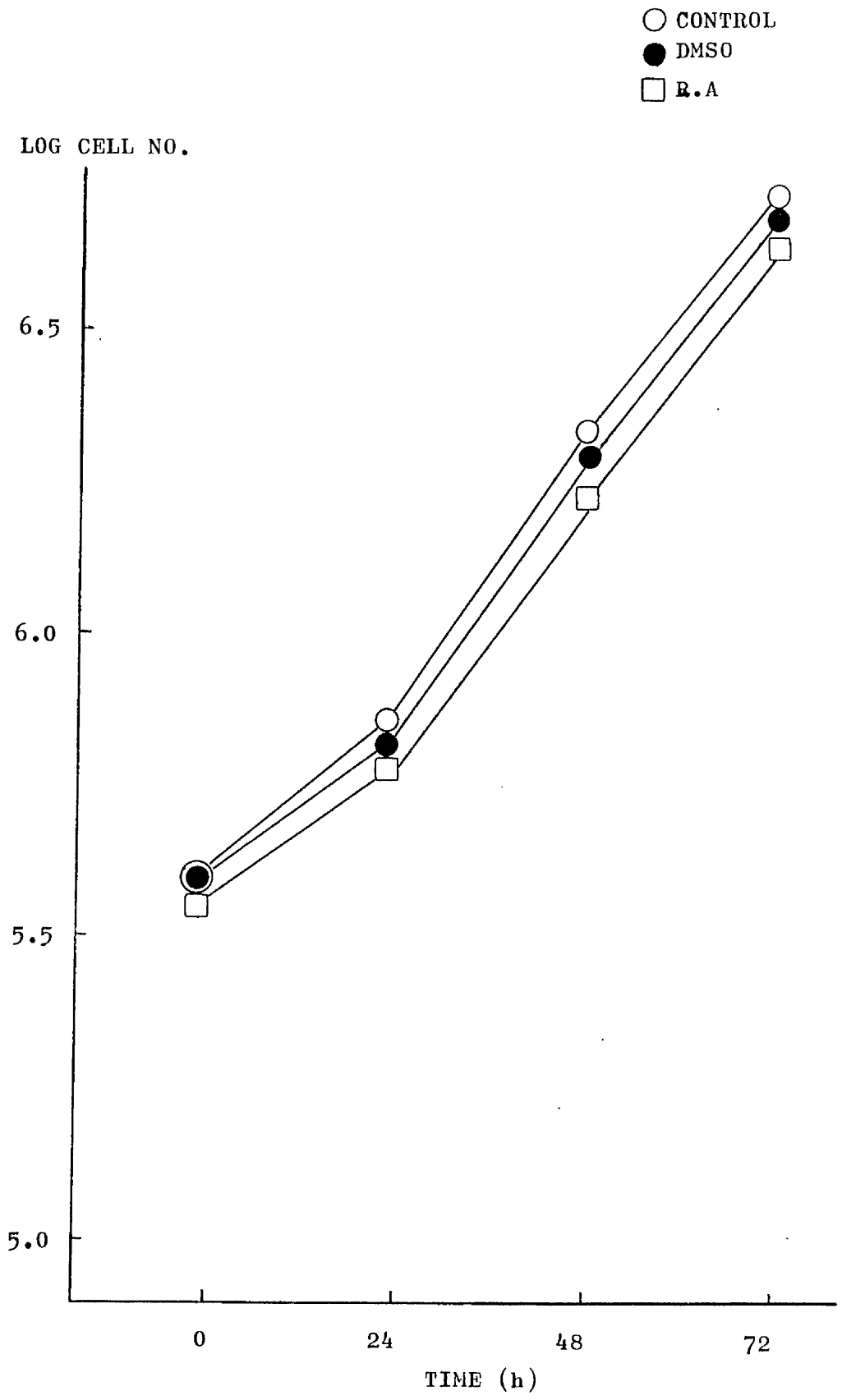

CONTROL
DMSO
R.A
LOG CELL NO.
6.5
6.0
5.5
5.0
0
24
48
72
TIME (h)

Figure 22 Effect of 10^{-4}M Trans Retinoic Acid on Growth of C13 Cells.

For experimental details see section 2.2.12.

Each point represents the average value of duplicate estimations of cell number.

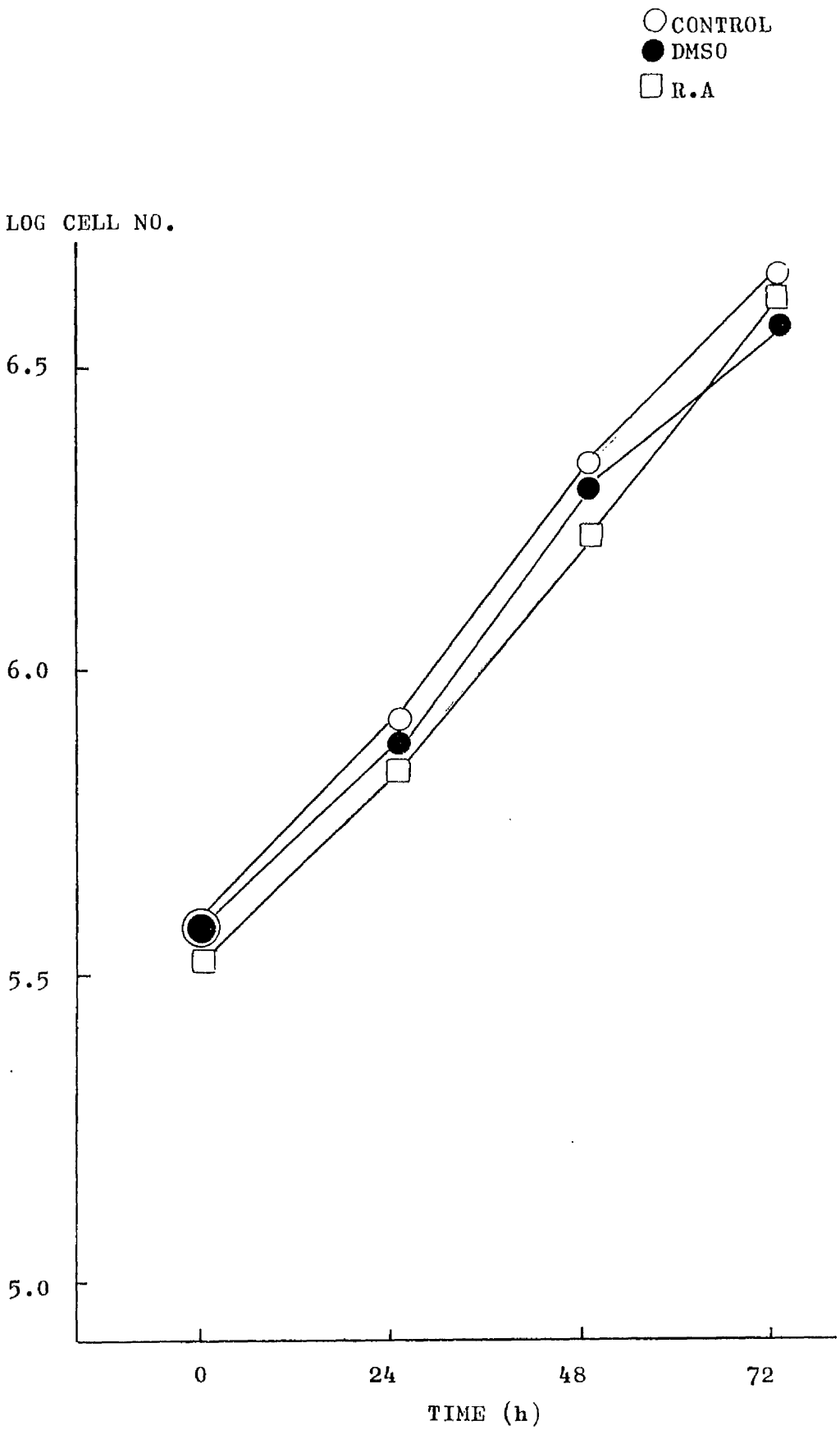

CONTROL
DMSO
R.A
LOG CELL NO.
6.5
6.0
5.5
5.0
0
24
48
72
TIME (h)

Table 9 Incorporation of [5-^{3}H] Uridine into Acid Soluble and Acid Insoluble Fractions in V79, BRL, C13, 3T3 and RTG_2 Cells in Presence and Absence of 10^{-4}M Trans Retinoic Acid.

Cell suspensions (10^5 cells/ml) in EC_{10} (RTG_2 cells in EFC_{10}) were dispensed in 2.0ml aliquots into 30mm plastic petri dishes and incubated at 37°C overnight. (RTG_2 cells were incubated at 18°C overnight). The medium was removed from the dishes and replaced with either 2.0ml fresh medium (control), 2.0ml medium + 10µl DMSO (DMSO) or 2.0ml medium + 10µl DMSO containing 2 x 10^{-2}M trans retinoic acid to give a final concentration of 10^{-4}M. This was followed either immediately or at later times, as indicated, by a 1h pulse of [5-^{3}H]uridine (2.0µl/ml;30Ci mmol $^{-1}$).

Incorporation of radioactivity into acid soluble and acid insoluble fractions was measured as described in section 2.2.11.

Each experiment was performed in duplicate and each experiment was repeated at least twice.

Replicate dishes in any experiment gave values differing by no more than 10%.

The values in Table 9 are the average cpm of two replicate cultures from one experiment.

The efficiency of counting, calculated from the channels ratio (i.e. quench correction) varied by no more than 5% between different samples using the same counting system.

Values in parenthesis are the percentage of control cpm at each time point.

ɔle 9.

	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
.d soluble cpm)	8718	8688(99.6)	8465(97.1)	7823	6639(84.8)	6459(82.5)	12618	11350(89.9)	9397(74.5)
.d insoluble cpm)	7155	7033(98.3)	6429(89.8)	9596	8282(86.0)	7021(73.0)	9062	8741(96.5)	5926(65.3)
d soluble cpm)	7017	6778(96.5)	5576(79.5)	9124	8644(94.7)	7185(79.6)	8527	7134(83.6)	3929(46.0)
d insoluble cpm)	4756	5111(107)	3731(78.4)	5236	5233(99.9)	5109(91.0)	11414	11348(99.3)	7924(69.0)
d soluble cpm)	7004	4896(69.9)	3325(47.7)	8512	7623(89.5)	6154(72.3)	9763	8766(89.7)	3619(37.1)
d insoluble cpm)	5435	5686(104)	3986(73.3)	10965	9986(91.0)	5684(53.3)	12123	10929(90.1)	5847(48.2)

le 9 (Continued)	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
d soluble cpm)	7005	5940(84.7)	5659(80.7)	5239	4791(91.4)	3636(69.9)	7175	6046(84.3)	3808(53.8)
d insoluble cpm)	6773	5357(78.9)	4131(60.9)	4556	3804(83.4)	3186(69.2)	3573	3075(86.1)	2137(59.8)
2 i soluble cpm)	4784	3957(82.5)	3886(81.2)	3193	3250(101)	2845(89.1)	4485	5172(115)	2962(66.6)
i insoluble cpm)	4156	3764(90.6)	3269(78.6)	3736	3384(90.5)	2686(71.9)	4925	5880(119)	3019(61.3)

Table 10 Incorporation of [Methyl -^{3}H] Thymidine into Acid Soluble and Acid Insoluble Fractions in V79, BRL, C13, 3T3 and RTG_2 Cells in Presence and Absence of 10^{-4}M Trans Retinoic Acid.

Cell suspension (10^5 cells/ml) in EC_{10} (RTG_2 cells in EFC_{10}) were dispensed in 2.0ml aliquots into 30mm plastic petri dishes and incubated at 37°C overnight. (RTG_2 cells were incubated at 18°C overnight). The medium was removed from the dishes and replaced with either 2.0ml fresh medium (control), 2.0ml medium + 10µl DMSO (DMSO) or 2.0ml medium + 10µl DMSO containing 2 x 10^{-2}M trans retinoic acid to give a final concentration of 10^{-4}M. This was followed either immediately or at later times, as indicated, by a 1h pulse of [Methyl -^{3}H]thymidine (2.0µli/ml 25 Ci mmol $^{-1}$).

Incorporation of radioactivity into acid soluble and acid insoluble fractions was measured as described in section 2.2.11.

Each experiment was performed in duplicate and each experiment was repeated at least twice.

Replicate dishes in any experiment gave values differing by no more than 10%.

The values in Table 10 are the average cpm of two replicate cultures from one representative experiment.

The efficiency of counting, calculated from the channels ratio (i.e. quench correction) varied by no more than 5% between different samples using the same counting system.

Values in parenthesis are the percentages of control cpm at each time point.

le 10.

	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
d soluble cpm)	3062	2041(66.6)	2579(84.2)	2090	2224(106)	2093(100)	3980	3625(91.0)	2836(71.2)
d insoluble cpm)	29439	19366(65.8)	20400(69.3)	35872	38320(107)	27309(76.0)	41610	36740(88.0)	26924(64.7)
d soluble cpm)	1600	1290(80.0)	1410(88.0)	3125	2891(92.5)	2185(69.9)	3065	3440(112.0)	2090(68.0)
d insoluble cpm)	2140	1760(82.0)	1715(79.9)	3736	3122(83.5)	2823(75.5)	4380	4525(103.0)	2629(61.4)
d soluble cpm)	2516	3321(131.9)	3754(149.0)	5871	5741(97.7)	5362(91.3)	5569	5124(92.0)	2356(42.3)
d insoluble cpm)	40123	38124(95.1)	31556(78.6)	87654	83169(94.8)	47489(54.5)	131458	120483(91.6)	41996(31.9)

›le 10 (Continued)	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
d soluble cpm)	6474	6270(96.8)	6420(99.1)	104930	117028(111.0)	81362(77.5)	11459	10423(90.9)	7507(65.5)
d insoluble cpm)	6360	6118(96.2)	5890(92.6)	63739	62632(98.3)	56780(89.1)	49469	40214(81.3)	31719(64.1)
2									
d soluble cpm)	1595	2199(137.0)	1198(75.1)	8107	7692(94.9)	7199(88.8)	5095	4916(96.5)	2754(54.1)
d insoluble cpm)	1964	2494(126.9)	1436(73.1)	13858	14519(104.9)	9205(66.6)	68833	60533(87.9)	40698(59.1)

Table 11 Incorporation of [G- ^{3}H] Hypoxanthine into Acid Soluble and Acid Insoluble Fractions in V79, BRL, C13, 3T3 and RTG_2 Cells in Presence and Absence of 10^{-4}M Trans Retinoic Acid.

Cell suspensions (10^5 cells/ml) in EC_{10} (RTG_2 cells in EFC_{10}) were dispensed in 2.0ml aliquots into 30mm plastic petri dishes and incubated at 37°C overnight. (RTG_2 cells were incubated at 18°C overnight). The medium was removed and replaced with either 2.oml fresh medium (control), 2.0ml medium + 10µl DMSO (DMSO) or 2.0ml medium + 10µl DMSO containing 2 x 10^{-2}M trans retinoic acid, to give a final concentration of 10^{-4}M. This was followed either immediately or at later times, as indicated, by a 1h pulse of [G - ^{3}H] hypoxanthine (2.0µli/ml 1.0 ci mmol $^{-1}$).

Incorporation of radioactivity into acid soluble and acid insoluble fractions was measured as described in section 2.2.11.

Each experiment was performed in duplicate and each experiment was repeated at least twice.

Replicate dishes in any experiment gave values differing by no more than 10%.

The values in Table 11 are the average cpm of two replicate cultures from one representative experiment.

The efficiency of counting, calculated from the channels ratio (i.e. quench correction) varied by no more than 5% between different samples using the same counting system.

Values in parenthesis are the percentage of control cpm at each time point.

Table 11

	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
d soluble cpm)	17009	17583 (103.0)	16250 (95.5)	20870	19052 (91.2)	15451 (74.0)	50774	45183 (88.9)	27133 (53.4)
d insoluble cpm)	13647	13589 (99.5)	11522 (84.4)	17572	15972 (90.8)	11984 (68.2)	20202	17586 (87.0)	11336 (56.1)
d soluble cpm)	24811	20282 (81.7)	19604 (79.0)	38494	35088 (91.1)	32030 (83.0)	72666	63040 (86.7)	46241 (63.6)
d insoluble cpm)	16959	14595 (88.2)	13734 (80.9)	31449	26949 (85.6)	19472 (61.9)	68168	58674 (86.0)	38625 (56.6)
d soluble cpm)	53641	48617 (90.6)	52817 (98.5)	71004	68547 (96.5)	51541 (72.6)	113652	10,068 (88.0)	35624 (31.3)
d insoluble cpm)	50396	45916 (91.1)	40238 (79.8)	60874	59123 (97.1)	32182 (52.8)	88741	91253 (102.0)	3075 (34.6)

Table 11 (Continued)

	0h			3h			24h		
	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA	control	DMSO	10^{-4}M RA
3									
.d soluble (cpm)	96236	88432 (91.8)	75493 (78.4)	58129	52491 (90.3)	33759 (58.1)	162187	152052 (93.7)	85451 (52.6)
.d insoluble (cpm)	85343	88029 (103.0)	63049 (73.4)	43709	40841 (93.4)	36861 (84.3)	45635	40508 (88.7)	34987 (76.6)
2									
.d soluble cpm)	6895	6691 (97.0)	5648 (81.9)	2808	2456 (87.4)	2014 (71.7)	9270	8768 (94.5)	6138 (66.2)
d insoluble cpm)	7952	7125 (89.6)	6666 (83.8)	3030	2911 (96.1)	2569 (84.8)	4190	3497 (83.4)	2307 (55.1)

Table 12 Rate of Inhibition of Junctional Communication Between C13 Cells by 10^{-4}M Trans Retinoic Acid.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing 2 x 10^{-2}M trans retinoic acid to give a final concentration of 10^{-4}M.

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.

Autoradiographs were exposed for 48h and 360h.

Recipient Cells in Contact(S.D.)/Isolated Recipient Cells (S.D

	48h exposure	360h exposure
control	25.0(8.0)/1.7(1.73)	174.0(31.6)/18.2(8.5)
10^{-4}M Trans Retinoic Acid	2.7(2.2)/2.7(2.0)	15.5(8.4)/12.3(4.2)

Table 13 Reversal of Retinoic Acid Inhibition of Junctional Communication Between C13 Cells and TG_2 Cells.

Cell suspensions of C13 and TG_2 cells (C13:TG_2 1:50) (10^5 cells/2ml) in EC_{10} were dispensed in 2.0ml aliquots into 30mm plastic petri dishes and incubated at 37°C overnight.

The medium was removed, 2.0ml medium + 10µl DMSO added to control dishes and 2.0ml medium + 10µl DMSO containing 2×10^{-2}M trans retinoic acid (to give a final concentration of 10^{-4}M) added to experimental dishes.

Forty-five minutes later the cultures were pulsed with [G - ^{3}H] hypoxanthine (10µCi/ml 1 Ci mmol $^{-1}$) for 15min. Labelled medium was removed and the cultures washed twice with 2.0ml neutralised BSS. 2.0ml medium + 10µl DMSO was added to all cultures and the cells were fixed and prepared for autoradiography (see section 2.2.9.) either immediately (time zero) or at various times later as indicated.

Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10).

The results shown are from one experiment of duplicate experiments.

Time (min)	Control Mean (S.D.)	10^{-4}M Trans RA Mean (S.D.)
0		
recipients in contact	70.0(30.6)	2.1(1.8)
isolated recipients	1.6(1.2)	2.5(1.9)
5		
recipients in contact	69.8(26.3)	31.3(20.0)
isolated recipients	3.0(1.5)	5.7(2.9)
10		
recipients in contact	85.9(26.2)	53.0(30.2)
isolated recipients	3.1(2.5)	4.0(2.5)
20		
recipients in contact	114.5(41.5)	93.0(44.6)
isolated recipients	2.0(1.6)	3.0(1.5)
30		
recipients in contact	146.4(43.1)	123.8(28.2)
isolated recipients	2.0(1.6)	3.1(1.7)
60		
recipients in contact	258.0(65.6)	239.0(65.4)
isolated recipients	3.1(2.2)	4.3(1.8)

Figure 23 Reversal of Retinoic Acid Inhibition of Junctional Communication Between C13 and TG_2 Cells.

For experimental details see legend to Table 13.

Values shown are the mean grain counts over 50 recipient cells in contact with donor cells, minus the mean grain counts over 50 isolated recipient cells.

The bars show the standard deviation of the grain counts over recipient cells in contact with donor cells.

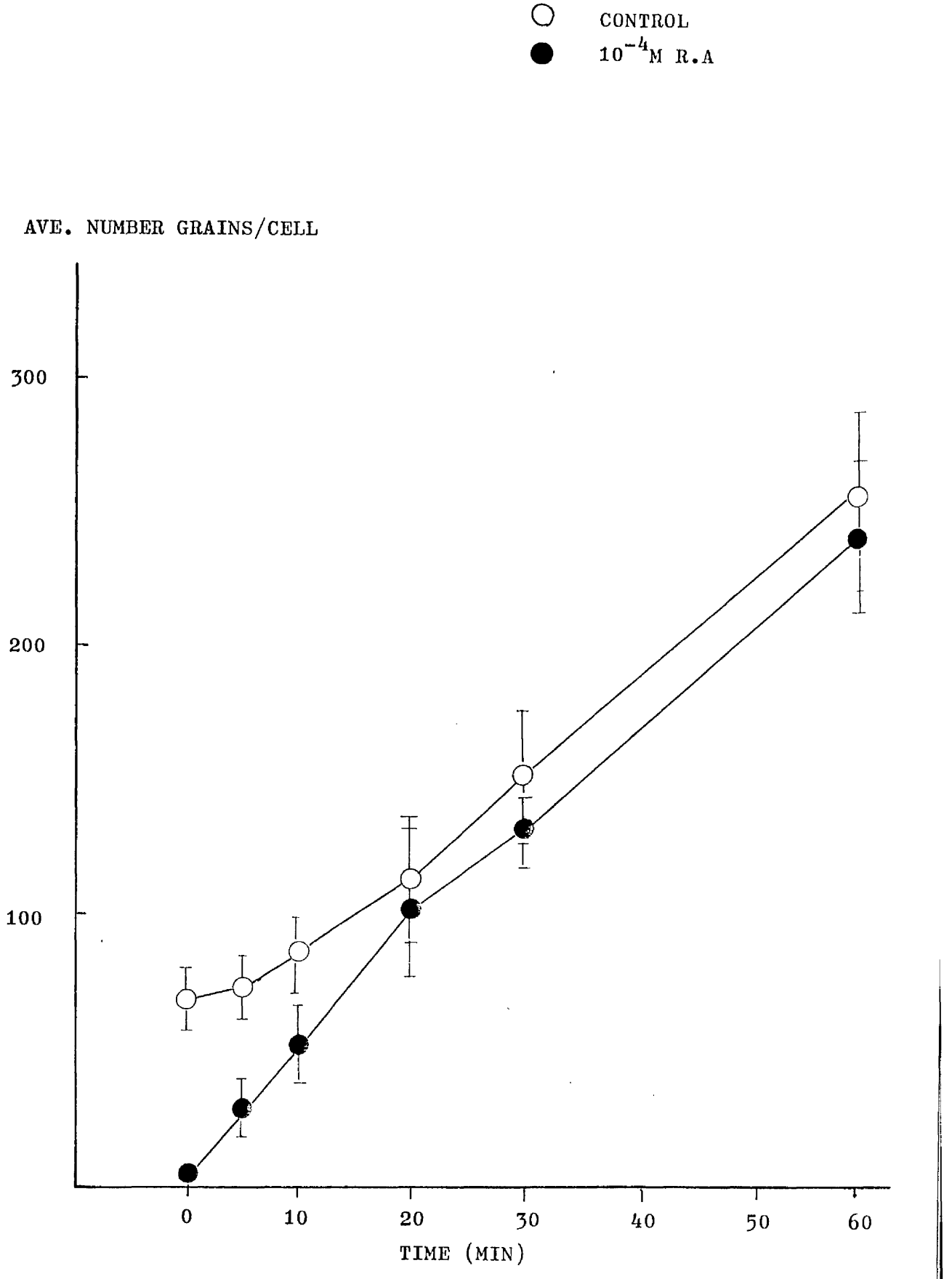

CONTROL
10^{-4}M R.A
AVE. NUMBER GRAINS/CELL
300
200
100
0
10
20
30
40
50
60
TIME (MIN)

Table 14 The Effect of Different Concentration of Trans Retinoic Acid (10^{-4}M - 10^{-7}M) on Uridine Nucleotide Transfer Between Cells in Homologous Cultures of BRL, C13 and V79 Cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing trans retinoic acid to give the final concentrations indicated.

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.). The data was analysed as described in section 2.2.10.

Grain Counts Recipients in Contact (S.D.)/Isolated Recipients (S.D.)			
Trans Retinoic Acid conc (M)	BRL -> BRL cells	C13 -> C13 cells	V79 -> V79 cells
10^{-4}	2.1(1.7)/1.6(1.4)	3.7(1.3)/34.(1.5)	1.3(1.1)/1.6(1.1)
10^{-5}	34.9(3.6)/4.7(2.7)	41.4(8.4)/4.1(1.9)	13.4(27.4)/1.6(0.7)
10^{-6}	35.7(3.2)/3.8(3.2)	42.4(3.8)/5.1(2.9)	56.3(25.7)/1.1(4.6)
10^{-7}	34.8(9.8)/4.6(2.3)	44.1(3.2)/5.8(3.1)	56.4(36.0)/1.4(1.8)
control	36.1(5.4)/3.5(1.7)	43.5(3.1)/4.0(1.9)	57.4(31.0)/1.9(1.6)

Table 15 Effect of Different Concentrations of Trans Retinoic Acid on Uridine Nucleotide Transfer Between C13 and BRL Cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing trans retinoic acid to give the final concentrations indicated.

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donors cells and over 50 isolated recipient cells (see section 2.2.10.

The results shown are from one experiment and are representative of duplicate experiments.

BRL	Recipients in Contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t	d.f.	P
control	91.4 (10.9)	11.8(5.2)	46.0	98	<0.001
10^{-5}M	93.6 (21.3)	12.2(5.2)	25.9	98	<0.001
2 x 10^{-5}M	83.6 (23.2)	9.3(5.3)	8.0	98	<0.001
4 x 10^{-5}M	51.2 (14.7)	12.9(4.5)	17.4	98	<0.001
6 x 10^{-5}M	33.1 (7.0)	13.9(4.0)	16.6	98	<0.001
8 x 10^{-5}M	21.2 (9.9)	12.9(4.5)	5.3	98	<0.001
10^{-4}M	11.1 (4.6)	11.3(4.0)	0.26	98	N.S.

C13

control	110.3(27.5)	6.8(3.0)	26.2	98	<0.001
10^{-5}M	108.2(30.8)	8.5(4.4)	22.4	98	<0.001
2 x 10^{-5}M	86.5(30.1)	6.3(5.3)	18.4	98	<0.001
4 x 10^{-5}M	59.6(25.6)	3.1(2.0)	15.4	98	<0.001
6 x 10^{-5}M	47.2(28.4)	7.1(3.4)	9.8	98	<0.001
8 x 10^{-5}M	14.1(10.0)	11.8(5.2)	1.4	98	N.S.
10^{-4}M	10.4(5.3)	10.1(3.7)	0.36	98	N.S.

Table 16 Statistical Comparison of Grain Counts Presented in Table 15.

For experimental details see legend to Table 15. The grain counts presented in Table 15 were paired as shown in Table 16 and analysed using the student's T-test.

BRL	t.	d.f.	P.
control : 10^{-5}M trans RA	0.5	198	N.S.
10^{-5}M trans RA : 2 x 10^{-5}M trans RA	1.5	198	N.S.
2 x 10^{-5}M trans RA : 4 x 10^{-5}M trans RA	9.2	198	<0.001
4 x 10^{-5}M trans RA : 6 x 10^{-5}M trans RA	8.2	198	<0.001
6 x 10^{-5}M trans RA : 8 x 10^{-5}M trans RA	6.4	198	<0.001
8 x 10^{-5}M trans RA : 10^{-4}M trans RA	5.8	198	<0.001

C13			
control : 10^{-5}M trans RA	0.6	198	N.S.
10^{-5}M trans RA : 2 x 10^{-5}M trans RA	3.2	198	<0.01
2 x 10^{-5}M trans RA : 4 x 10^{-5}M trans RA	5.4	198	<0.001
4 x 10^{-5}M trans RA : 6 x 10^{-5}M trans RA	3.0	198	<0.01
6 x 10^{-5}M trans RA : 8 x 10^{-5}M trans RA	8.8	198	<0.001
8 x 10^{-5}M trans RA : 10^{-4}M trans RA	1.2	198	N.S.

Figure 24 Effect of Different Concentrations of Trans Retinoic Acid on Uridine Nucleotide Transfer Between C13 and BRL Cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing trans retinoic acid to give the final concentrations indicated.

After 3h co-culture, the cells were fixed and prepared for autoradiography as described in section 2.2.9.

Values shown are the mean grain counts over 50 recipient cells in contact with donor cells minus the mean grain counts over 50 of the isolated recipient cells. The bars show the standard deviation of the grain counts over recipient cells in contact with donor cells.

The results shown are from one experiment and are representative of results obtained in duplicate experiments.

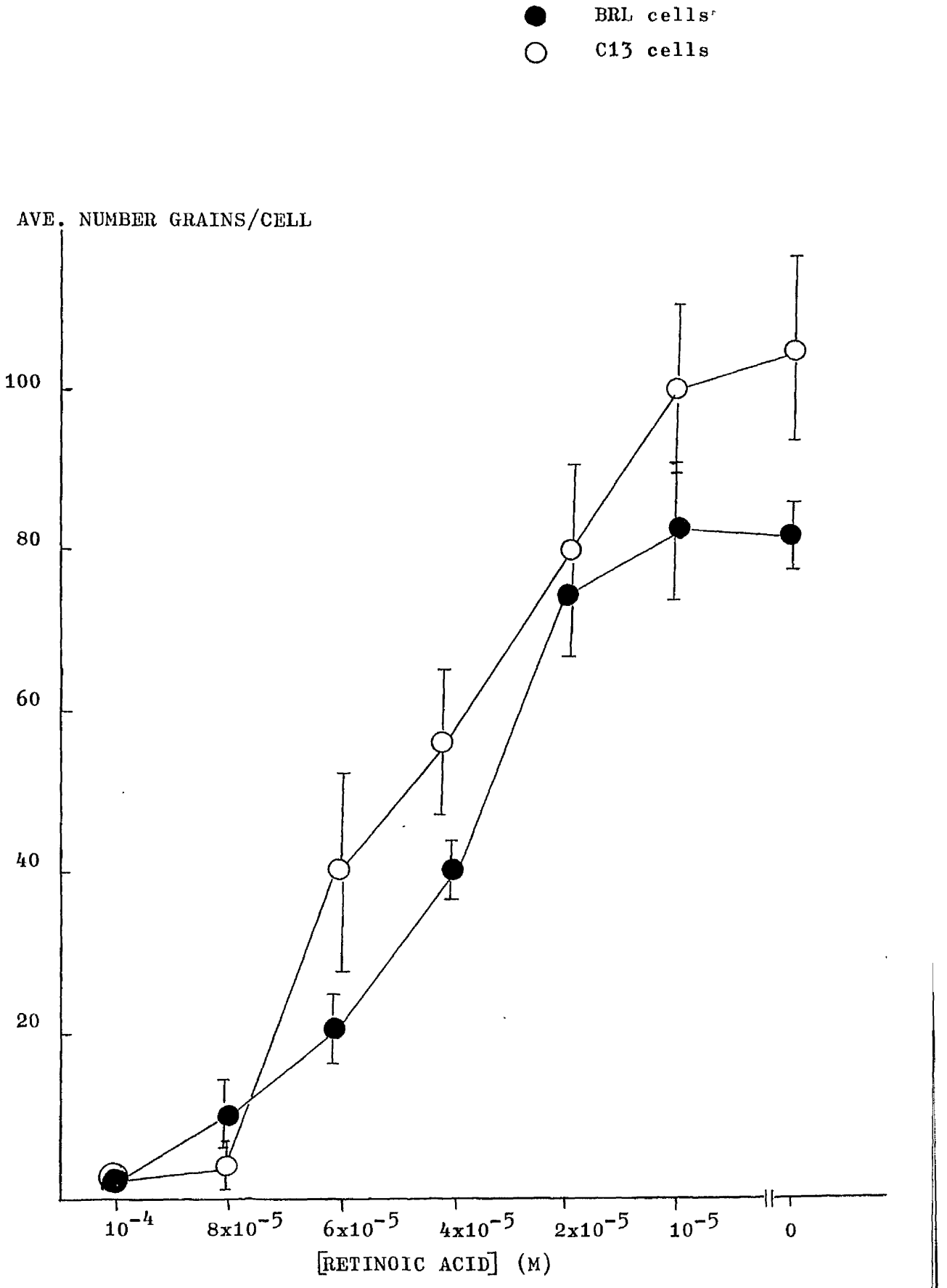

BRL cells
C13 cells
AVE. NUMBER GRAINS/CELL
100
80
60
40
20
10^-4
8x10^-5
6x10^-5
4x10^-5
2x10^-5
10^-5
0
[RETINOIC ACID] (M)

Figure 25 Effect of Different Concentrations of Trans Retinoic Acid on Uridine Nucleotide Transfer Between Primary and Secondary Recipient Cells.

For experimental details see legend to Fig 24.

Grains were counted over 20 secondary recipient cells in contact with primary recipient cells (see section 2.2.10.). Primary recipients are recipient cells in contact with donor cells and secondary recipients are recipient cells in contact with primary recipient cells (but not in contact directly with donor cells).

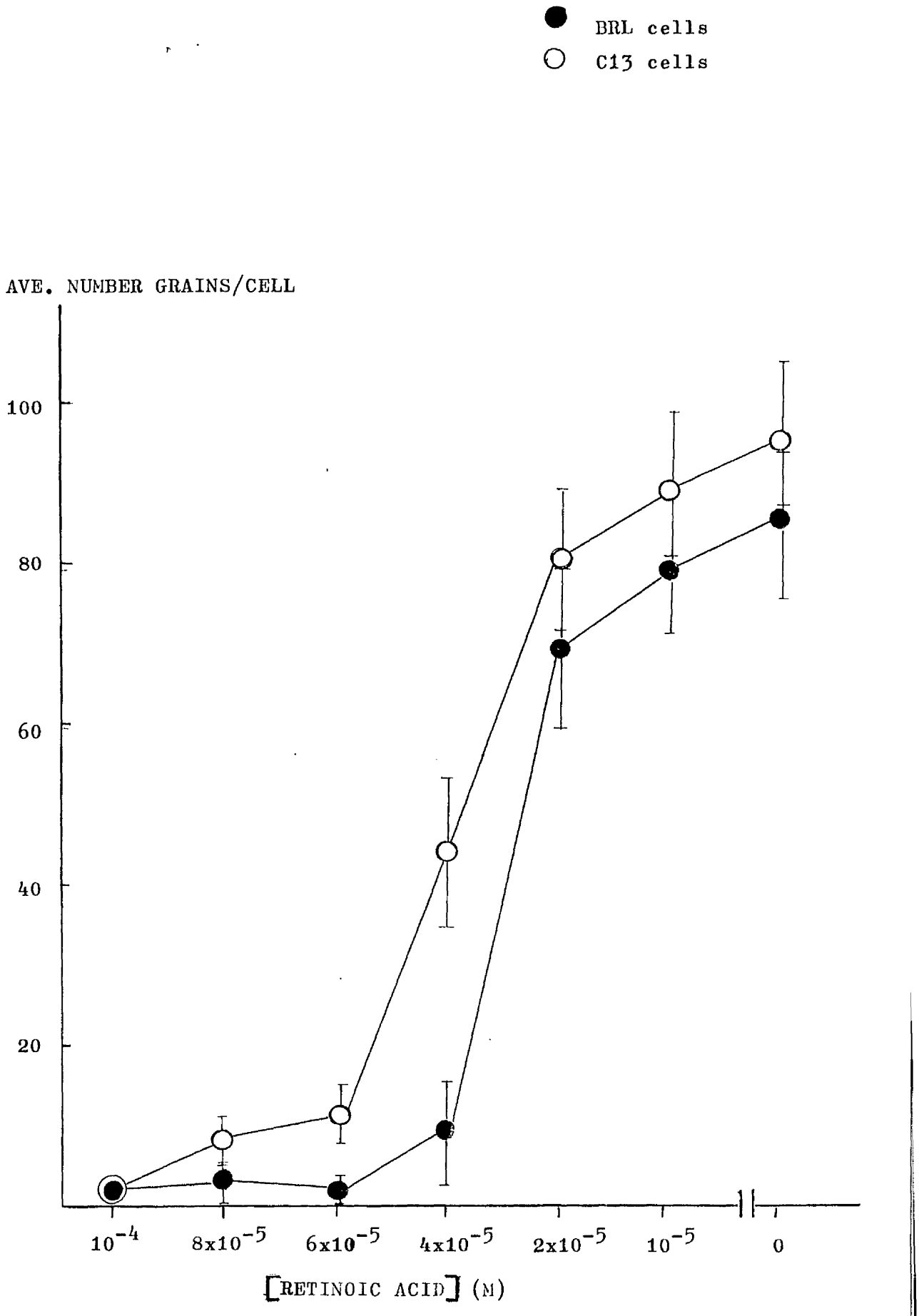

BRL cells
C13 cells
AVE. NUMBER GRAINS/CELL
100
80
60
40
20
10^-4
8x10^-5
6x10^-5
4x10^-5
2x10^-5
10^-5
0
[RETINOIC ACID] (M)

Table 17 Effect of Different Concentrations of Trans and 13 cis Retinoic Acid (10^{-4}M - 10^{-7}M) on Uridine Nucleotide Transfer Between C13 cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing 13 cis retinoic acid or trans retinoic acid to give the final concentrations indicated.

After co-culturing for 30min, 60min or 180min as indicated, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.).

able 17

	Control Mean (S.D.)	10^{-4}M Mean (S.D.)	10^{-5}M Mean (S.D.)	10^{-6}M Mean (S.D.)	10^{-7}M Mean (S.D.)
Trans RA					
)min					
ecipients in ontact	38.8(7.2)	1.2(1.6)	42.8(7.8)	47.5(9.7)	44.0(7.3)
olated recipients	1.6(1.8)	1.7(1.9)	1.4(1.9)	2.0(2.1)	2.9(2.6)
min					
cipients in ntact	56.4(9.0)	2.8(2.4)	53.9(9.4)	55.4(9.3)	56.2(9.9)
olated recipients	2.7(2.4)	2.0(2.2)	2.2(2.2)	2.1(2.2)	4.4(2.1)
0min					
cipients in ntact	84.7(13.3)	4.5(2.1)	83.9(13.9)	89.2(9.7)	90.2(8.0)
olated recipients	4.6(1.7)	5.3(1.7)	5.3(2.0)	5.2(1.8)	5.6(2.6)

able 17 (cont)

	Control Mean (S.D.)	10^{-4}M Mean (S.D.)	10^{-5}M Mean (S.D.)	10^{-6}M Mean (S.D.)	10^{-7}M Mean (S.D.)
13cis RA					
)min					
ecipients in ontact	40.9(6.5)	3.3(1.9)	38.5(8.6)	43.5(10.8)	40.8(9.1)
solated recipients	2.4(2.0)	3.3(1.9)	3.6(1.9)	4.6(1.1)	3.9(1.7)
)min					
ecipients in ntact	53.9(7.4)	4.1(1.2)	52.5(7.6)	59.0(6.0)	55.8(5.2)
solated recipients	4.2(1.1)	4.1(1.5)	4.1(1.8)	4.0(1.9)	4.2(1.5)
0min					
cipients in ntact	84.4(12.5)	4.7(1.1)	79.6(13.6)	87.5(6.1)	86.5(14.0)
olated recipients	5.0(1.2)	4.7(1.1)	4.9(1.3)	4.9(1.4)	4.8(1.4)

Table 18 Effect of Different Concentrations of Trans and 13 cis Retinoic Acid (10^{-4}M - 10^{-7}M) on Uridine Nucleotide Transfer Between V79 cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml fresh medium containing 13 cis retinoic acid or trans retinoic acid to give the final concentrations indicated.

After co-culturing for 30min, 60min or 180min as indicated, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 100 recipient cells in contact with donor cells and over 100 isolated recipient cells (see section 2.2.10.).

able 18

	Control Mean (S.D.)	10^{-4}M Mean (S.D.)	10^{-5}M Mean (S.D.)	10^{-6}M Mean (S.D.)	10^{-7}, Mean (S.D.)
Trans RA					
)min					
:cipients in)ntact	24.3(28.4)	2.2(4.5)	3.4(8.8)	17.4(15.1)	27.4(22.8)
:olated recipients	1.1(1.6)	2.5(2.1)	1.4(2.3)	1.1(1.5)	1.0(1.3)
min					
:cipients in)ntact	41.8(30.0)	2.3(2.0)	7.7(9.0)	32.8(33.8)	34.1(22.7)
olated recipients	2.4(5.2)	2.5(2.5)	1.9(5.7)	1.4(1.9)	1.1(2.0)
0min					
cipients in ntact	57.4(31.0)	1.3(1.1)	13.4(27.4)	56.3(25.7)	56.4(36.0)
olated recipients	1.9(1.6)	1.6(1.1)	1.6(0.7)	1.1(4.6)	1.4(1.8)

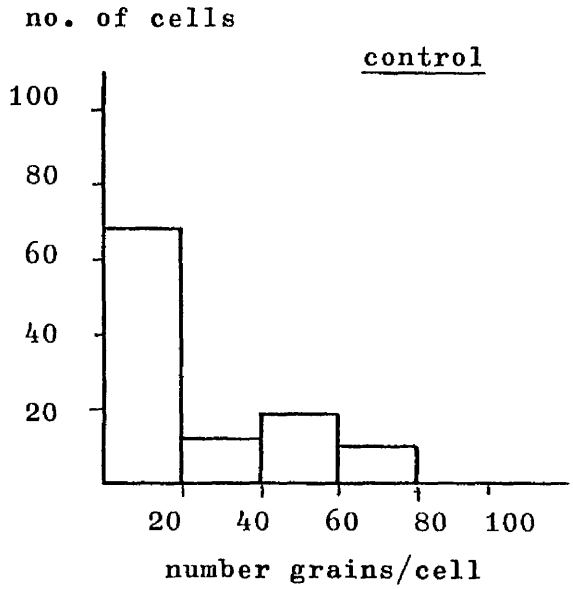
able 18 (cont)

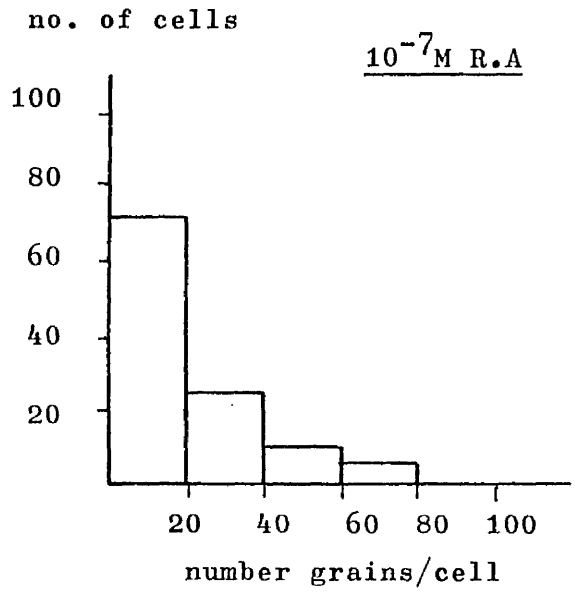
	Control Mean (S.D.)	10^{-4}M Mean (S.D.)	10^{-5}M Mean (S.D.)	10^{-6}M Mean (S.D.)	10^{-7}M Mean (S.D.)
3cis RA					
)min					
ecipients in ontact	27.9(32.0)	1.6(1.2)	4.3(6.2)	10.1(18.3)	26.7(30.2)
olated recipients	1.5(1.6)	1.6(1.3)	1.4(2.2)	1.4(1.4)	1.2(1.4)
)min					
cipients in ntact	36.5(43.8)	2.0(1.9)	29.0(36.4)	43.5(29.3)	42.8(25.9)
olated recipients	1.4(2.1)	1.1(1.6)	1.6(2.1)	1.6(2.0)	2.0(1.7)
0min					
cipients in ntact	55.4(29.7)	1.3(2.1)	50.8(30.8)	59.7(25.7)	60.9(26.4)
olated recipients	1.5(3.6)	1.8(1.8)	1.2(2.1)	1.4(4.0)	1.5(4.9)

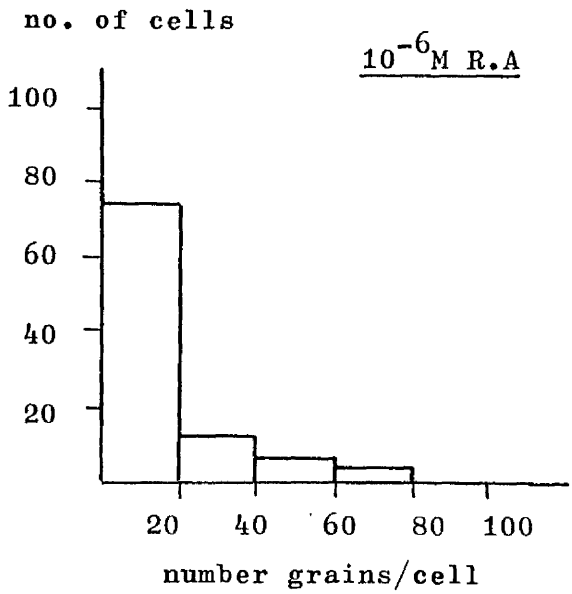
Figure 26 Histograms of Grain Counts over V79 and C13 Cells.

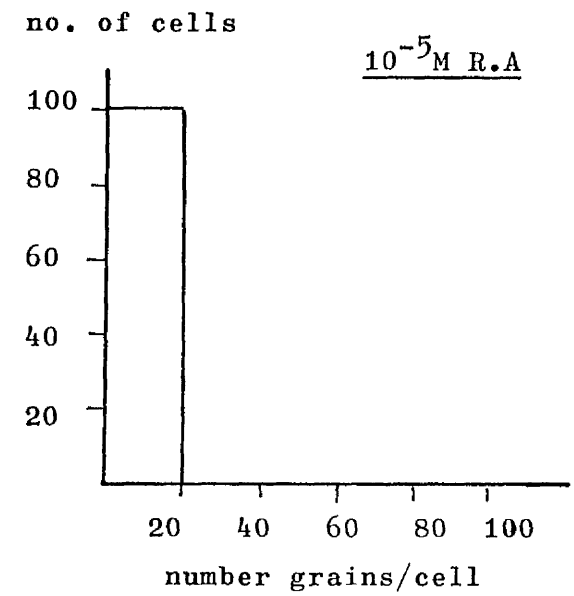
For experimental details see legend to Table 17 and Table 18.

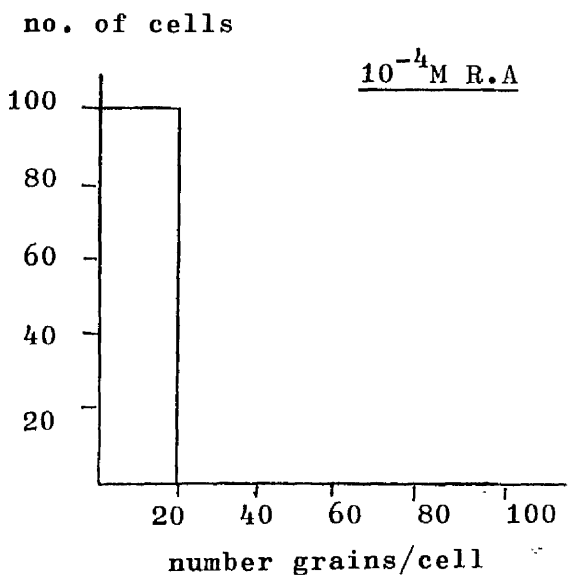
V79 CELLS: +TRANS RETINOIC ACID : 30 MIN



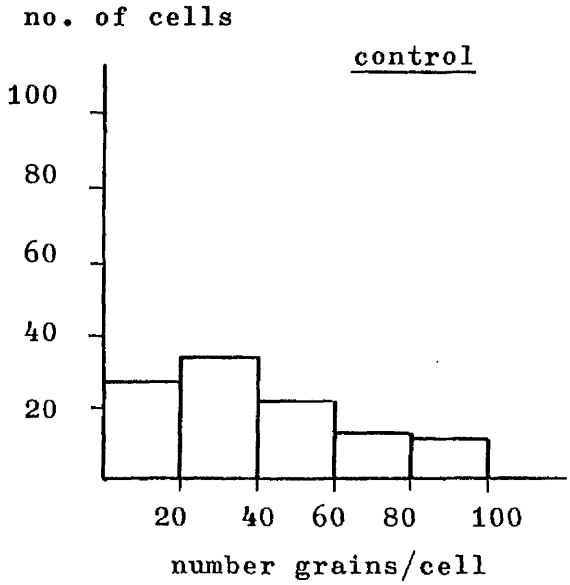


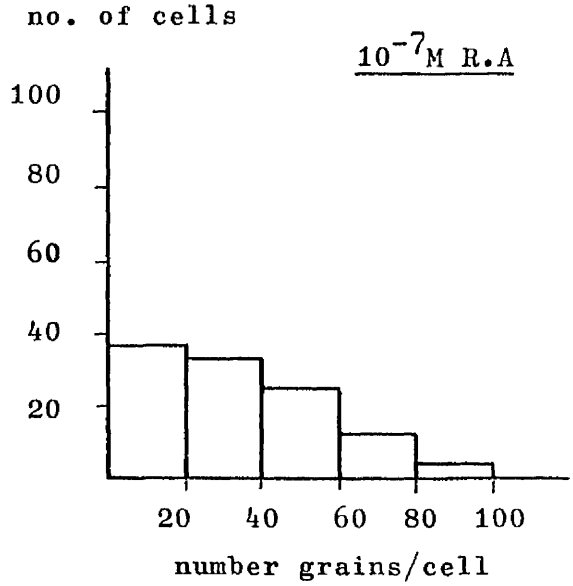


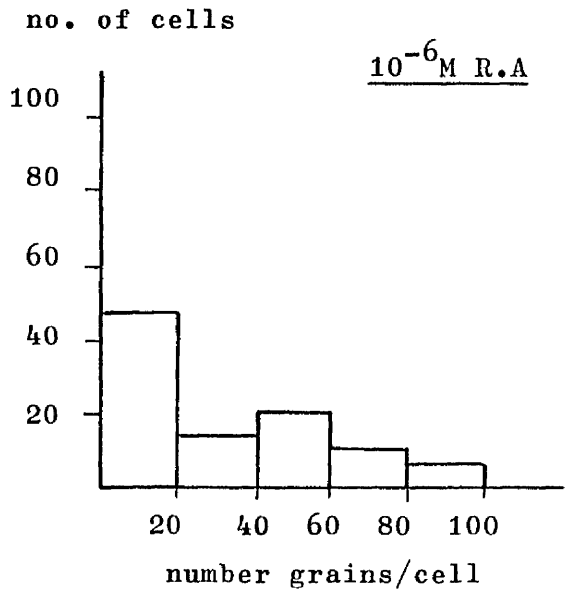


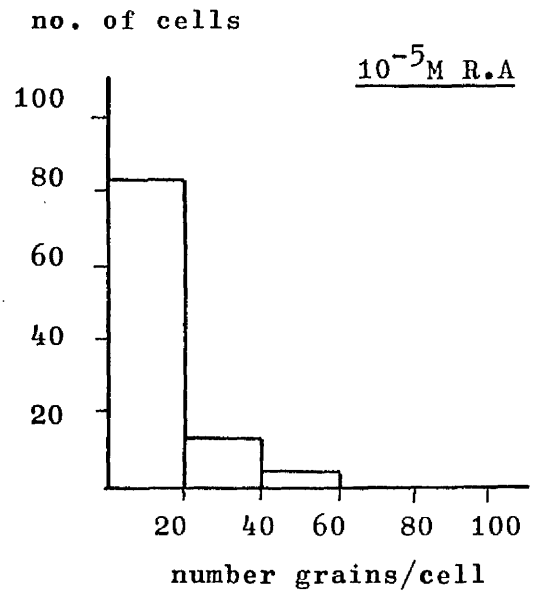


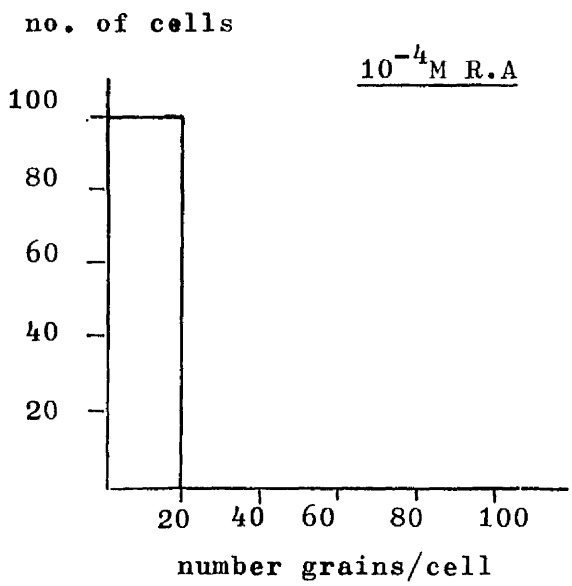
V79 CELLS +TRANS RETINOIC ACID : 60 MIN



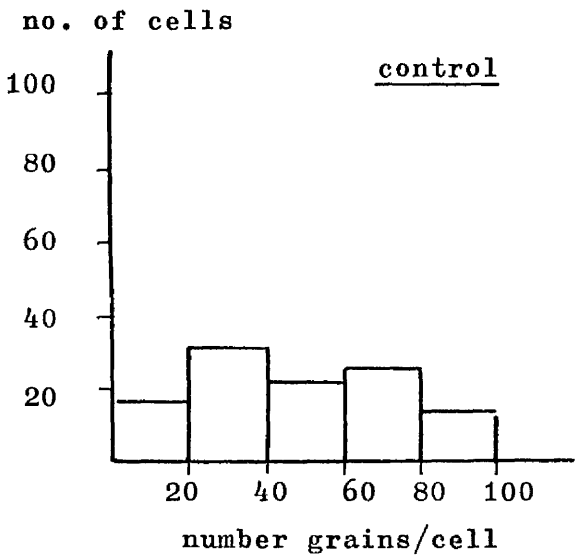


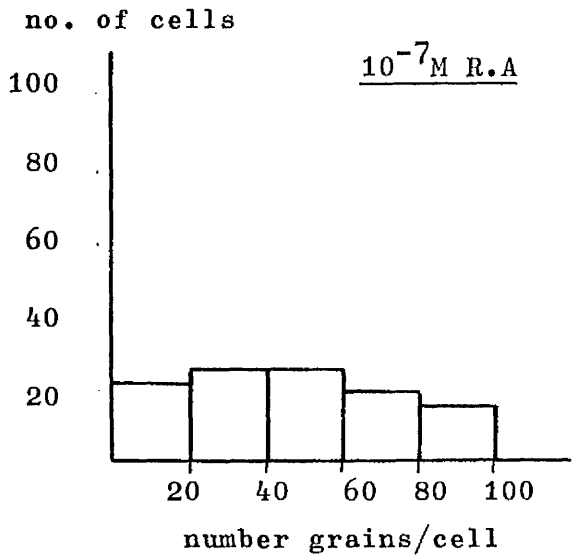


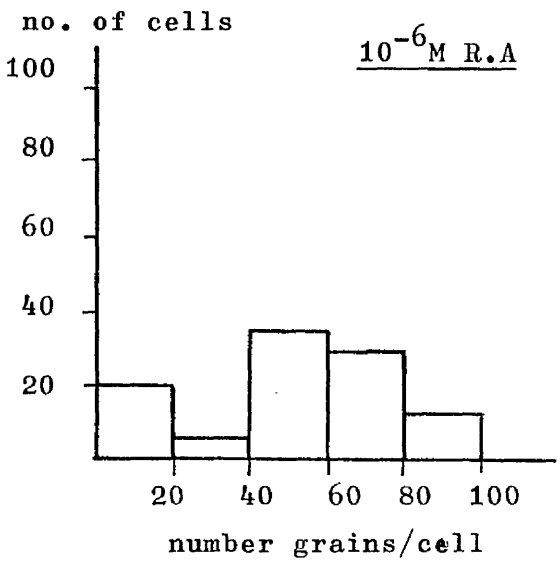


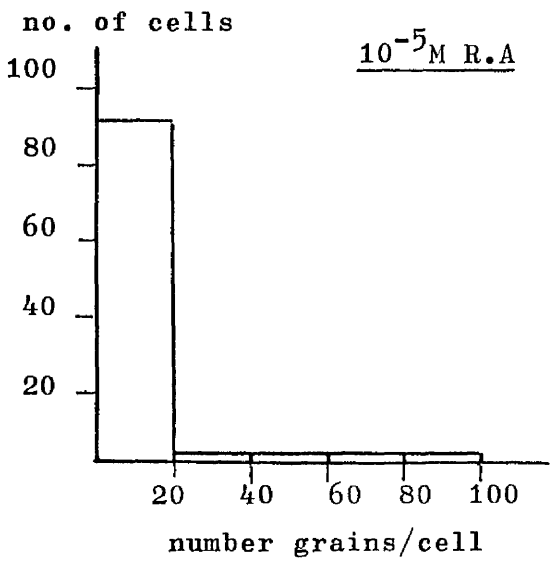


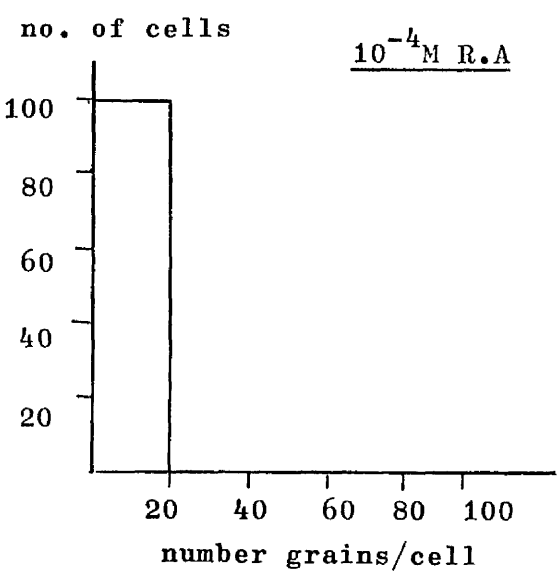
V79 +TRANS RETINOIC ACID : 180 MIN



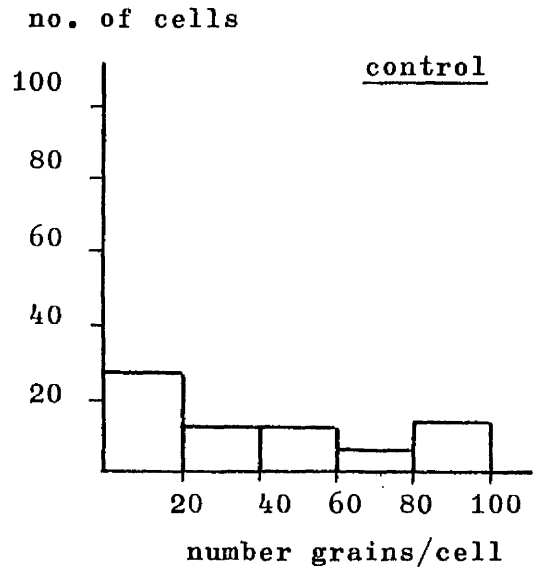


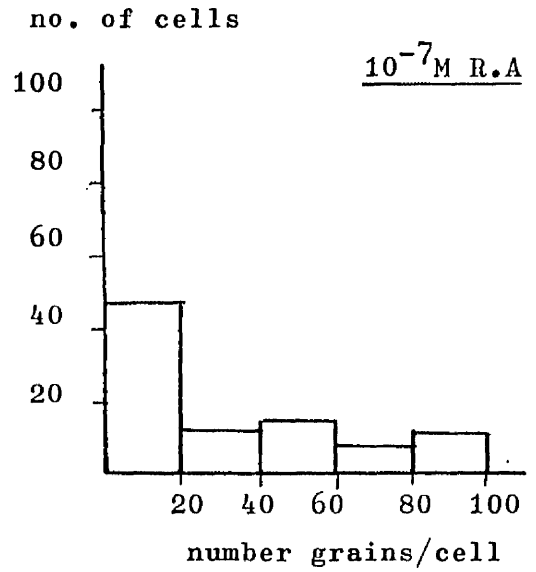


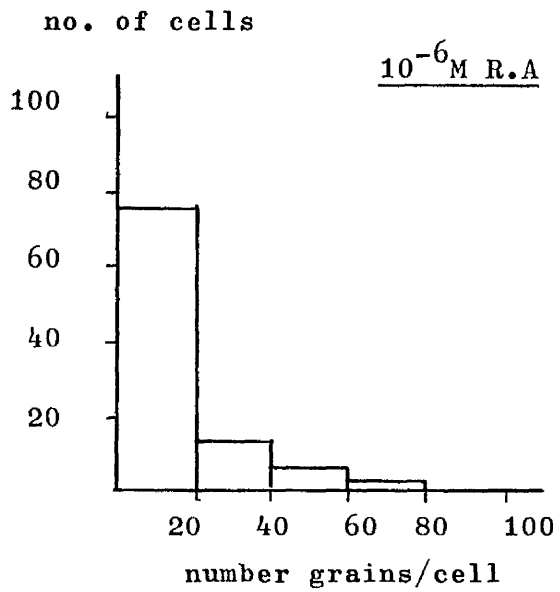


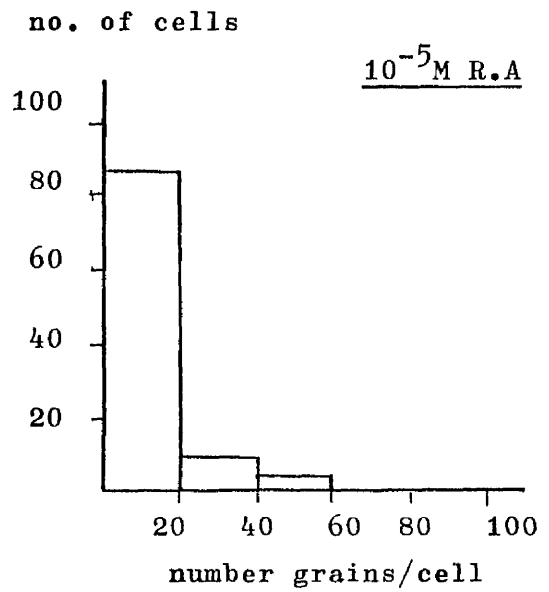


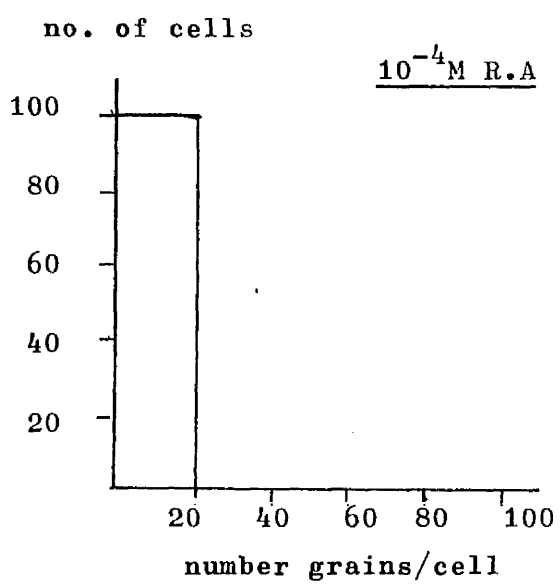
V79 CELLS +CIS RETINOIC ACID : 30 MIN



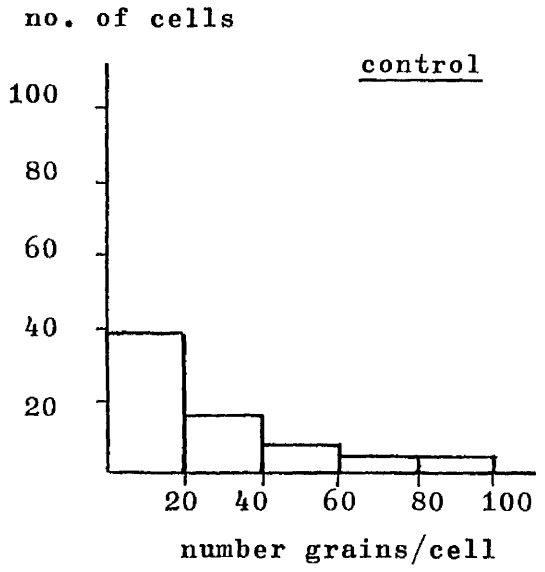


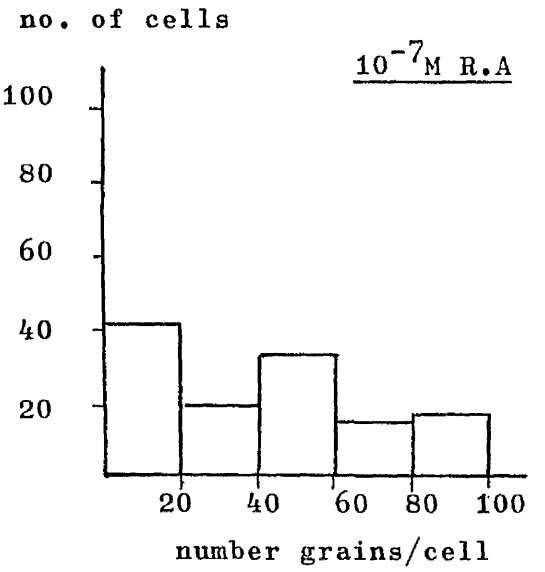


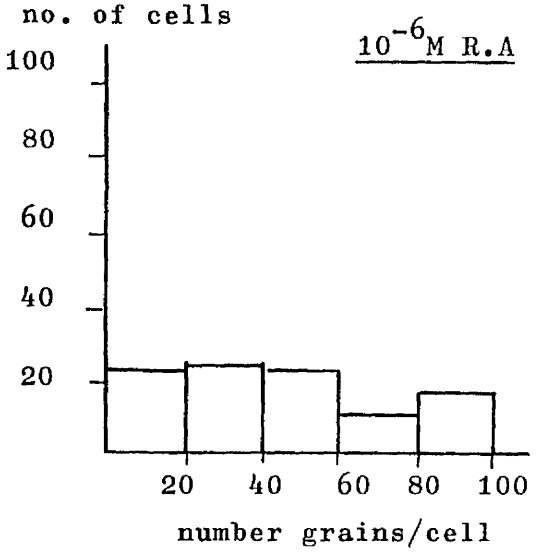


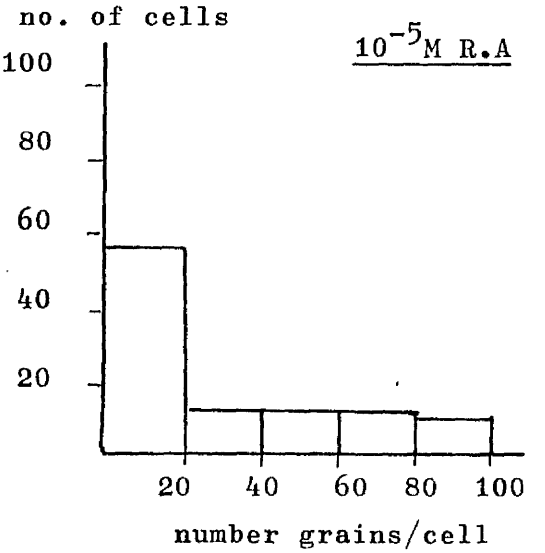


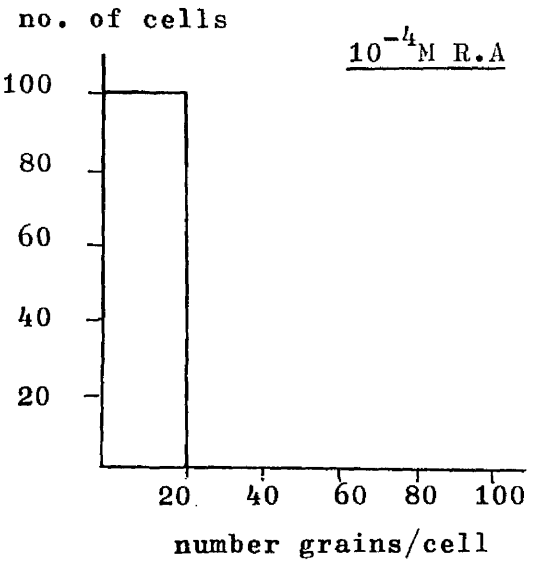
V79 CELLS +CIS RETINOIC ACID : 60 MIN



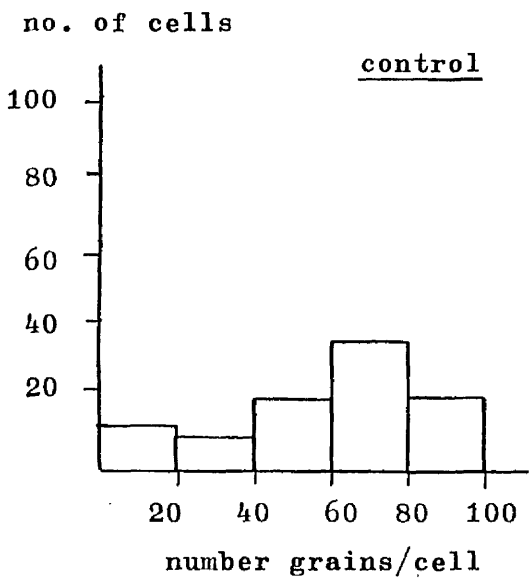


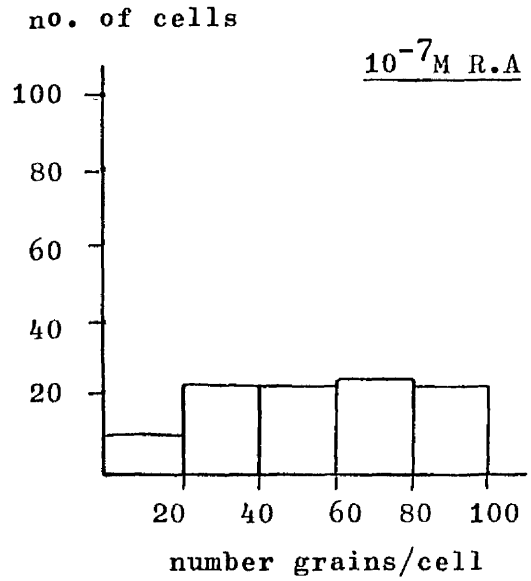


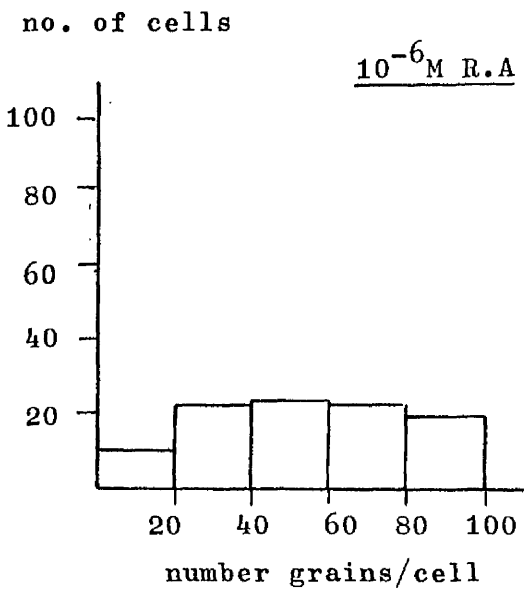


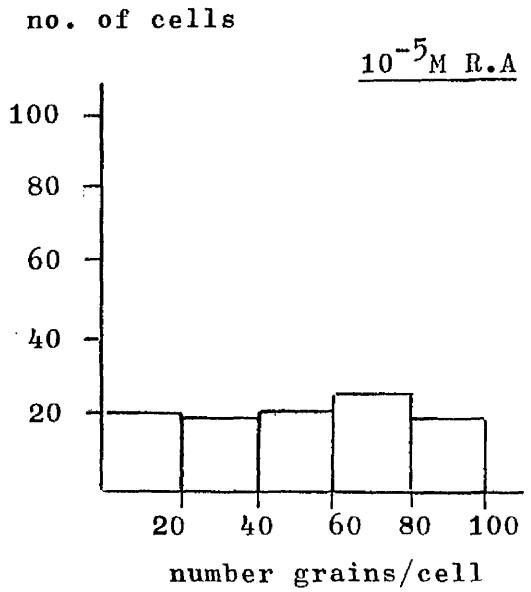


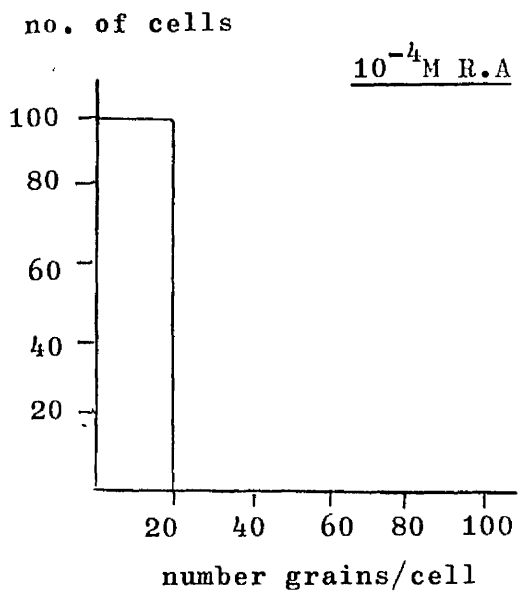
V79 CELLS +CIS RETINOIC ACID : 180 MIN



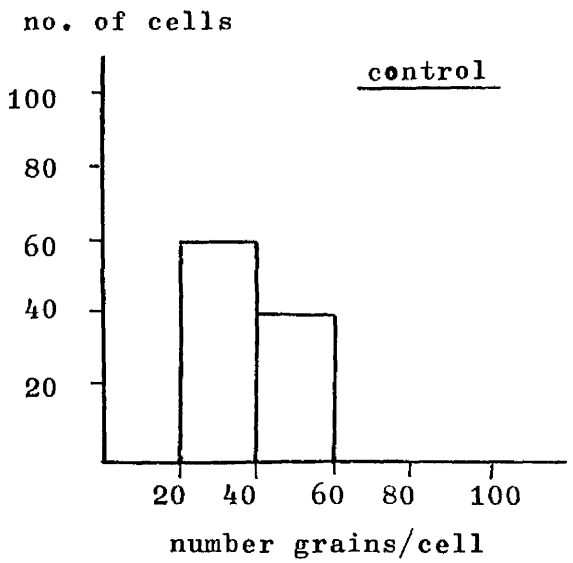


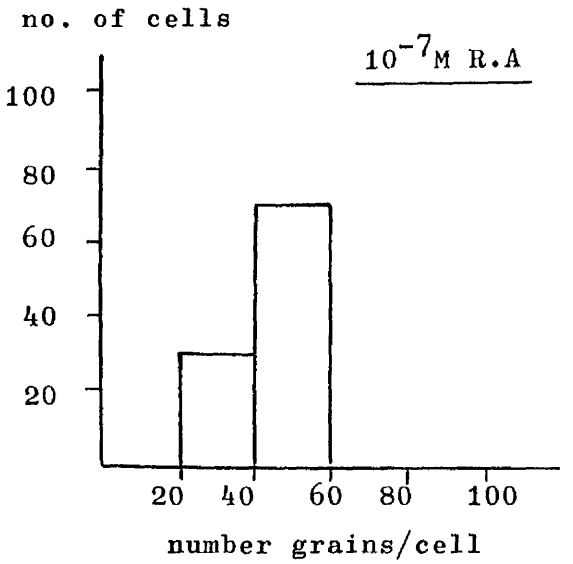


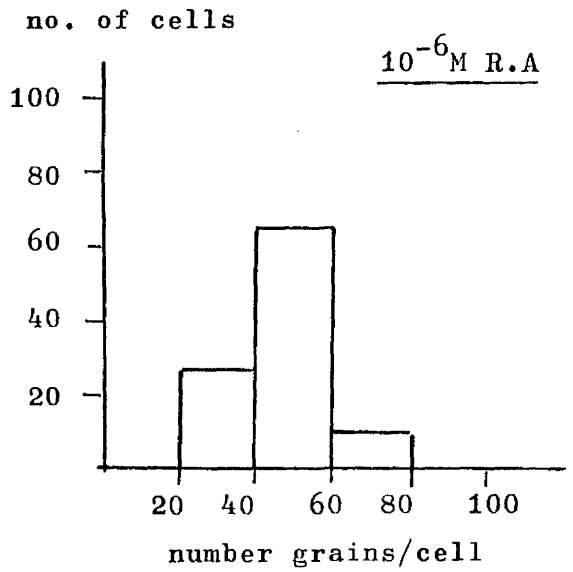


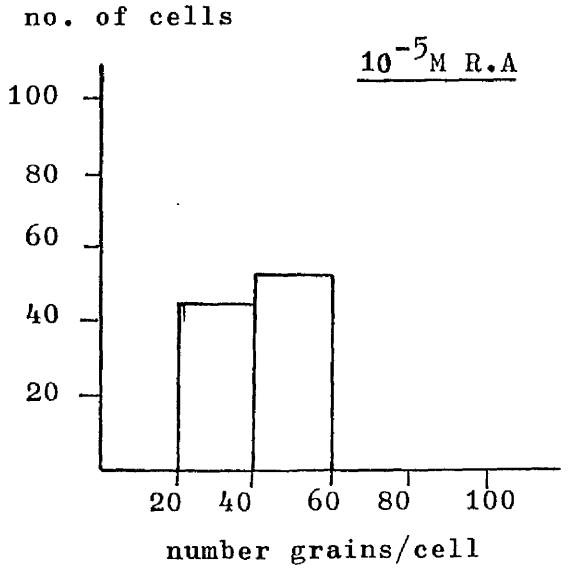


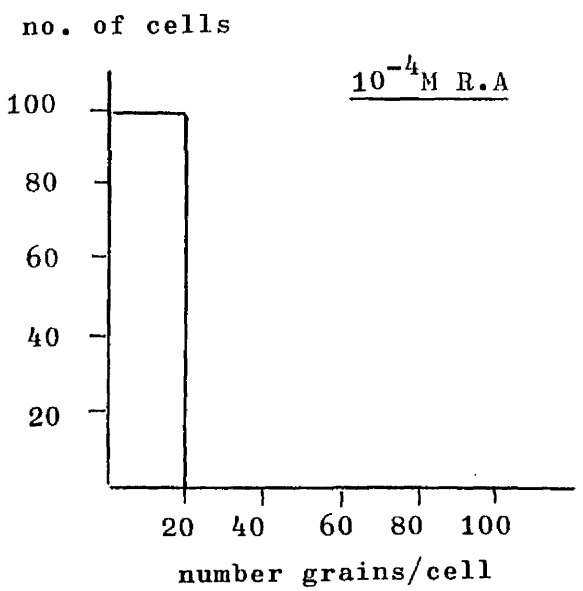
C13 CELLS +TRANS RETINOIC ACID : 30 MIN



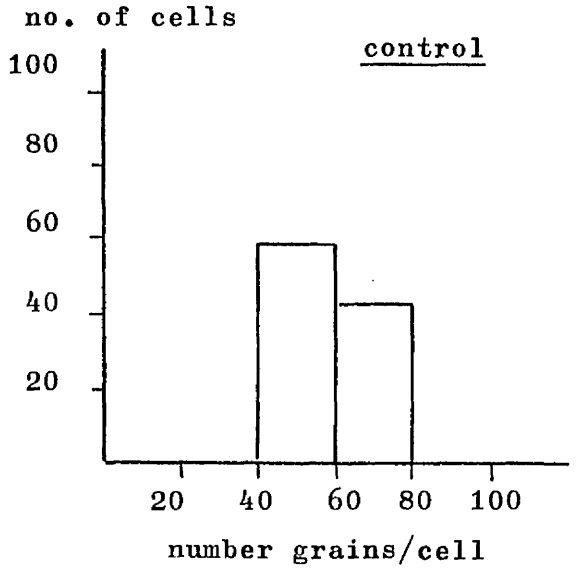


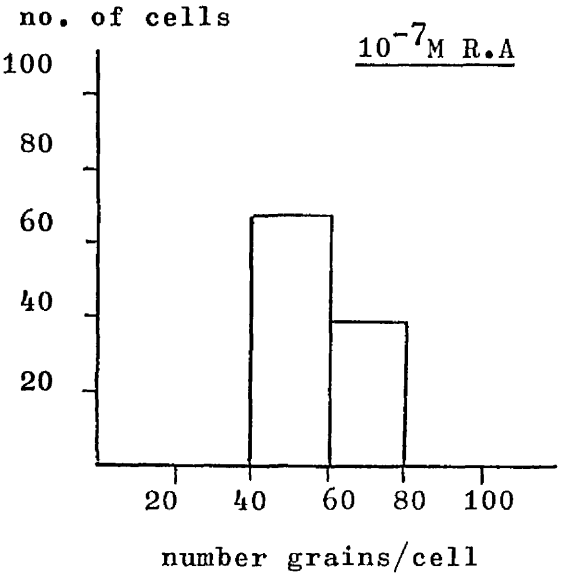


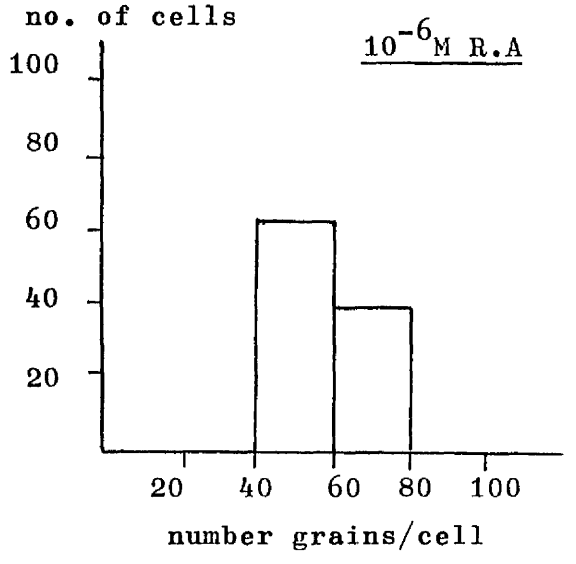


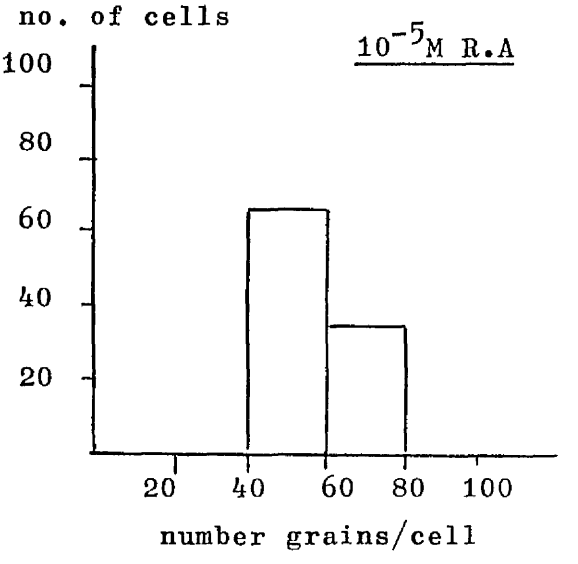


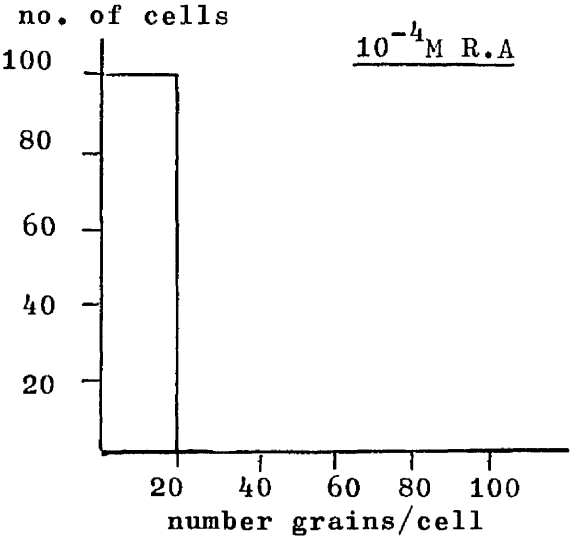
C13 CELLS +TRANS RETINOIC ACID : 60 MIN



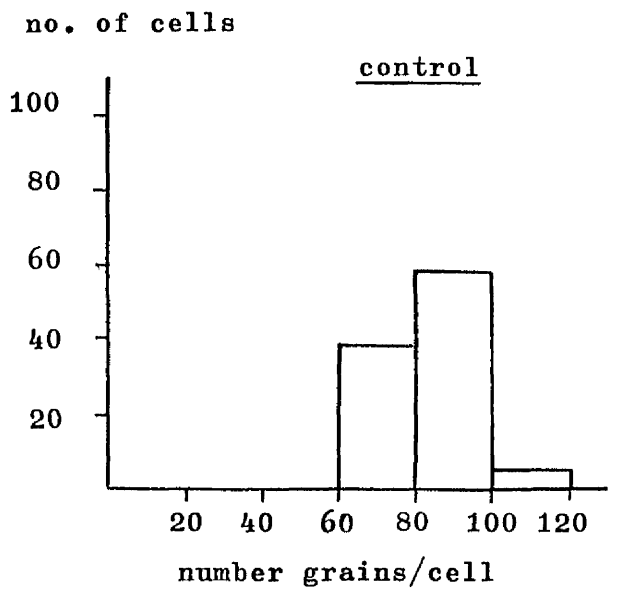


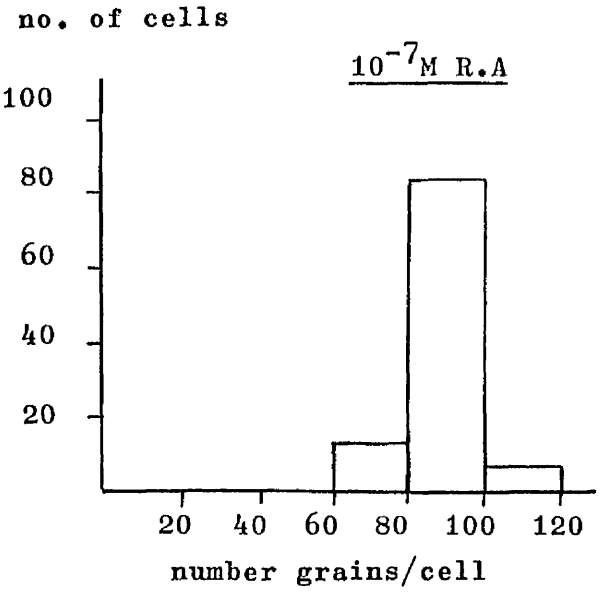


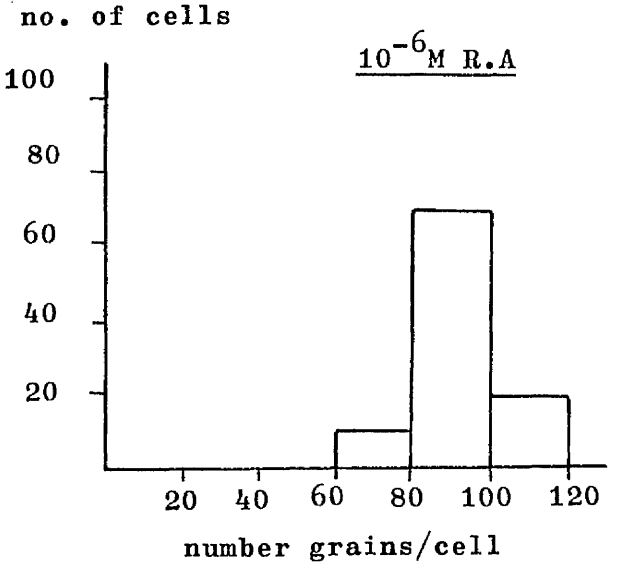


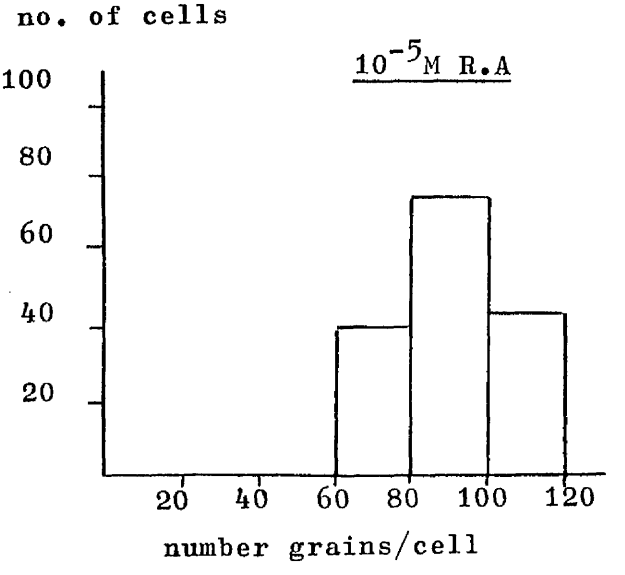


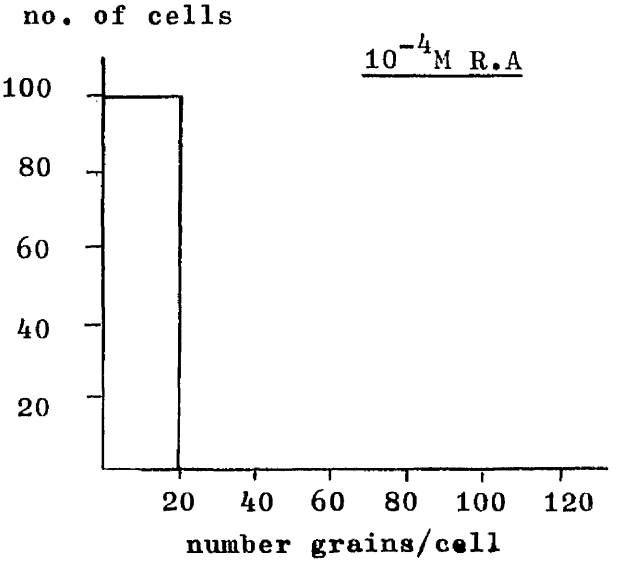
C13 CELLS +TRANS RETINOIC ACID : 180 MIN



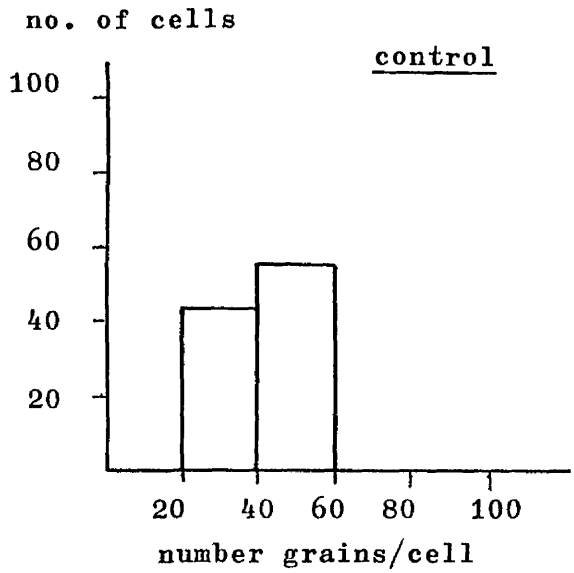


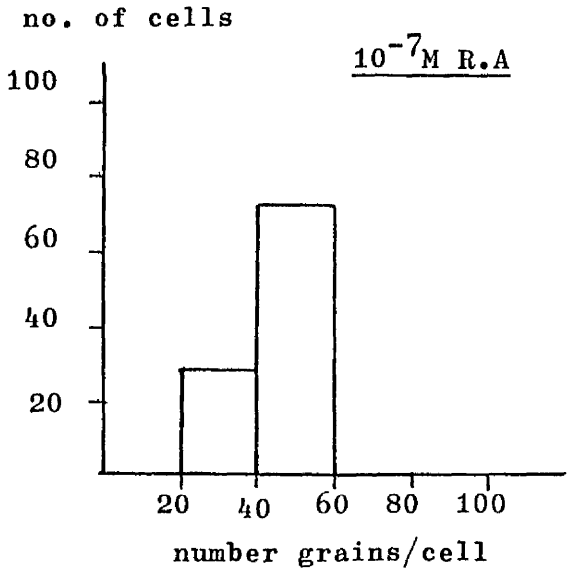


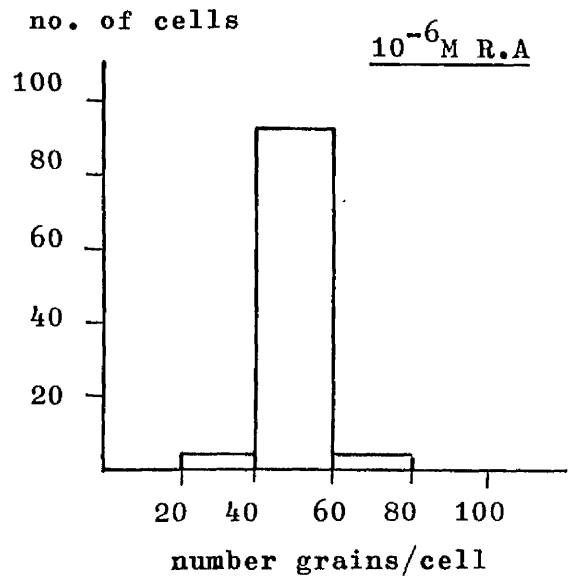


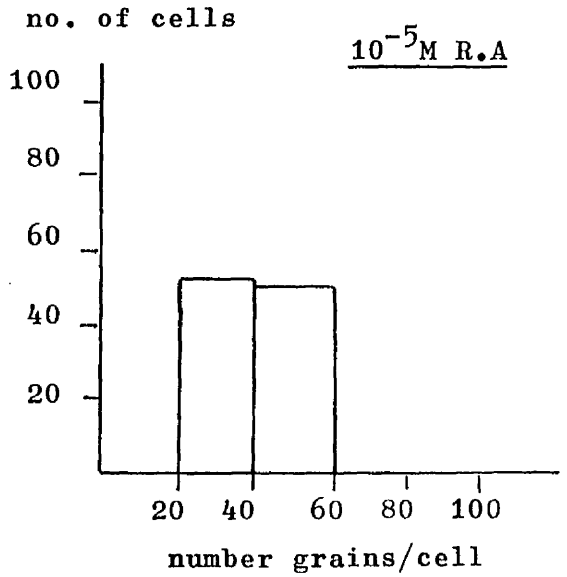


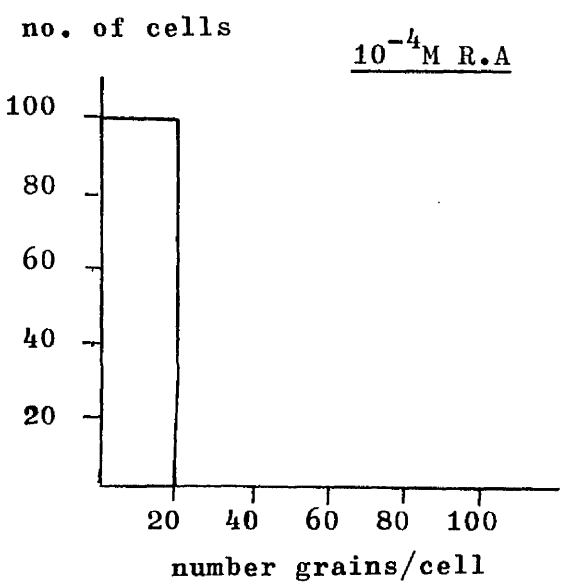
C13 CELLS +CIS RETINOIC ACID : 30 MIN



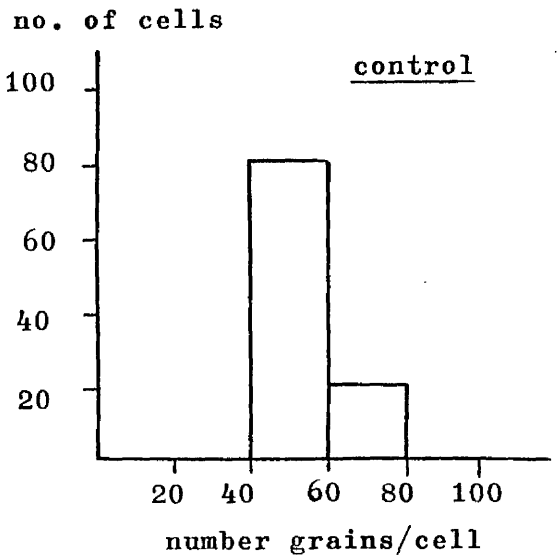


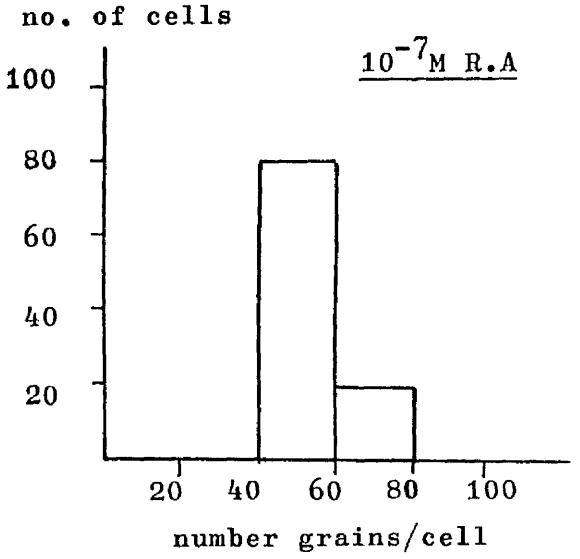


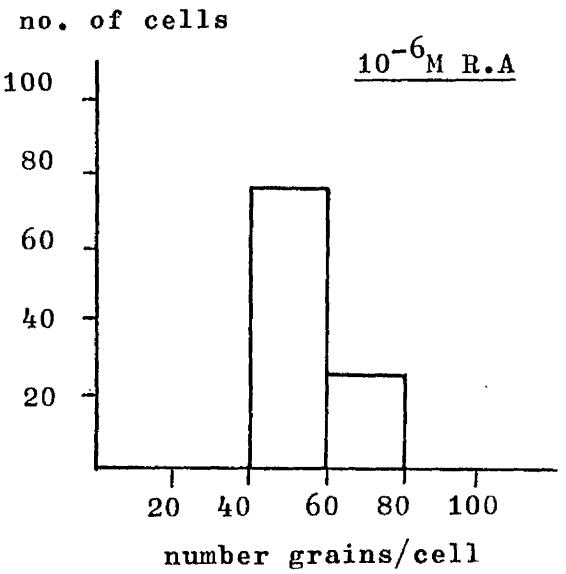


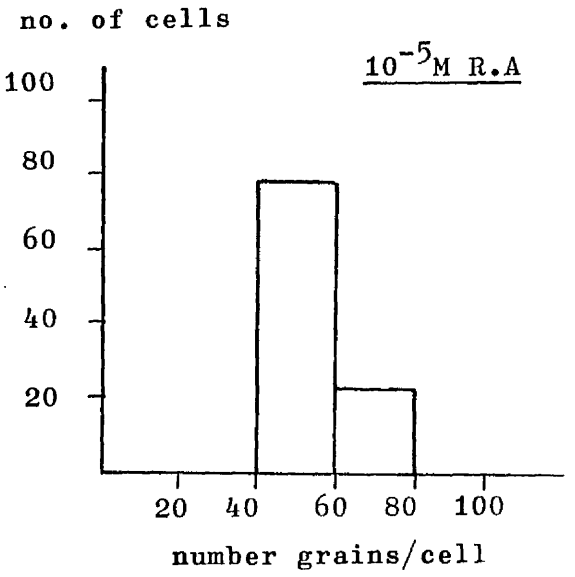


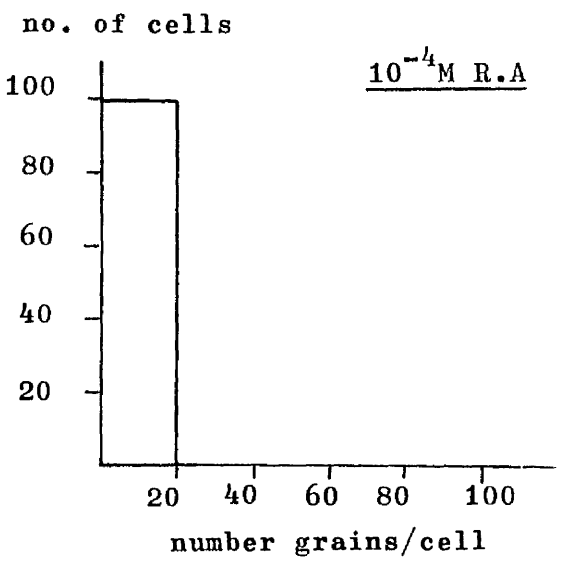
C13 CELLS +CIS RETINOIC ACID : 60 MIN



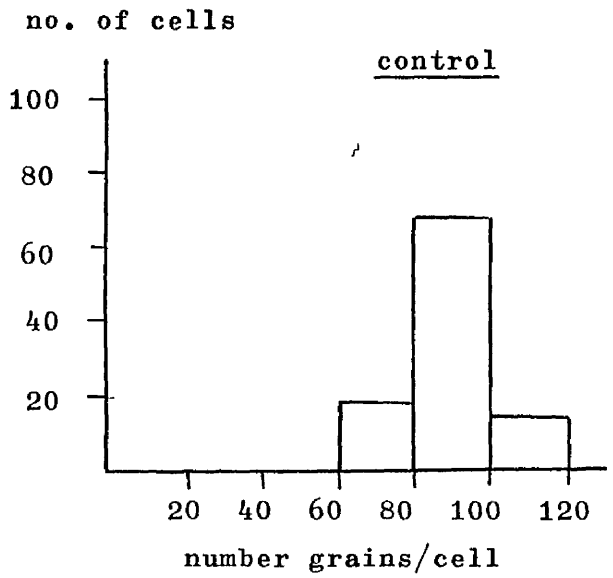


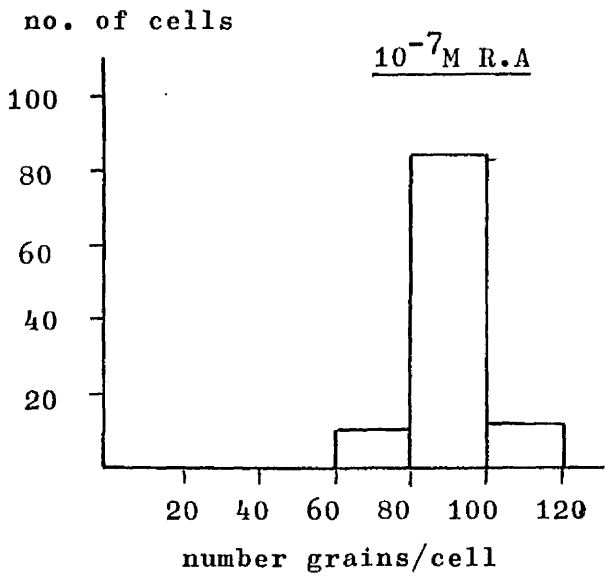


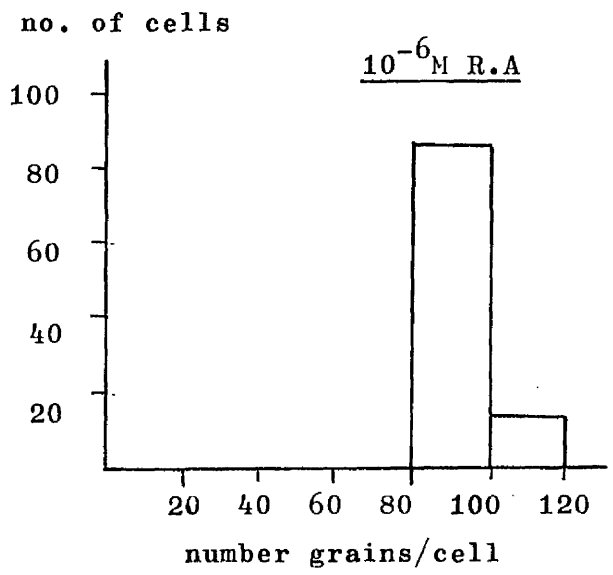


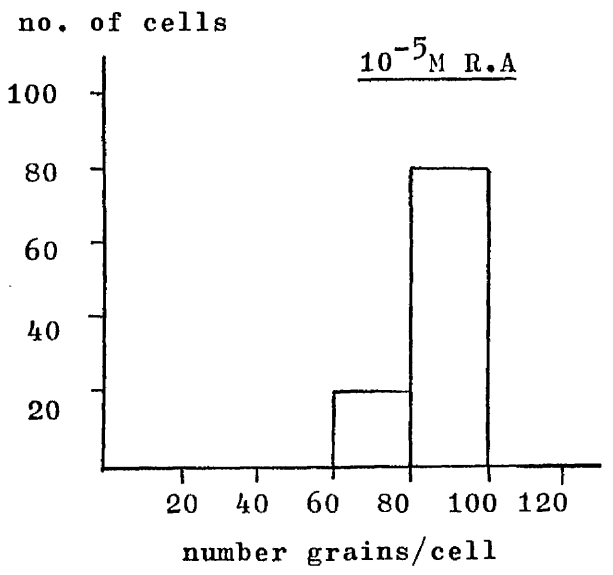


C13 CELLS +CIS RETINOIC ACID : 180 MIN









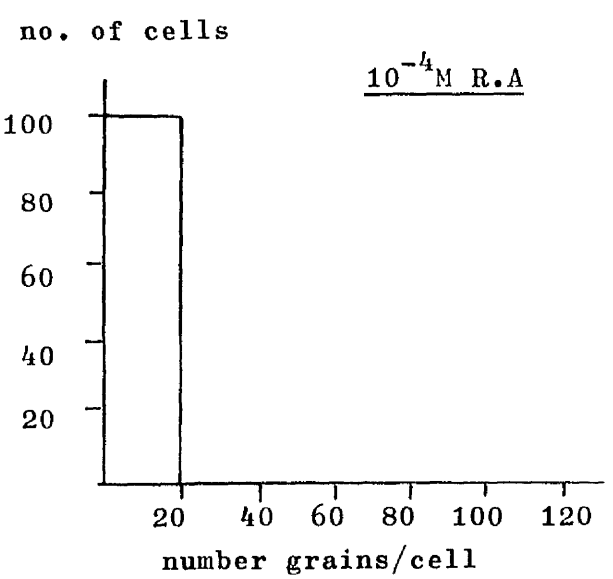


Table 19 Effect of Different Concentrations of Trans and 13 cis Retinoic Acid (10^{-4}M - 10^{-7}M) on Uridine Nucleotide Transfer between GCCM and NORT Cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 10µl DMSO (control) or with 2.0ml medium + 10µl DMSO containing 13 cis or all trans retinoic acid to give the final concentrations indicated.

After co-culturing for 60min or 180min as shown in Table 19, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 25 recipient cells in contact with donor cells and over 25 isolated recipient cells (see section 2.2.10.).

	Donor	Recipient	Donor	Recipient	Donor	Recipient
	GCCM	GCCM	GCCM	GCCM	GCCM	NORT
	(1h coculture)		(3h coculture)		(3h coculture)	
	Recipients in Contact	Isolated Recipients	Recipients in Contact	Isolated Recipients	Recipients in contact	Isolated Recipients
	Mean(S.D.)	Mean (S.D.)	Mean(S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
ANS RA						
$^{-4}$M	4.5(3.4)	5.5(2.8)	6.0(3.4)	6.3(3.5)	6.3(2.4)	7.4(3.0)
$^{-5}$M	3.8(3.2)	1.9(2.3)	6.0(2.9)	5.6(2.5)	106.4(20.3)	7.7(3.5)
$^{-6}$M	5.4(3.5)	4.1(2.8)	7.8(11.8)	6.1(1.2)	115.0(18.4)	6.5(2.1)
$^{-7}$M	4.4(3.4)	2.3(2.0)	25.4(38.7)	5.6(2.5)	100.8(34.6)	7.0(3.1)
ntrol	2.9(1.9)	2.6(2.5)	29.6(28.6)	5.8(3.7)	102.6(30.6)	5.1(2.5)

	Donor	Recipient	Donor	Recipient	Donor	Recipient
	GCCM	GCCM	GCCM	GCCM	GCCM	NORT
	(1h coculture)		(3h coculture)		(3h coculture)	
	Recipients in Contact Mean(S.D.)	Isolated Recipients Mean(S.D.)	Recipients in Contact Mean (S.D.)	Isolated Recipients Mean(S.D.)	Recipients in contact Mean(S.D.)	Isolated Recipients Mean(S.D.)
s RA						
-4_M	6.0(1.4)	3.2(3.3)	6.9(4.1)	8.4(3.8)	5.8(3.5)	4.7(2.5)
-5_M	6.0(4.1)	5.0(3.9)	12.4(21.0)	6.8(4.4)	98.8(36.4)	3.7(3.1)
-6_M	8.5(6.4)	5.2(2.9)	13.9(13.0)	8.6(3.7)	104.6(38.8)	6.1(2.3)
-7_M	6.4(3.7)	3.4(3.4)	27.2(29.7)	7.5(1.8)	98.0(21.0)	6.2(2.9)
ntrol	6.7(7.2)	6.6(2.1)	22.2(27.6)	5.8(3.2)	103.7(59.3)	6.6(3.0)

Table 20 Uridine Nucleotide Transfer Between Combinations of BRL, CK and C13 Cells.

Labelled donor cells were prepared as described in section 2.2.8. and added to recipient cells (see section 2.2.8.).

After 3h co-culture the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10.).

The results shown are from one experiment and are representative of duplicate experiments.

Donor	Recipient	Recipients in Contact Mean(S.D.)	Isolated Recipients Mean(S.D.)	t	d.f.	P.
CK	CK	19.9(1.5)	3.4(1.0)	49.3	98	<0.001
CK	C13	4.2(0.9)	3.8(0.9)	1.7	98	N.S.
CK	BRL	3.1(0.7)	3.2(0.7)	0.5	98	N.S.
CK	A9	3.6(0.9)	3.7(1.1)	0.4	98	N.S.
C13	C13	28.7(2.5)	3.1(1.1)	50.6	98	<0.001
C13	CK	5.2(0.9)	4.8(0.9)	1.7	98	N.S.
C13	BRL	3.9(0.8)	3.5(0.5)	0.8	98	N.S.
C13	A9	3.5(1.1)	3.3(0.9)	2.2	98	N.S.
BRL	BRL	13.6(1.3)	3.3(0.8)	35.1	98	<0.001
BRL	CK	4.3(0.9)	3.9(0.7)	1.9	98	N.S.
BRL	C13	3.3(0.6)	2.9(0.6)	2.6	98	N.S.
BRL	A9	2.9(0.8)	3.2(0.7)	1.5	98	N.S.

Table 21 Chromatographic Separation of 13 cis Retinoic Acid, All Trans Retinoic Acid and Tigason.

For experimental details see section 2.2.13.

Solvent 1	chloroform : petroleum ether (4:1)
Solvent 2	chloroform : acetone (4:1)
Solvent 3	chloroform : acetone (99:1)

r.f value = distance migrated from origin (cm).

compound	r.f. value		
	Solvent 1	Solvent 2	Solvent 3
13cis Retinoic Acid	5.4	5.7	5.6
All Trans Retinoic Acid	5.4	5.8	6.0
Tigason	9.6	10.4	10.5

Table 22 Effect of Eluted All Trans Retinoic Acid on Uridine Nucleotide Transfer Between C13 Cells.

13 cis retinoic acid, all trans retinoic acid and tigason were applied to thin layer silica gel plates (see section 2.2.13.) and separated in the solvent system chloroform : acetone (99:1). The developed chromatogram was viewed under u.v. light and the position of the spots marked. All trans retinoic acid was eluted as described in section 2.2.14.

The cells used as donor and recipients were C13 cells. Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml fresh medium + 50µl Etoh (control) or with 2.0ml medium containing the eluted all trans retinoic acid. (final concentration retinoic acid 10^{-4}M).

After 3h coculture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 25 recipient cells in contact with donor cells and over 25 isolated recipient cells (see section 2.2.10.).

	Recipients in Contact Mean(S.D.)	Isolated Recipients Mean(S.D.)	t	d.f.	P.
control	122.0(41.7)	3.2(2.1)	19.9	48	<0.001
eluted trans retinoic acid	3.7(3.5)	2.9(2.6)	1.3	48	N.S.

Figure 27 Effect of Eluted All Trans Retinoic Acid on Uridine Nucleotide Transfer Between C13 Cells.

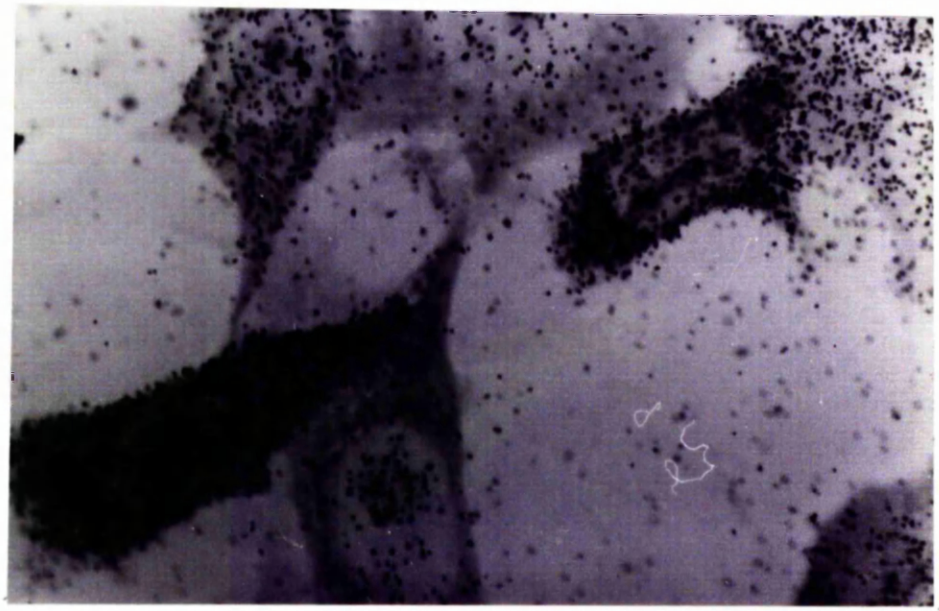
Experimental details as described in legend to Table 22. Autoradiographs were exposed for 1 week.

a) C13 donor and recipient cells in control culture

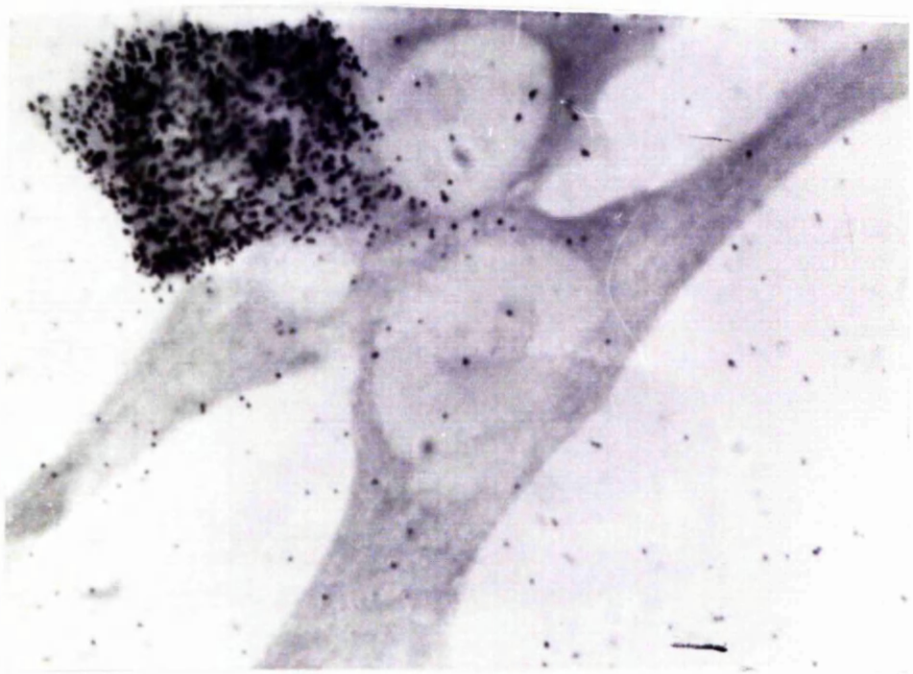
b) C13 donor and recipient cells in culture with eluted all trans retinoic acid.

Magnification x 1460

a)



b)



4.3. CONCLUSIONS.

Retinoic acid (10^{-4}M) inhibits junctional communication between all cells in culture which were tested. This inhibition occurs rapidly (within minutes) and is also rapidly and fully reversible. Some cells were found to be more sensitive to retinoic acid, (e.g. V79 cells) and junctional communication between such cells was inhibited by concentrations as low as 10^{-6}M trans retinoic acid. There appears to be two mechanisms of inhibition of junctional communication. Retinoic acid probably inhibits junctional communication between all the cells tested by reducing junctional permeability, either by reducing the proportion of channels open or by reducing the number of channels. Junction formation between some cells (e.g. V79 cells) also appears to be reduced by retinoic acid.

Chapter 5 - The Effect of Retinoic Acid on the Isolation of Junctional Protein From Cells in Culture.

5.1. INTRODUCTION.

Retinoic acid has been shown to inhibit junctional communication between cells in culture (Pitts et al, 1981; Hamilton et al, 1982). This appears to contradict morphological observations made after the application of retinoic acid to chick embryo shank skin and to skin tumours which show an increased proliferation of gap junctions (Prutkin, 1975; Elias and Friend, 1976; Elias et al, 1981) and which have been interpreted as a retinoid induced increase in junctional communication.

The next section attempts to relate the morphological and functional observations by looking at the effect of retinoic acid on the isolation of the junctional protein from cells in culture using conditions where a parallel investigation of intercellular communication is possible.

5.2. THE EFFECT OF RETINOIC ACID ON ISOLATION OF JUNCTIONAL PROTEIN FROM CELLS IN CULTURE.

Retinoic acid has been shown to inhibit junctional communication, measured by [^{3}H]-uridine nucleotide transfer, between cells in culture (see chapter 4). This inhibition could be due to retinoic acid acting as a detergent and causing dispersal of junctional plaques in the cell surface, to inhibition of the formation or breakdown of junctions between contacting cells or to inhibition of channel permeability. This section examines the effect of retinoic acid on junctional plaques at the cell surface. For a discussion of the other possible mechanisms of inhibition of intercellular communication see section 4.2.

BRL cells were treated with 10^{-4}M retinoic acid for 1h before junction isolation to determine if junctional plaques were still present in treated cells. The junction rich fraction was analysed by SDS PAGE and the effect of retinoic acid on the junctional plaques quantitated by measuring the amount of the junctional protein present (Figs 28, 29 and Table 23). The isolation method will isolate only those junctions present as junctional plaques (i.e. aggregates of junctional particles) and individual junctional particles will be lost during the isolation procedure (see section 3.1.). Figure 28 shows that it is still possible to isolate junctional protein from retinoic acid treated cells and it therefore appears likely that the junctional plaques are still present. Junctional protein isolated from retinoic acid treated cells, even after 24h treatment, retains the characteristic property of heat induced aggregation in SDS buffers (Fig 32). Junctional morphology also appears

unaffected by treatment with retinoic acid (Fig 33).

The increase in the number of proteins present in junction enriched fractions from BRL cells treated with retinoic acid (10^{-4}M) is due to the use of an old batch of trypsin in the isolation, believed to have lost much of its activity (Fig 32). The control isolation was carried out some days later using a fresh supply of trypsin.

These results suggest that the inhibition of junctional communication between BRL cells by retinoic acid is not due to either dispersal of junctional plaques or to inhibition of junction formation. The retinoic acid containing medium removed from cells prior to junction isolation was tested in a uridine nucleotide transfer experiment to check the stability of the retinoic acid after the 1h incubation. The medium was added to recipient cells before the addition of donor cells prelabelled with [^{3}H]-uridine. Donor and recipient cells were co-cultured for 3h and the cells fixed and prepared for autoradiography. Grains were counted over recipient cells in contact with donor cells. Recipient cells become labelled only if they form permeable junctions with donor cells. It was found that the retinoic acid containing medium inhibited uridine nucleotide transfer between these BRL cells (Table 24). This suggests that the gap junctions isolated from retinoic acid treated cells were in a closed form. Fig 29 and Table 23 show that the amount of junctional protein isolated from 1h retinoic acid treated cells is increased by 30%. This was the maximum increase observed in cells treated with retinoic acid, other increases in 4 separate experiments ranged from 5% - 20% (results not

shown).

When the length of time of exposure to retinoic acid is increased from 1h to 24h, there is a further increase in the amount of junctional protein which can be isolated from retinoic acid treated BRL cells (Figs 30 and 31, Table 23). The increase in junctional protein in 4 different experiments varied between 1.9 and 3.1 times that isolated from control BRL cells (results not shown). Since there is a greater possibility that the retinoic acid may become unstable over a 24h period, again the retinoic acid containing medium was removed and samples added to cultures of recipient cells before the addition of pre-labelled donor cells and the effect on uridine nucleotide transfer examined (Table 24). The results in Table 24 show the retinoic acid containing medium still inhibits uridine nucleotide transfer between recipient cells and donor cells in contact.

These results suggest that prolonged exposure (24h) of cells to 10^{-4}M retinoic acid leads to an increase in the number of junctional plaques present, which are in a closed state. Elias et al, (1981) have reported a quantitatively similar increase in the number of gap junctions in basal cell carcinomas in response to topical application of cream containing 1% retinoic acid. This was interpreted by these authors as a retinoic acid induced increase in junctional communication although no assay for communication was performed. In every experiment the medium containing retinoic acid to which the cells were exposed was effective in inhibiting junctional communication, and it is therefore possible that the junctions seen by Elias et al, (1981) were also in a closed state.

The purification procedure isolates junctional plaques but it is not possible to say whether the plaques originated from the cell surface or from internalised (annular) gap junctions. Annular gap junctions are believed to represent one stage in the turnover of gap junctions and since they retain their plaque structure should therefore be isolated along with cell surface junction plaques. Retinoic acid treatment of BRL cells might induce the formation of annular gap junctions (by promoting turnover, for example) and such an effect has been observed in retinoic acid treated keratocanthoma of rabbit skin (Prutkin, 1975). Annular gap junctions were not observed however by Elias et al, (1981) in the basal cell carcinomas treated with retinoic acid. The results presented here cannot distinguish between a retinoic acid induced increase in cell surface junctions and an increase in annular gap junctions. This would require thin-section or freeze-fracture EM analysis of BRL cells treated with retinoic acid.

A preliminary result (Fig 34) suggests that the retinoic acid induced increase in the number of gap junctions may be an effect which can be dissociated from the inhibition of junctional communication. BRL cells were treated with a concentration of retinoic acid which has no detectable effect on junctional communication (10^{-7}M) and the junctions isolated. The junction enriched pellet was analysed by SDS PAGE, and this showed an apparent increase in the amount of the junctional protein compared to that isolated from control cells. The increase is similar to that in BRL cells treated with 10^{-4}M retinoic acid. The

implications of this result will be discussed in Chapter 6.

V79 cells behave differently on prolonged treatment with retinoic acid. Gap junctional protein can still be isolated from retinoic acid treated (for 1h and 24h) V79 cells (Figs 35, 36 and Table 15) but there is a decrease of 15% compared to the levels of junctional protein in control cultures (Table 25). The retinoic acid containing medium removed from these cells before junction isolation was also added to cultures of recipient cells prior to the addition of prelabelled donor cells and uridine nucleotide transfer was again found to be inhibited (Table 26), suggesting that the junctions isolated from retinoic acid treated V79 cells are also in a closed state. Again junctional morphology appears unaffected by the retinoic acid treatment (Fig 37).

Figure 28 Effect of Trans Retinoic Acid (10^{-4}M for 1h) on the Isolation of the 16K Protein From BRL Cells.

Gap junctions were isolated from 3 x 3 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

1h before junction isolation, the medium was removed and replaced with 150ml EC_{10} + 750µl DMSO (control) or with 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M).

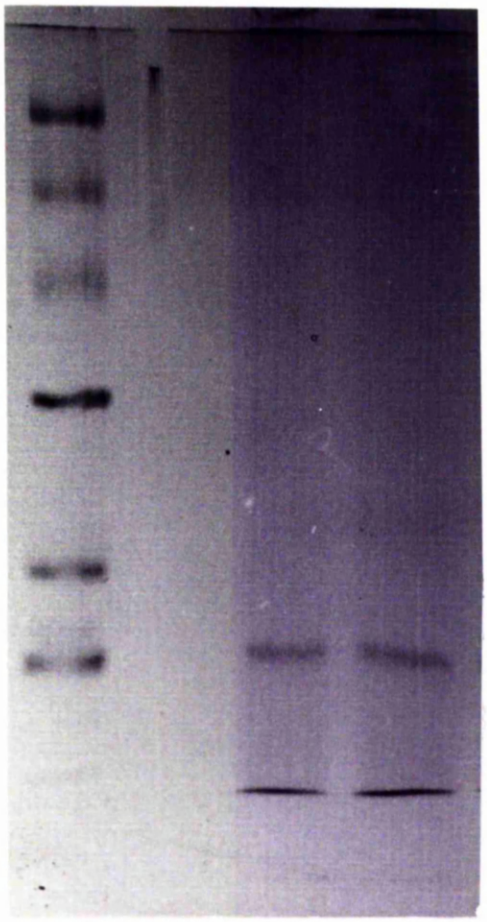
The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio Rad M.Wt Marker Proteins

Lane 2 : junction enriched pellet - control BRL

Lane 3 : junction enriched pellet BRL + 10^{-4}M trans RA (1h)

For quantitation of relative amounts of protein see Fig 29 and Table 23.



LANE 1 2 3

Figure 29 Treatment of BRL cells with 10^{-4}M Trans RA (for 1h): Quantitation of Relative Amounts of 16K Protein.

For experimental details see legend to Fig 28.

The relative amount of the 16K protein in control and trans retinoic acid treated BRL cells was measured by the absorption of Coomassie stain in the gel bands using a Joyce-Loebel microdensitometer (see section 2.2.5.).

a) BRL cells + 10^{-4}M trans retinoic acid (1h)

b) control BRL cells.

For relative amounts see Table 23.

a)

b)

Figure 30. Effect of Trans Retinoic Acid (10^{-4}M for 24h) on the Isolation of the 16K Protein From BRL Cells.

Gap junctions were isolated from 2 x 5 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation, the medium was removed and replaced with 150ml EC_{10} + 750µl DMSO (control) or with 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M).

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

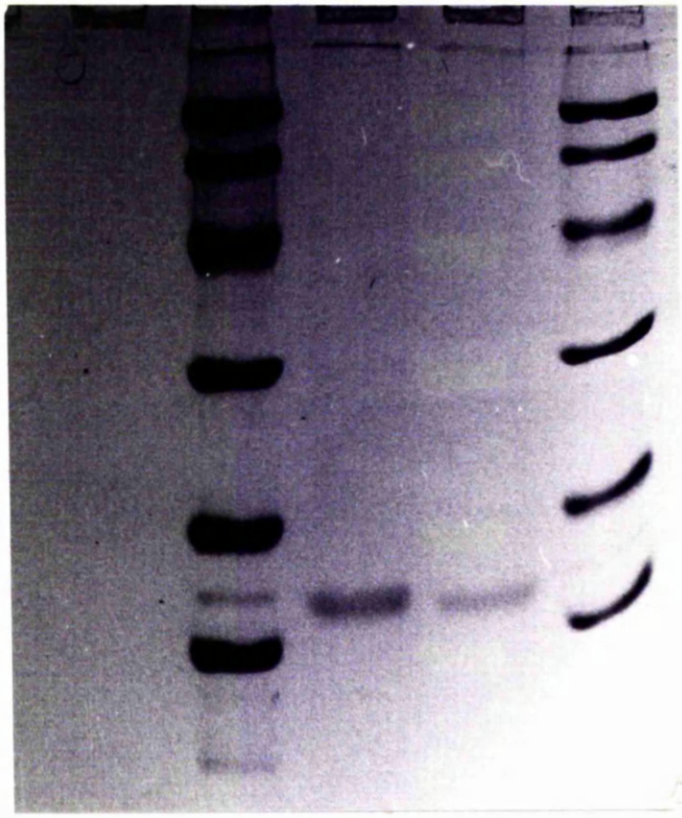
Lane 1 : Bio-Rad M.Wt marker proteins (10µg protein)

Lane 2 : junction enriched pellet : BRL + 10^{-4}M trans RA (24h)

Lane 3 : junction enriched pellet : control BRL

Lane 4 : Bio-Rad M.Wt marker proteins (5µg protein)

For quantitation of relative amounts of protein see Fig 31 and Table 23.



LANE 1 2 3 4

Figure 31. Treatment of BRL Cells with 10^{-4}M Trans RA (for 24h): Quantitation of Relative Amount of 16K Protein.

For experimental details see legend to Fig 30.

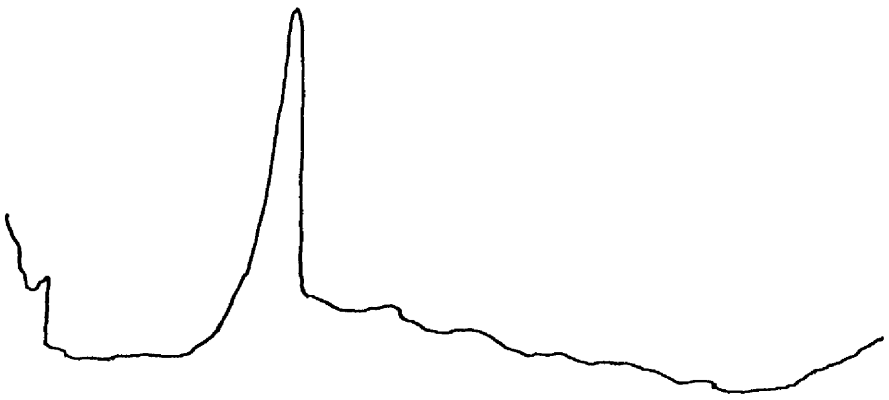
The relative amount of the 16K protein in control and trans retinoic acid treated BRL cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

a) control BRL cells

b) BRL cells + 10^{-4}M trans retinoic acid (24h)

For relative amounts see Table 23.

a)



b)

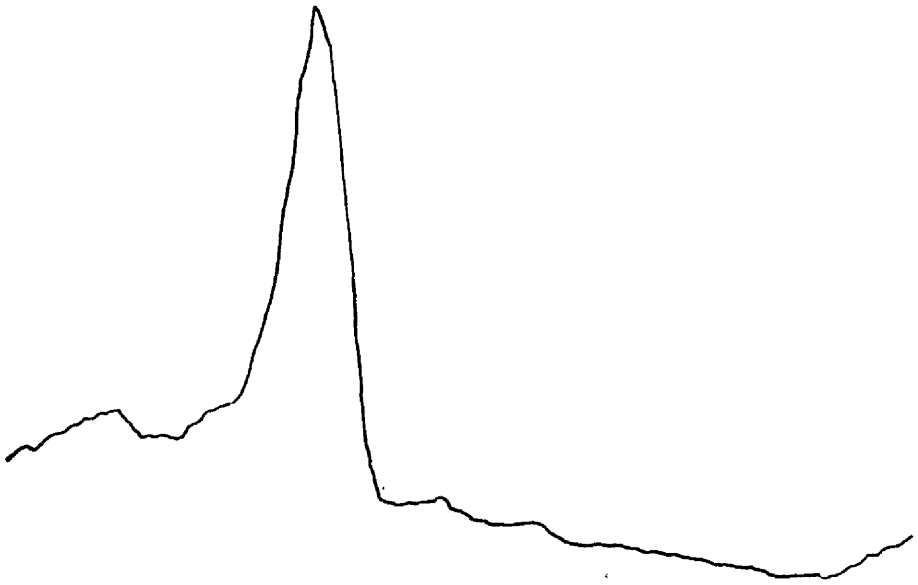


Table 23 Quantitation of Relative Amounts of 16K Protein From Retinoic Acid Treated (1h and 24h)BRL Cells.

For experimental details see legend to Fig 28 and Fig 30.

The relative amount of the 16K protein in control and trans retinoic acid treated BRL cells (1h and 24h) was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

The mean % increase (standard deviation) in the amount of the 16K protein isolated from BRL cells treated with retinoic acid (10^{-4}M) for 1h compared to control amounts from 4 separate experiments = 12.3(8.9). Range of values in the 4 experiments = 5% - 20%.

The mean % increase (standard deviation) in the amount of the 16K protein isolated from BRL cells treated with retinoic acid (10^{-4}M) for 24h compared to control amounts from 4 separate experiments = 157.5%(51.2). Range of values in the 4 experiments = 90% - 210%.

Treatment	Area Under 16K Peak (Arbitory Units)	Ratio Control : RA treated
Control	0.024	
10^{-4}M trans RA (1h)	0.032	1 : 1.3
Control	0.0120	
10^{-4}M trans RA (24h)	0.0379	1 : 3.1

Figure 32. Heat Induced Aggregation of the 16K Protein Isolated From BRL Cells Treated with 10^{-4}M Trans Retinoic Acid for 24h.

Gap junctions were isolated from 2 x 10 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation the medium was removed and replaced with 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M), or with 150ml EC_{10} + 750µl DMSO (control).

After dissolving the final junction enriched pellet in 40µl 1% SDS, the sample was split into two equal aliquots, 20µl were left at room temperature for 10min and the other 20µl heated to 100°C for 5min. The samples were then analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

This experiment was carried out only once.

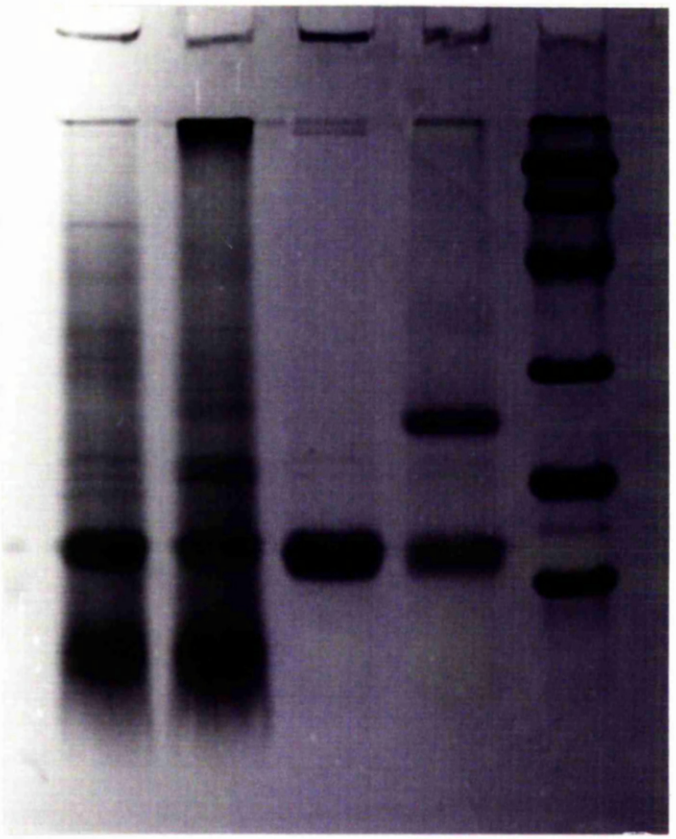
Lane 1 : junction enriched pellet BRL + 10^{-4}M RA (24h) - solubilised at room temperature

Lane 2 : junction enriched pellet BRL + 10^{-4}M RA (24h) - heated to 100°C

Lane 3 : junction enriched pellet BRL (control) - solubilised at room temperature

Lane 4 : junction enriched pellet BRL (control) - heated to 100°C

Lane 5 : Bio-Rad M.Wt marker proteins



LANE 1 2 3 4 5

Figure 33 Electron Micrograph of Junction Enriched Pellet Isolated from Retinoic Acid Treated BRL Cells.

Gap junctions were isolated from 10 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation, the medium was removed and replaced with 150ml EC_{10} + 750µl DMSO containing 2×10^{-2}M trans RA (final concentration 10^{-4}M).

The final junction enriched pellet was prepared for electron microscopy as described in Section 2.2.7.

Magnification x 100,000

a)

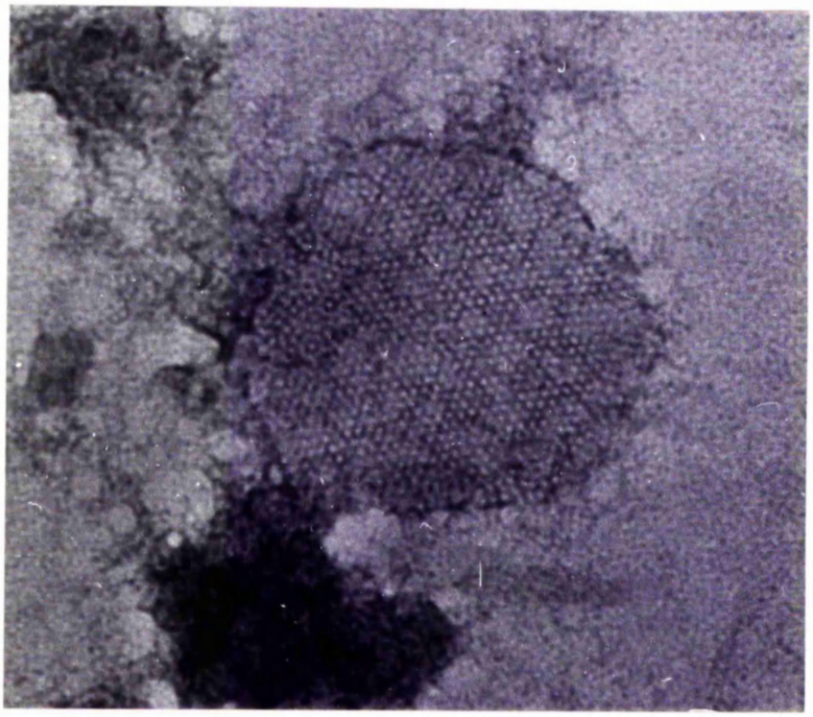


Table 24 Effect of Trans Retinoic Acid From Treated Cultures on Uridine Nucleotide Transfer Between BRL Cells.

BRL cells were treated with 10^{-4}M trans retinoic acid for 1h and 24h before gap junction isolation (as described in legend to Fig 28 and Fig 30).

The retinoic acid containing medium and control medium were removed and their effect tested on uridine nucleotide transfer between BRL cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml control medium, or 2.0ml medium + 10^{-4}M trans retinoic acid.

After 3h co-culture the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10).

	Recipients in Contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t.	d.f.	P.
Control	22.9(1.9)	4.7(0.6)	63.6	98	<0.001
10^{-4}M Trans RA (1h)	4.1(0.7)	4.1(0.7)	0.1	98	N.S.
Control	26.8(1.9)	5.7(1.0)	68.3	98	<0.001
10^{-4}M Trans RA (24h)	6.1(0.9	5.9(0.8)	1.7	98	N.S.

Figure 34 Effect of 10^{-4}M and 10^{-7}M Trans Retinoic Acid on The Isolation of the 16K Protein From BRL Cells.

Gap junctions were isolated from 3 x 3 confluent roller bottles of BRL cells using Isolation Protocol II as described in section 2.2.3.

24h before isolation, the medium was removed and replaced with either 150ml EC_{10} + 750µl DMSO (control), 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M or with 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-5}M trans RA (final concentration 10^{-7}M).

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio Rad M.Wt. Marker proteins

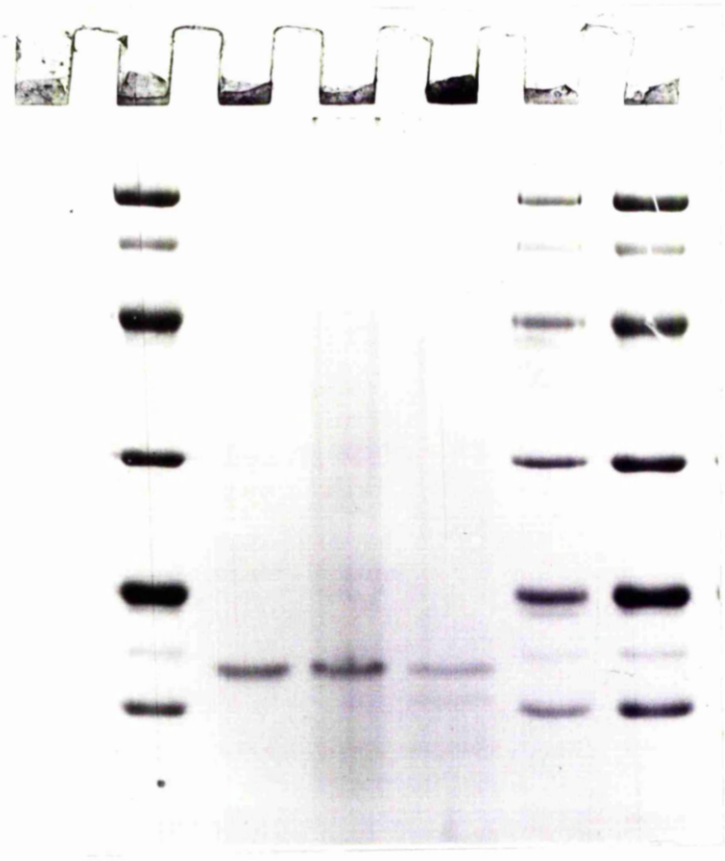
Lane 2 : junction enriched pellet - BRL + 10^{-7}M trans RA

Lane 3 : junction enriched pellet - BRL + 10^{-4}M trans RA

Lane 4 : junction enriched pellet - control BRL

Lane 5 : Bio Rad M.Wt. marker proteins

Lane 6 : Bio Rad M. Wt. marker proteins



LANE 1 2 3 4 5 6

Figure 35 Effect of Trans Retinoic Acid on Isolation of the 16K Protein from V79 Cells.

Gap junctions were isolated from 3 x 5 confluent roller bottles of V79 cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation the medium was removed from 5 roller bottles and replaced with 15oml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M). The medium was removed from the remaining 10 roller bottles, 1h before junction isolation and replaced with 150ml EC_{10} + 750µl DMSO (control) or with 150ml EC_{10} + 750µl DMSO containing 2 x 10^{-2}M trans RA (final concentration 10^{-4}M).

The final junction enriched pellet was dissolved in 20µl 1% SDS at room temperature and analysed by SDS polyacrylamide gel electrophoresis as described in section 2.2.4.

Lane 1 : Bio-Rad M.Wt. marker proteins

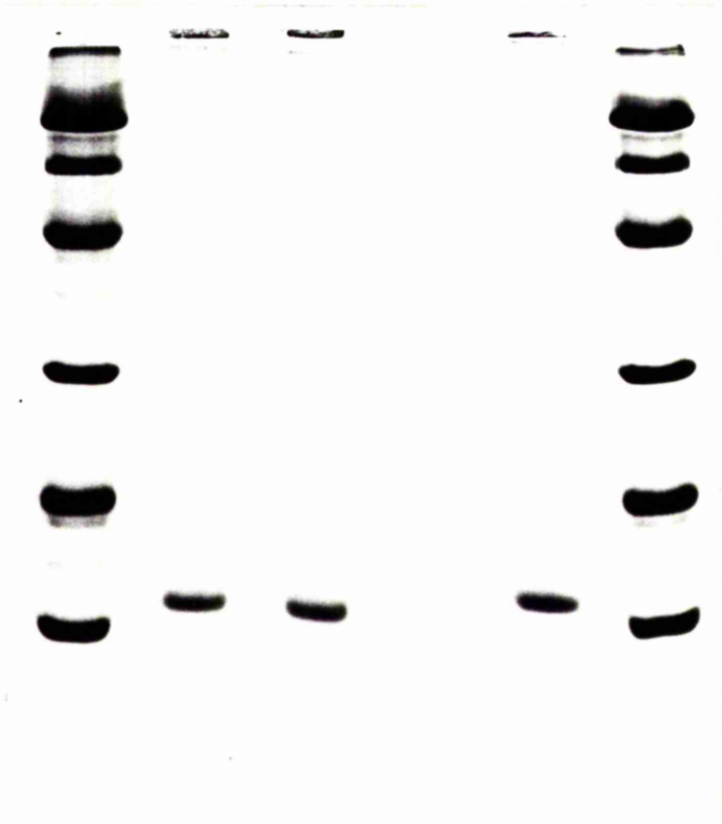
Lane 2 : junction enriched pellet - V79 + 10^{-4}M RA (24h)

Lane 3 : junction enriched pellet - V79 + 10^{-4}M RA (1h)

Lane 4 : junction enriched pellet - control V79

Lane 5 : Bio-Rad M.Wt. marker proteins

For quantitation of relative amounts of protein see Fig 36 and Table 25.



LANE 1 2 3 4 5

Figure 36 Treatment of V79 Cells with 10^{-4}M Trans Retinoic Acid - Quantitation of Relative Amounts of the 16K Protein.

For experimental details see legend to Fig 35.

The relative amount of the 16K protein in control and trans retinoic acid treated V79 cells was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitometer (see section 2.2.5.).

a) control V79 cells

b) V79 cells + 10^{-4}M trans RA (1h)

c) V79 cells + 10^{-4}M trans RA (24h)

For relative amounts see Table 25.

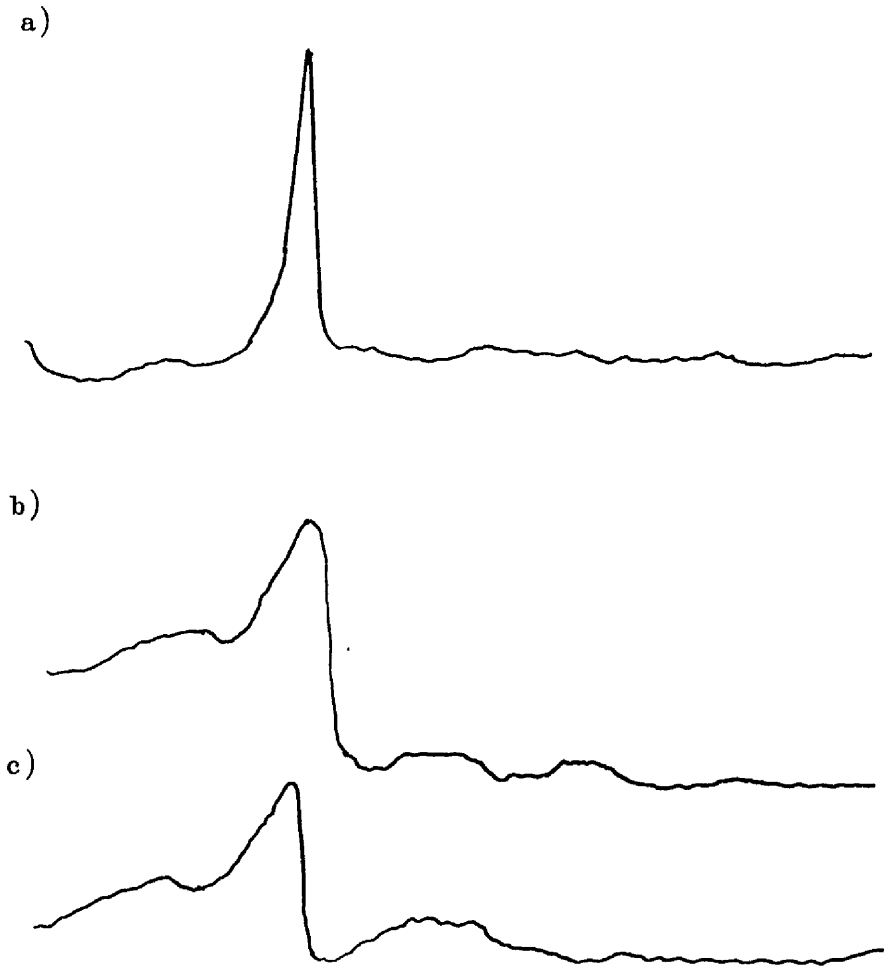

a)
b)
c)

Table 25 Quantitation of Relative Amounts of 16K Protein from Retinoic Acid Treated (1h and 24h) V79 Cells.

For experimental details see legend to Fig 35.

The relative amount of the 16K protein in control and trans retinoic acid V79 cells (1h and 24h) was measured by the absorption of Coomassie stain using a Joyce-Loebel microdensitomet (see section 2.2.5.).

The mean % decrease (standard deviation) in the amount of the 16K protein isolated from V79 cells treated with retinoic acid (10^{-4}M) for 1h compared to control amounts in 3 separate experiments = 7.5(9.6). Range in the 4 experiments = 0 - 20%.

The mean % decrease (standard deviation) in the amount of the 16K protein isolated from V79 cells treated with retinoic acid (10^{-4}M) for 24h compared to control amounts in 3 separate experiments = 15.0(12.9). Range in the 4 experiments = 0 - 30%.

Treatment	Area Under 16K Peak (Arbitary Units)	Control : RA treated
Control	0.096	-
10^{-4}M trans RA 1h	0.0086	1.1 : 1
10^{-4}M trans RA 24h	0.0081	1.2 : 1

Table 26 Effect of Trans Retinoic Acid From Treated Cultures on Uridine Nucleotide Transfer Between V79 Cells.

V79 cells were treated with 10^{-4}M trans retinoic acid for 1h and 24h before gap junction isolation (as described in legend to Fig 35).

The retinoic acid containing medium and control medium were removed and their effect tested on uridine nucleotide transfer between V79 cells.

Prior to the addition of labelled donor cells prepared as described in section 2.2.8., the medium was removed from the recipient cells (see section 2.2.8.) and replaced by 2.0ml control medium or 2.0ml medium + 10^{-4}M trans retinoic acid.

After 3h co-culture, the cells were fixed and prepared for autoradiography as described in section 2.2.9. Grains were counted over 50 recipient cells in contact with donor cells and over 50 isolated recipient cells (see section 2.2.10).

	Recipients in Contact Mean (S.D.)	Isolated Recipients Mean (S.D.)	t.	d.f.	P.
Control	24.0(3.4)	4.4(1.1)	38.1	98	<0.001
10^{-4}M trans RA (1h)	4.1(1.1)	4.3(1.3)	0.5	98	N.S.
10^{-4}M trans RA (24h)	4.3(1.8)	4.5(1.8)	0.6	98	N.S.

Figure 37 - Electron Micrograph of Junction Enriched Pellet Isolated from Retinoic Acid Treated V79 Cells.

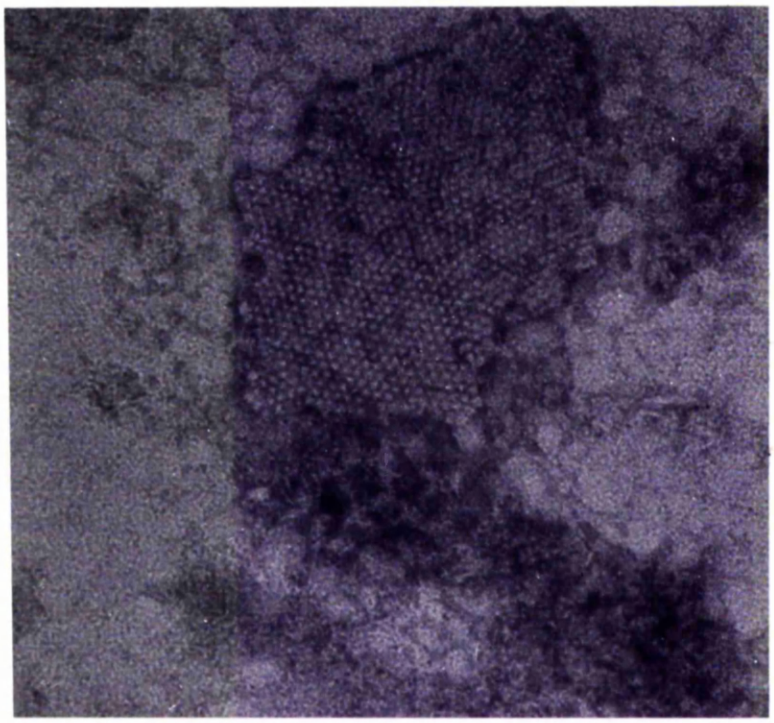
Gap junctions were isolated from 10 confluent roller bottles of V79 cells using Isolation Protocol II as described in section 2.2.3.

24h before junction isolation, the medium was removed and replaced with 150ml EC_{10} +750µl DMSO containing 2×10^{-2}M trans RA (final concentration 10^{-4}M).

The final junction enriched pellet was prepared for electron microscopy as described in section 2.2.7.

Magnification x 100,000

a)



5.3. CONCLUSIONS.

Retinoic acid treatment of BRL and V79 cells does not appear to cause dispersal of the junctional plaques. The junctional plaques isolated from BRL cells treated with 10^{-4}M retinoic acid appear to be in a closed conformation, and retinoic acid appears to have no effect on their morphology. The characteristic property of the junctional protein of heat induced aggregation in SDS also appears unaffected by 10^{-4}M retinoic acid. The number of junctional plaques present in BRL cell cultures appears to increase when treated with retinoic acid (10^{-4}M) for 24h. The reason for the increase is not understood, it could be that retinoic acid induces an increase in the number of junctional plaques present at the surface of BRL cells or that it causes increased turnover and internalisation of junctional plaques (which then accumulate as annular gap junctions) or, that it induces both. Treatment of BRL cells with concentrations of retinoic acid which have no effect on junctional communication may also cause an increase in the amount of junctional protein isolated.

The number of junctional plaques present in V79 cells decreases when V79 cell cultures are treated with 10^{-4}M retinoic acid.

Chapter 6 - General Discussion.

6.1. Isolation and Identification of the Junctional Protein.

A major difficulty of any biochemical analysis of gap junctions is the lack of a definitive assay by which to monitor enrichment during isolation. For example, there is no enzyme or binding activity known to be associated with gap junctions. The only criterion of purity available has been a morphological one, whereby the purity of gap junction enriched fractions isolated from various sources has been assessed by electron microscopy. Since gap junctions are repetitive structures, they are easily seen after staining while any amorphous material would be difficult to resolve from unevenly deposited stain. It is therefore possible to determine if gap junctions are present in enriched fractions but it is not possible to say that other components are not present. Although several laboratories (see section 1.2.2.) have made preparations of gap junctions which appeared uncontaminated morphologically, SDS polyacrylamide gel electrophoresis has,in many of these studies,shown the presence of different protein species and it has been extremely difficult to decide which is the junctional protein. It is also conceivable that the junctional protein is insoluble in SDS thus resulting in analysis of only contaminating proteins present in the junction enriched fractions. The consequent uncertainties confirm that morphological purity is an inadequate indicator of the junctional origin of any particular protein species and it is therefore important to find some additional approach to unambiguously identify the junctional protein.

One such approach used the regenerating rat liver system and another,described in this report, uses TPA treated V79 cells. In both these systems it is possible to correlate biochemical and morphological information. This has been discussed previously (see section 3.2.) but since it is of major importance the systems will be redescribed briefly. Quantitative freeze-fracture analysis has shown that in both the above systems the area of gap junctions between cells decreases (Yancey et al, 1979; Yancey et al, 1982). If the 16K protein is of junctional origin, the amount of protein recovered should parallel the observed changes in the junctional area. After removing $^{2}/3$ of the liver from three to four week old weanling rats, the area of gap junctions between the remaining hepatocytes is reduced by more than 95% between 24 and 28 hours post-operatively (Yee and Revel, 1978; Yancey et al, 1979). Analysis of junction enriched pellets isolated from livers during this period show a corresponding decrease in the amount of the 26K protein (isolated via plasma membranes (Finbow et al, 1980) and of the 16K protein isolated directly with triton (Finbow et al, 1982).

Partial hepatectomy causes tissue reorganisation and involves large scale cell division. This may produce changes in the amounts of several proteins present and not only in the junctional protein. The changes monitored in the 26K and 16K proteins although suggestive of, do not prove their junctional origin.

Exposure of V79 cells to 2×10^{-7}M TPA causes loss of junctional communication (Yotti et al, 1979). Freeze-fracture analysis of TPA treated V79 cells shows that this

is due to loss of gap junctions from the cell surface (Yancey et al, 1982). After treating V79 cells with TPA for 18h there is a 95% reduction in the area of gap junctions between cells. SDS polyacrylamide gel electrophoresis of junction enriched pellets isolated from TPA treated V79 cells shows a large decrease (67-80%) in the 16K protein. The reduction in the 16K protein is smaller than that observed by Yancey et al,(1982). This could be due to the difference in the two techniques used. In the freeze-fracture analysis, by necessity, a small number of cells were screened for the presence of gap junctions while, in the isolation method,junctions are isolated from very large numbers of cells ($2-3 \times 10^9$). The isolation method may therefore provide a more accurate indication of the effect of TPA.

It is possible that other V79 cell proteins may change in amount after TPA treatment. TPA does not cause by itself cell division, and it is unlikely that the other proteins affected would be the same as those affected in the regenerating liver system. Neither study in isolation confirms the junctional origin of the 16K protein, but taken together they provide the best evidence presently available that the 16K protein is derived from gap junctions.

In both systems the number of junctions present at the cell surface is greatly reduced. The freeze-fracture data of Yancey et al, (1982) suggest that when junctions are present they are the same size as those in control cells. It is however possible that single junctional channels (ie. channels not in plaques) can allow functional

coupling. Neither freeze-fracture nor biochemical analysis will detect individual channels since in the former, these particles will be indistinguishable from other intramembranous particles and in the isolation procedure individual junctional particles should remain free in the detergent and should not sediment into junctional pellets under the conditions used. Functional assays, however, in regenerating liver (electrical coupling and dye transfer) and in TPA treated V79 cells (electrical coupling) show that functional coupling fluctuates with junctional plaque area and suggest that active single channels present do not make a significant contribution to junctional permeability.

The effect of TPA on electrical coupling between epithelial cells from human amniotic membrane (FL cells) has been studied (Enomoto et al, 1981). Addition of 2×10^{-7}M TPA to culture medium results in an 84% decrease in the number of cells which are electrically coupled. Those cells which were coupled were fully coupled (i.e. the effect of TPA by this criterion appears to be all or nothing). When TPA is removed 90% of the cells become coupled within 4h. These results agree with the data presented in section 3.2. on the isolation of the 16K protein before, after and during treatment of V79 cells with TPA. Since these epithelial cells are, like V79 cells, sensitive to TPA, it would be interesting to extend the biochemical analysis to these cells in the presence and absence of TPA which would allow more direct

correlation with the electrophysiological data.

In studies using FL cells (Enomoto et al, 1981) and using V79 cells (Pitts, personal communcation) it has been observed that in untreated cultures, not all cells in contact communicate, and that TPA does not cause total inhibition of junctional communication. In this study however,(see section 4.2.) junctional communication between V79 cells was found to be completely inhibited by TPA and all V79 cells in contact were observed to form permeable junctions. The reason for these (small) quantitative differences found in the patterns of communication is not understood but may be due to minor variations in culture conditions.

Finbow et al, (1982) have been able to relate the 16K protein to proteins isolated by other groups from junction rich fractions by examining its aggregation properties in SDS and its sensitivity to trypsin. A number of other studies have suggested that the native junctional protein has an apparent M_r of 26,000 (Henderson et al, 1979; Hertzberg and Gilula, 1979; Finbow et al, 1980). The 16K protein does not appear to be a breakdown product of the 26K protein for a number of reasons. Firstly, the yield of the 16K protein isolated from mouse livers is unaltered by the inclusion of protease inhibitors in all isolation buffers or by the omission of a trypsin step to remove any contaminating proteins (Finbow et al, 1982). Secondly, heat induced aggregation in SDS solutions offers an alternative explanation which is that the 26K protein is a dimer derived from the 16K protein. The discrepancy in

the molecular weight i.e. the fact that the dimer molecular weight appears not to be twice that of the monomer, may be due to the two forms having different conformations in SDS or different binding capacities for SDS. There are several examples of proteins which migrate anomalously (especially low molecular weight hydrophobic and glycosylated proteins) and the molecular weight markers used in this work provide one instance. Lysozyme (14.3K) migrates between cytochrome C (11.5K) and ribonuclease A (13.4K).

Other workers (Hertzberg and Gilula, 1979; Henderson et al, 1979; Nicholson et al, 1981) have reported the aggregation of the liver 26K component to a 47-50K form which is again believed to be a dimerisation.

A third reason which suggests that the 16K component is not a breakdown product is that when the isolation procedure is modified such that trypsinisation is carried out on extracted plasma membrane preparations (which have not been exposed to triton), the 16K component can be extensively degraded (Finbow et al, 1982). It may be that triton treatment of membranes containing gap junctions increases the resistance of the 16K component to trypsin by changing the conformation in some way such that the trypsin sensitive regions are protected.

While none of the above reasons in isolation offers conclusive proof of the relationship between the 16K and 26K components, taken together they provide reasonable evidence that the 16K component is the native junctional protein and the 26K component a stable dimeric form. If

however, this conclusion is incorrect and the 16K component is really a breakdown product, the gels presented in this thesis show that there is a quantitative conversion, and so the conclusions drawn in this work from differences in the recovery of the 16K protein are not affected.

A direct relationship between the 26K and 16K protein could be determined in several ways such as amino acid sequence analysis, antibody cross reactivity, and by peptide map analysis. Such analysis has recently been applied to the 26K component (Nicholson et al, 1982) and preliminary results described in this work (see section 3.2.) show it is possible to digest the 16K protein into a suitable number of discrete peptides for analysis. This technique could be extended to look at the 16K and 26K protein isolated by different procedures and from different cell types and different tissues.

The junctional protein is thought to be highly conserved among vertebrates as indicated by functional studies which have shown that very different cell types are able to form junctions (Pitts, 1976). Protein conservation is also suggested by the difficulties several laboratories have had with attempts to raise antibodies to gap junctions. Furthermore, this study has shown that by the criterion of mobility in SDS gels, a similar 16K protein can be isolated from a wide range of cultured cells (amphibian, fish, hamster, rat and mouse cells). A protein with indistinguishable mobility on SDS gels can also be isolated from vertebrate tissues such as liver, heart, and uterus (Finbow, personal communication). Indeed,

a similar band, but with slightly reduced mobility consistent with observed structural differences (see section 1.2.1.), has been observed in junction fractions from an arthropod tissue (Finbow, personal communication). Peptide mapping and amino acid analysis will provide further information on the conservation of the junctional protein in different species. Amino acid sequence data which is already available for the 26K component (Nicholson et al, 1981) opens up the possibility of synthesising an oligonucleotide probe which can then be used to isolate the cDNA corresponding to junctional protein mRNA. In turn, this should allow the gene to be isolated which will lead to better understanding of species variation.

The relationship between the different proteins could also be determined if antisera could be raised against gap junctional protein. A recent report suggests that an antibody has been produced against the 26K protein isolated from mouse hepatocyte gap junctions (Traub et al, 1982). Antisera were raised in rats and rabbits against junctional plaques and also against protein bands 44-49K, 26K and 21K which have all been reported to be of junctional origin. Of these, the anti-26K antisera was found to be the most reactive towards junctional plaques. No inhibition of metabolic cooperation between mouse 3T6 cells was however observed in the presence of Fab fragments prepared from rabbit anti-plaque antiserum or from rabbit anti-26K antiserum. Conservation of the protein was also suggested in this study by this apparent failure to raise antibodies against that part of junctional protein which is exposed

to the cell surface and which should be required for functional interaction. The immune systems of rabbits and rats may not recognise the cell surface part of mouse gap junction proteins as foreign because it is very similar to their own protein.

The 16K protein has been isolated in small amounts from L cells (Finbow, personal communication) and in this study from A9 cells (a derivative of L cells) neither of which form functional junctions as determined by nucleotide transfer (Pitts, 1971; Pitts and Simms, 1977). Other studies have however found that in certain combinations with other cell types, L cells can exchange nucleotides (Gaunt and Subak-Sharp, 1979). The reason for these differences may perhaps be related to the origins of the cell types. Previous studies have also failed to detect junctional plaques in freeze-fracture replicas of L cells (Gilula, 1972). It is possible that the number of junctional plaques present in L cells is sufficiently small that they were missed in freeze-fracture analysis which examines only a limited number of cell interfaces. Since the isolation method described in section 3 screens large numbers of cells,it may be a more sensitive method for detecting junctional plaques. The presence of the 16K band could be used as an argument for the 16K protein not being of junctional origin but it is also possible either, that L cells have only very few junctions, or, more interestingly, that the L cells have the cellular machinery necessary for assembling junctional plaques at the cell surface but that for some reason the channels remain in the closed conformation.

6.2. The Effect of Retinoic Acid on Gap Junctional Mediated Communication.

Retinoic acid at a concentration of 10^{-4}M inhibits junctional communication between all cell types tested in culture. This inhibition could be the result of 1) the loss of junctions at the cell surface, 2) closure of junctional channels, 3) an effect on formation or breakdown of gap junctions, or a combination of two or more of the above effects.

Retinoic acid treatment of cells does not however appear to cause loss of gap junctions from the cell surface (see section 5.2.) and this will be discussed fully in section 6.3.

When Xenopus and RTG_2 cells are treated with retinoic acid two populations of cells are found. One which can communicate normally at levels comparable to that observed in control cultures and a second population where communication is completely inhibited. A certain concentration of retinoic acid may be required to bind directly to junctional protein or to stimulate binding or action of an inhibitor of channel permeability. The initial binding (or reaction) of retinoic acid (or the retinoic acid stimulated inhibitor) may make it easier for subsequent molecules to bind (or react). A critical threshold concentration of retinoic acid molecules may be required to close the channels and this may be reached in some cells and not in others, giving rise to two populations of cells which are identifiable by the level of autoradiographic grains. Cells within a population may differ in their abilities to take up retinoic

acid (or produce the retinoic acid induced inhibitor) due to lack of receptor proteins, intracellular binding proteins or a required enzymic activity. This effect is very difficult to explain but a very similar effect is seen in V79 cells at low retinoic acid concentrations (see later).

When the level of communication between primary and secondary recipient cells is examined the same pattern is observed. Junctions between primary and secondary recipient cells are established before the addition of the drug and thus we can dissociate the effects of retinoic acid on formation and on permeability. The similarity of the pattern i.e. some secondary recipient cells communicating some not, suggests that retinoic acid is not affecting junction formation between RTG_2 cells and between Xenopus cells but is affecting communication by altering junctional permeability.

The above considerations do not apply to BRL and C13 cells where there appears to be only one population of cells at concentrations which cause intermediate levels of inhibition (10^{-5}M - 10^{-4}M). Uridine nucleotide transfer between BRL cells and between C13 cells in homologous cultures is partially inhibited, but all the primary recipients appear to be equally affected. Again, as with Xenopus and RTG_2 cells, the communication pattern between primary and secondary recipient cells is indistinguishable from that between donor and primary recipient cells, suggesting that retinoic acid does not affect junction formation.

If the junctional channels are in equilibrium between the open and closed states, it may be that retinoic acid

pushes the equilibrium towards the closed state complex, so that the proportion of open channels is directly related to the retinoic acid concentration.

[open state] + RA $\rightleftharpoons$ [closed state - RA]

Whatever the explanation, the effect of retinoic acid in this system appears to be at least subtly different from that in the RTG_2, Xen, and V79 systems.

There are several possible mechanisms for the interaction of retinoic acid with the junctional protein : direct binding of retinoic acid or a derivative of retinoic acid, or binding of another molecule which is produced as a result of retinoic acid treatment, or perhaps a covalent modification induced directly or indirectly by retinoic acid. Recent experiments (Finbow and Pitts, personal communication) have shown that the 16K protein can be phosphorylated. Phosphorylation and dephosphorylation may be triggered directly or indirectly by retinoic acid which could result in changes in conformation of the gap junctional protein switching the junctions from an open to a closed state. Retinoids are believed to affect glycoprotein synthesis (see section 1.9.2.) and it is possible that retinoic acid treatment alters the glycosylation of the junctional protein producing conformational changes causing junctional closure. However, since analysis of junctional protein has not as yet revealed any association with carbohydrate (Griepp and Revel, 1977; Revel et al, 1978), this mechanism seems unlikely.

Treatment with retinoic acid may cause an increase in

the intracellular [Ca^{2+}] or [H^+] both of which lead to junction closure (see section 1.5.1.). This could be determined by measuring the intracellular pH and [Ca^{2+}] before and after retinoic acid treatment. There are however practical difficulties associated with measurement of $[Ca^{2+}]_i$ (see section 1.5.1.) and indeed it may be difficult to dissociate the Ca^{2+} and pH effects.

V79 cells have unusual properties compared to the majority of cells which have so far been studied, (most cells behave like BRL and C13 cells) with regard to junctional communication in that they appear to form junctions more slowly and also subsequently move away from each other and break communication. The results from V79 cells and from tumour cell experiments show that retinoic acid is more active (i.e. effective at lower concentrations), partially inhibiting junctional communication at concentrations as low as 10^{-6}M. As the length of coculture increases there is an increase in the proportion of cells communicating. These results can be interpreted as an effect of retinoic acid on junction formation between the cells.

Gaunt and Subak-Sharp (1979) have proposed an extension of the frequency of interaction model (Pitts and Bürk, 1976). They suggest that contact of two cells leads to an initial state that does not establish junctional communication but from which, with a certain probability per unit time, they can progress to permeable junction formation. Retinoic acid could reduce the probability of junction formation after the initial cell contact between

cells such that after a given time, fewer of the cells in a population will have formed permeable intercellular junctions. This may also operate in C13 cultures and BRL cultures but, because these cells perhaps have an overcapacity for junctional communication, reduction of the frequency may not produce detectable functional differences.

Inhibition of [^{3}H]-uridine nucleotide transfer between coupled cells by 10^{-4}M trans retinoic acid is observed with BRL, C13, V79 cells and also with 3T3 cells (Pitts, personal communication). Inhibition at lower concentrations is only observed between V79 cells and between glioma cells. The explanation may lie partly in differences in the mobility of the cells. V79 cells are known to be very mobile cells. The cells were selected for studies on mutation rate and the strain was chosen partly because daughter cells separated quickly after division, thus reducing interference by "kiss of death" between wild type cells and mutant cells produced after the first round of DNA synthesis. (Mutant cells which are HGPRT$^-$ and TK$^-$ are resistant to toxic base or nucleotide analogues when cultured alone, but are killed by nucleotide exchange in mixed culture with wild-type cells, if junctions are formed between the mutant and wild-type cells. This junction-mediated cell killing has been termed the "kiss of death" (Subak-Sharp et al, 1966; 69)). Retinoic acid may either increase cell movement such that cells come into contact but move away before junctions can be established or conversely, decrease cell movement such that cells take longer to make contact than in control cultures, both of which could reduce the chance of transfer of detectable

levels of labelled nucleotides.

Inhibition of junctional communication between cells which show low levels of communication may be important *in vivo*. Different cells in the body may have varying abilities to communicate with homologous and/or heterologous cell types. Retinoic acid may therefore have a differential effect on communication with different pathways of junctional communication responding at different concentrations. Indeed,perhaps *in vivo* only more sensitive junctional communication interfaces are affected and the high (toxic) concentrations needed to break the less sensitive coupling may never be reached.

The activity of retinoic acid would appear to be stable over 24h since medium removed from cells 24h after adding the drug is still effective in inhibiting [^{3}H]-uridine nucleotide transfer between fresh cells. Since the retinoic acid was not extracted and analysed, it is possible that it had been wholly or partially converted to some other substance which is the active form required for inhibition of junctional communication.

The concentration of trans retinoic acid required to cause complete inhibition of junctional communication is high (10^{-4}M). Experiments completed in the absence of serum (Murphy, personal communication) have shown that inhibition of junctional communication is complete at concentrations of 10^{-6}M trans retinoic acid. Concentrations higher than 10^{-6}M are toxic, under these conditions. Retinoic acid in the blood is bound to serum albumin and it may be that it is the free concentration of retinoic acid which is important in the inhibition of intercellular

communication and that this concentration is of the order of 10^{-6}M. The teratogenic effect of trans retinoic acid on the development of embryonic limbs *in vivo* is however also observed at high concentrations. Maternal administration of single doses of retinoic acid (initial concentration ~3 x 10^{-4}M) produces limb defects in mouse fetuses (Kwasigroch and Kocchar, 1980). Limb defects in rat fetuses are also produced by administration of high maternal doses of trans retinoic acid (initial concentration ~4 x 10^{-4}M) (Kistler, 1981). Injection into the amniotic cavity in chick embryos of trans retinoic acid (initial concentration ~4 x 10^{-5}M) produces developmental abnormalities with the formation of feathers on the scaled foot integument (Dhouailly *et al*, 1980). Lower doses of retinoic acid (initial concentrations 5 x 10^{-6}M - 5 x 10^{-7}M) are effective in preventing fusion of foetal mouse palates *in vivo* (Newall and Edwards, 1981).

Retinoic acid over a low concentration range (10^{-9}M - 10^{-5}M) stimulates several murine embryonal carcinoma cell lines to differentiate (Jetten *et al*, 1979). There is as yet however, insufficient data to determine what role retinoic acid inhibition of junctional communication plays in these processes.

6.3. The Effect of Retinoic Acid on the Isolation of Gap Junctions.

It is still possible to isolate junctional protein from BRL cells treated with 10^{-4}M retinoic acid and therefore there must be junctional plaques present which, since retinoic acid inhibits junctional communication, are

most likely in a closed form. Prolonged exposure to 10^{-4}M trans retinoic acid leads to an increase in the amount of the 16K component isolated. The observed increase in junctional protein isolated from retinoic acid treated BRL cells should reflect an increase in the number of junctional plaques (see section 3.2.). There is however a problem in the interpretation of the data. The isolation technique does not involve preparation of plasma membranes and so it is possible that the 16K protein is derived, either principally, or in part, from junctional plaques located in internalised cytoplasmic vesicles (see section 1.3.2.). Internalisation of gap junctions in retinoic acid treated skin epithelium has been reported (Prutkin, 1975) but no internalisation has been reported in the studies of Elias et al, (1976; 1980; 1981) on the effect of retinoic acid on chick shank and on human basal cell carcinomas. The internalisation of gap junctions in retinoic acid treated cells would be consistent with the blocking of junctional communication as this might deplete the cell surface forms and hence reduce communication. Arguing against this interpretation is the observation that retinoic acid inhibition of junctional communication and its reversal occur within minutes. Morphological evidence (Albertini et al, 1975; Larsen et al, 1979) suggests that internalisation and reformation of gap junctions occurs over a much longer time course. The results to date, therefore favour the idea that the junctional plaques at the cell surface can be switched to an impermeable form.

Although no definitive evidence exists, there is considerable correlative evidence (see section 1.4.4.) that it is these plaques which are active in intercellular communication. Recent experiments carried out by Sheridan and Larson (1982) however,suggest that functional communication can occur in at least one system in the absence of morphologically identifiable gap junctional plaques. They suggest that single paired particles may be responsible for intercellular communication. Such communicating units would not be distinguished from other intramembranous particles in freeze-fracture replicas and, unless they aggregated during isolation,they would not appear in junction enriched pellets.

If single paired junctional particles are the active form of communication, then the aggregates of junctional particles could represent an inactive form, perhaps a stage in a process of normal turnover. Particles may aggregate at the cell surface prior to their degradation by internalisation and it is possible that retinoic acid treatment of BRL cells blocks junctional communication either by causing rapid aggregation and internalisation, or by causing the individual, previously open, junctional particles to close,which would lead to the normal process of aggregation. If the second alternative is correct and if aggregation of junctional particles takes a long time, (measured in tens of minutes or hours rather than seconds or minutes), it would explain rapid loss of coupling but a slow increase in the recovery of 16K in the presence of retinoic acid. After 1 hour exposure the amount of junctional protein isolated from retinoic acid treated

cells is not significantly increased compared to control cultures and this may reflect the normal equilibrium in the level of junctional plaques in the turnover process. Retinoic acid may therefore have two different effects: it may have a direct effect on the junctional protein causing conformational change, switching the junctions to an impermeable form, and it may either directly, or as a consequence of channel closure,stimulate the aggregation of junctional particles into plaques. Inhibition of communication is reversed within minutes of removal of retinoic acid and so either junctional plaques could disassemble into individual (open) particles in this time or a pool of precursor units could be available to form new channels.

As yet there is no information on the precursor protein. For example, it is not known whether the junctional particles are assembled prior to junction formation.

The increase in junctional plaques may be explained by retinoic acid inhibition of junction degradation. Junctional plaques would accumulate at the cell surface and this would be reflected in an increase in the amount of junctional protein isolated. Alternatively, there may be a feedback mechanism which results in an increased production of junctional plaques in response to the blockage of communication. However, preliminary experiments with lower concentrations of retinoic acid suggest this observed increase in junctional protein may be an effect which is unrelated to the inhibition of junctional communication. Isolation and analysis of gap junctions

from BRL cells treated with 10^{-7}M retinoic acid, i.e. under conditions where junctional communication is unaffected, also show increased levels of the 16K protein. This result supports retinoic acid having two separate effects on gap junctions in BRL cells. The first would result in inhibition of junctional communication by a direct effect on channel closure and would occur at high concentrations of retinoic acid. The second effect would be on the extent of aggregation, which would cause a build up of junctional plaques at the cell surface and/or promote internalisation of the plaques to stable annular structures.

Isolation of junctional protein from V79 cells treated with 10^{-4}M retinoic acid shows a slightly decreased level of 16K protein when compared to control V79 cells. It is however possible that perhaps in V79 cells, retinoic acid may cause aggregation of particles into plaques but that the normal turnover rate of junctions may prevent any build up of plaques at the cell surface.

From present data it is impossible to say whether the junctional plaques are at the cell surface. Further work with freeze-fracture and thin section EM analysis of cells is required to determine the location of plaques in retinoic acid treated cells.

It seems most likely, however, in view of the rates at which the changes occur (see above) that retinoic acid directly or indirectly blocks the junctional channel in a fully reversible manner.

As described in the introduction (see section 1.9.) retinoic acid has a wide variety of effects on cell

behaviour *in vivo* and in culture. It is possible that some or many of these effects are due to perturbations of junctional communication, either between sensitive cell types or at sensitive interfaces between groups of cells. If so, this may be an important way of understanding the functions of junctional communication in animal tissues.

Chapter 7 - References.

Adamo S., De Luca L.M., Silverman-Jones C.S. and Yuspa S. (1979). J. Biol. Chem. 254 (9) 3279-3287.

Albertini D.F. and Anderson E. (1974). J. Cell. Biol. 63 234-50.

Albertini D.F., Fawcett P.W. and Old P.J. (1975). Tissue & Cell 7 389-405.

Amos W.B. (1976). Anal. Biochem. 70 612-615.

Anderson E, and Albertini D.F. (1976) J. Cell Biol. 71 680-686.

Anderson W.B., Johnson G.S. and Pastan I. (1973 a) Proc. Nat. Acad. Sci. 70 1055-1059.

Anderson W.B., Russell T.R., Carchnan R.A. and Pastan I. (1973 b) Proc. Nat. Acad. Sci. 70 3802-3805.

Atkinson M.M., Menko, A.S., Johnson R.G., Sheppard J.D. and Sheridan J.D. (1981) J. Cell Biol. 91 573-578.

Azarnia, R. and Loewenstein W.R. (1971) J. Membrane Biol. 6 368-385.

Azarnia R. and Loewenstein W.R. (1978) Nature 241 455-457.

Azarnia R. and Loewenstein W.R. (1976) J. Membrane Biol. 30 175-186.

Azarnia R. and Loewenstein W.R. (1977) J. Membrane Biol. 34 1-28.

Azarnia R., Dahl G. and Loewenstein W.R. (1981) J. Membrane Biol. 63 133-146.

Baker P.F., Hodgkin A.L. and Ridgeway E.B. (1971) J. Physiol. 218 709-755.

Baux G., Simonneau M., Tauc L. and Segundo J.P. (1978) Proc. Nat. Acad. Sci. 75 (9) 4577-81.

Bendich A., Visozo, A.D. and Harris K.G. (1967) Proc. Nat. Acad. Sci. 57 1029-1035.

Benedetti E. and Emmelot P. (1968) J. Cell Biol. 38 15-24.

Benedetti E., Dunia I. and Bloemendal H. (1974). Proc. Nat. Acad. Sci. 71 5073-5077.

Bennett M.V.L. (1972) in : "Structure and Function of Synapses". (Pappas G.D. and Purpura, D.P., eds) pp221-256, Raven Press: New York.

Bennett N.V.L., Dunham P.B. and Passas G.D. (1967) J. Gen. Physiol. 50 1094 Abstr.

Bergman R.A. (1968) J. Cell Biol. 36 639-648.

Bjersing L. and Cajander S. (1974) Cell Tissue Res. 153 1-14.

Bollag W. (1972) Europ. J. Cancer 8 689-693.

Bollag W. and Ott F. (1970) Agent Actions 1 172-175.

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

Borek G., Grab M. and Burger M.M. (1973) Exp. Cell Res. 77 207-215.

Brandes D., Sato T., Ueda H. and Rundell J.D. (1974) Cancer Res. 34 2151-2158.

Branton D. (1966) Proc. Nat. Acad. Sci. 55 1048-1056.

Brink P.R. and Dewey M.M. (1968) Nature 285 101-103.

Brockhuyse R.M. and Kuhlmann E.D. (1979) Exp. Eye Res. 28 615-618.

Burghardt R.C. (1980) J. Ultrastruct. Res. 71 162-172.

Burk R.R. (1968) Nature 219 1272-1275.

Burton A.C. (1971) Persp. Biol. Med. 14 301-308.

Caspar D.L.D., Goodenough D.A., Makowski I. and Phillips W.C. (1977) J. Cell Biol. 14 605-628.

Chopra D.P. and Wilkoff L.J. (1977 a) J. Natl. Cancer Inst. 58 923-930.

Chopra D.P. and Wilkoff L.J. (1977 b) Nature 265 339-341.

Choo-Chung Y.S. and Gillino P.M. (1974) Science 183 87-89.

Chytil F. and Ong D.E. (1976) Nature 260 49-51.

Chytil F. and Ong D.E. (1979 a) Fed. Proc. 38 2510-12.

Chytil F. and Ong D.E. (1979 b) In : "Receptors and Hormone Action II" (O'Malley B.W. and Birnbaumer L.B. eds.). pp 573-591, Academic Press: New York.

Cone M.F. and Nettesheim D. (1973) J. Natl. Cancer Inst. 5 1599-1616.

Coon H.G. (1968) J. Cell Biol. 39 29a.

Corsaro C.M. and Migeon B.R. (1977) Nature 268 737-739.

Crick F.H.C. (1970) Nature 225 420-22.

Culvenor J.G. and Evans W.H. (1977) Biochem. J. 168 475-481.

Dahl I.M.S. and Axelsson I. (1980) Exp. Eye Res. 31 (4) 443-450.

Daniel E.E., Daniel V.P., Duchan G., Garfield R.E., Nicols M., Halhotra J.K. and Oki M. (1976) J. Membrane Biol. 28 207-239.

Decker R.S. (1976) J. Cell Biol. 69 669-685.

Decker R.S. and Friend D.S. (1974) J. Cell Biol. 62 32-47.

De Laat S.W., Tertoolen L.G.S., Dorresteijn A.W.C. and Van Den Bigelaar S.M. (1980) Nature 287 546-548.

Daleze J. and Loewenstein W.R. (1976) J. Membrane Biol. 28 71-86.

De Luca L. (1977) Vitamine and Hormones 35 1-54.

De Luca L., Maestri M., Bonanni F. and Nelson D. (1972) Cancer 30 1326-1331.

De Mello W.C. (1975) Experimentia 15 460-462.

Dewey M.M. and Barr L. (1962) Science 137 670-672.

Dhouailly D., Hardy M. and Sengel P. (1980) J. Embryol. Exp. Morph. 58 63-78.

Dicker P. and Rozengurt E. (1979) Biochem. Biophys. Res. Commun. 91 1203-1210.

Dion L.D., Blalock J.E. and Gifford G.E. (1977) J. Natl. Cancer Inst. 58 795-800.

Dorsey J.K. and Roth S. (1974) In : "Control of Proliferation in Animal Cells" (Clarkson B and Baserga R. eds.) pp 533-539 Cold Spring Harbor Laboratory: New York.

Drummond J.C. (1920) Biochem. J. 14 660.

Duguid J.R. and Revel J.P. (1975) Cold Spring Harbor Sym. Quant. Biol. 40 45-47.

Dulcibella T. and Anderson E. (1975) Devel. Biol. 47 45-47.

Dunganin P.E. Jr., Meadows E.H. Jr. and Olson J.A. (1965) Science 148 86-87.

Ehrhart J.C. and Chaveau J. (1977) Febs. Lett. 78 295-299.

Elias P.M. and Friend D. (1976) J. Cell Biol. 68 173-188.

Elias P.M., Grayson S., Caldwell T.M. and McNutt N.S. (1980) Laboratory Investigation 42 (4) 469-474.

Elias P.M., Grayson, S., Gross E.G., Peck G.L. and McNutt N.S. (1981) Cancer (Phila) 48 932-938.

Emerick R.J., Zile M. and De Luca H.F. (1967) Biochem. J. 102 606-611.

Enomoto T., Sasaki Y., Shiba Y., Kanno Y. and Yamasaki H. (1981) Proc. Nat. Acad. Sci. 78 (9) 5628-5632.

Evard J.P. and Bollag J.W. (1972) Schwerz. Med. Wochenschr. 102 1880-1883.

Fallon R.F. and Goodenough D.A. (1981) J. Cell Biol. 90 521-526.

Fentiman I., Taylor-Papadimitrou J. and Stocker M.G.P. (1976) Nature 264 760-762.

Fentiman I. and Taylor-Papadimitrou J. (1977) Nature 269 156-157.

Finbow M.E., Yancey S.B., Johnson R. and Revel J.P. (1979) Proc. Nat. Acad. Sci. 77 970-974.

Finbow M.E. and Pitts J.D. (1981) Exp. Cell Res. 131 1-13.

Finbow M.E. and Yancey S.B. (1981) In : "Biochemistry of Cellular Recognition, Vol IV : "The Cell Surface" (Knox P. ed.) pp 215-249 CRC Press Inc.

Finbow M.E. (1982) In : "Functional Integration of Cells in Animal Tissue (Pitts J.D. and Finbow M.E. eds.) pp 4-23 Cambridge University Press.

Finbow M.E., Hamilton A.E. and Pitts J.D. (1982) Paper submitted.

Flagg-Newton J., Simpson I. and Loewenstein W.R. (1979) Science 205 404-407.

Flagg-Newton J., Dahl G. and Loewenstein W.R. (1981) J. Membrane Biol. 63 105-121.

Flower N.E. (1972) J. Cell Sci. 10 683-691.

Flower N.E. (1977) J. Cell Sci. 25 163-171.

Ford D.K. and Yerganian K.L. (1958) J. Natl. Cancer Inst. 21 1065-1066.

Friend D.S. and Gilula N.B. (1972) J. Cell Biol. 53 755-776.

Furshpan E.J. (1964) Science 144 878-880.

Furshpan E.J. and Potter D.D. (1959) J. Physiol. 145 280-325.

Furshpan E.J. and Potter D.D. (1968) Curr. Top. Dev. Biol. 3 95-127.

Garfield R.E. and Hayashi R.J. (1980) Am. J. Obstet. Gynaecol. 138 (5) 569-574.

Garfield R.E., Merrett D. and Grover A.K. (1980) Am. J. Physiol. 239 (5) 217-228.

Gaunt S.J. and Subak-Sharpe J.H. (1979) Exp. Cell Res. 120 307-320.

Gilula N.B. (1972) J. Ultrastruct. Res. 38 215-216.

Gilula N.B. (1974) J. Cell Biol. 63 1119.

Gilula N.B. and Satir P. (1972) J. Cell Biol. 51 869-872.

Gilula N.B., Reeves D.R. and Steinbach A. (1972) Nature 135 262-265.

Gilula N.B., Epstein M.L. and Beers W.H. (1978) J. Cell Biol. 78 58-75.

Goodenough D.A. (1974) J. Cell Biol. 61 557-563.

Goodenough D.A. (1976) J. Cell Biol. 68 220-231.

Goodenough D.A. and Revel J.P. (1970) J. Cell Biol. 45 272-290.

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

Goodenough D.A. and Gilula N.B. (1974) J. Cell Biol. 61 575-581.

Goodenough D.A., Paul L. and Culberk K.E. (1978) Birth Defects 14 (2) 83-97.

Goodman D.S., Blomstrand R., Werner G., Huang, H. and Shiratori T. (1966) J. Clin. Invest. 45 1615-1623.

Goodwin P.W., (1981) Proc. XIIIth Int. Congress Botany p35 Abstr.

Griepp E.B. and Revel J.P. (1977) In : "Intercellular Communication" (deMello W.C. ed.) p 1-32 Plenum Press: New York.

Hamilton A.E., Pitts J.D., Burk R.R. and Murphy J.P. (1982) Paper submitted.

Hand A.R. and Globel S. (1972) J. Cell Biol. 52 397-408.

Hanna R.B., Model P.H., Spray D.C., Bennett M.V.L. and Harris A.L. (1980) Am. J. Anat. 158 111-114.

Hardy M.H., Dhouailly D. and Sengel P. (1981) Annals of the New York Academy of Sciences 359 394-395.

Henderson D., Eibl H. and Weber K. (1979) J. Mol. Biol. 132 193-218.

Harmann A., Rieske E., Kreutzberg G.W. and Lux H.D. (1975) Brain Res. 95 125-131.

Hertzberg E.L. and Gilula N.B. (1979) J. Biol. Chem. 254 2138-2147.

Hogan-Ryan A. and Fennelly J.J. (1978) Eur. J. Cancer 14 113-116.

Hsie A.W. and Puck T.T. (1971) Proc. Nat. Acad. Sci. 68 358-361.

Huang H.S. and Goodman D.S. (1965) J. Biol. Chem. 240 2839-2844.

Huebner E. (1981) J. Ultrastruct Res. 74 (1) 95-104.

Hunter G.K. and Pitts J.D. (1981) J. Cell Sci. 49 163-175.

Ito Y., Zile M., De Luca H.F. and Ahrens H.M. (1974) Biochem. Biophys. Acta 369 338-350.

Iwatsuki N. and Peterson D.H. (1977) Nature 286 148-149.

Jetten A.M. and Jetten M.E.R. (1979) Nature 278 180-182.

Jetten A.M., Jetten M.E.R., Shapiro J.S. and Poon J.P. (1979 a) Exp. Cell Res. 119 289-299.

Jetten A.M., Jetten M.E.R. and Sherman M.I. (1979 b) Exp. Cell Res. 124 381-391.

Johnson R.J. and Sheridan J.D. (1971) Science 174 717-719.

Johnson G.S. and Pastan J. (1972) J. Natl. Cancer Inst. 48 1377-1387.

Johnson R.J., Hammer M., Sheridan J.D. and Revel J.P. (1974) Proc. Nat. Acad. Sci. 71 4536-4540.

Kanno Y. and Matsui H. (1968) Nature 218 775-776.

Kensler R.W. and Goodenough D.A. (1980) J. Cell Biol. 86 755-764.

Kistler A. (1981) Teratology 23 25-31.

Kochhar D.M., Dingle J.T. and Lucy J.A. (1968) Exp. Cell Res. 52 591-601.

Kocher D., Amaudruz M., Schindler A.M. and Gabbioni G. (1981) J. Submicroscop. Cytol. 13(2) 267-282.

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

Kreuger J.G., Wang E. and Goldberg A.R. (1980 a) Virology 101 25-40.

Kreuger J.G., Wang E., Garber E.A. and Goldberg A.R.(1980b) Proc. Nat. Acad. Sci. 77 (7) 4142-4146.

Krishna B.M. and Cama H.R. (1979) Biochem. Biophys. Acta 587 (2) 273-281.

Kuffler S.W. and Potter D.D. (1966) J. Neurophysiol. 27 290-320.

Kuffler S.W., Nicholls J.G. and Orklund R.G. (1966) J. Neurophysiol. 29 768-777.

Kwasigroch T.E. and Kocchar D.M. (1980) Anat. Embryol. 161 (1) 105-114.

Laemmli U.K. (1970) Nature 227 680-686.

Lane N.J. and Chandler H.J. (1980) J. Cell Biol. 86 765-774.

Lane N.J. and Swales L. (1980) Cell 18 579-586.

Lane N.J. (1982) In : "Functional Integration of Cells in Animal Tissues" (Pitts J.D. and Finbow M.E. eds.) pp 46-57 Cambridge University Press.

Larsen W.J. (1977) Tissue and Cell 9 (3) 373-394.

Larsen W.J. and Hai-Nan T. (1977) Tissue and Cell 10 585-598.

Larsen W.J., Azarnia R. and Loewenstein W.R. (1977) J. Membrane Biol. 34 39-54.

Larsen W.J., Hai-Nan T., Murray S.A. and Swenson C.A. (1979) J. Cell Biol. 83 576-587.

Lasnitski I. (1955) British J. Cancer 8 689-693.

Lasnitski I (1955) Exp. Cell Res. 8 121-125.

Lasnitski I. (1976) British J. Cancer 34 239-248.

Lawrence P.A. and Green S.M. (1975) J. Cell Biol. 49 173-188.

Lawrence T.S., Beers W.H. and Gilula N.B. (1978) Nature 272 501-506.

Ledbetter M.L.S. and Lubin M. (1977) Exp. Cell Res. 105 223-236.

Ledbetter M.L.S. and Lubin M. (1979) J. Cell Biol. 80 150-165.

Lenarz W.S. (1975) Science 188 986-991.

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

Lo C.W. and Gilula N.B. (1980 a) Cell 18 399-409.

Lo C.W. and Gilula N.B. (1980 b) Cell 18 411-422.

Loewenstein W.R. (1967) J. Colloid and Interface Sci. 26 34-36.

Loewenstein W.R. (1968) Devel. Biol. Suppl. 2 151-183.

Loewenstein W.R. (1975) Cold Spring Harbor Symp. Quant. Biol. 40 49-63.

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

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

Loewenstein W.R. and Kanno Y. (1967) J. Cell Biol. 33 225-230.

Loewenstein W.R., Nakas M. and Socolar S.J. (1967) J. Gen. Physiol. 50 1865-1891.

Loewenstein W.R., Kanno Y., and Socolar S.J. (1978) Nature 274 133-136.

McCollum E.V. and Kennedy C. (1916) J. Biol. Chem. 24 491-493.

McNutt W.S. and Weinstein R.S. (1970) J. Cell Biol. 47 666-668.

McNutt W.S. and Weinstein R.S. (1973) Prog. Biophys. Mol. Biol. 26 45-101.

McPherson I.A. and Stoker M.G.P. (1962) Virology 16 147-151.

Makowski L., Caspar D.L.D., Phillips W.C. and Goodenough D.A. (1977) J. Cell Biol. 74 629-645.

Maden M. (1982) Nature 295 672-675.

Marin G. and Littlefield J.W. (1968) J. Virology 2 69-77.

Matter A. and Bollag W. (1977) Eur. J. Cancer 13 831-838.

Mayer H., Bollag W., Hanni R. and Ruegg R. (1978) Experimentia 34 1105-1119.

Meda P., Perrelet A. and Orci L. (1982) In : "Functional Integration of Cells in Animal Tissues" (Pitts J.D. and Finbow M.E. eds.) pp 61-67 Cambridge University Press.

Meech R.W. and Thomas R.C. (1977) J. Physiol. 265 867-869.

Merk F.B., Botticelli C.R. and Albright J.T. (1978) Endocrinology 90 992-994.

Merril C.R., Switzer R.C. and Van Keuren M.L. (1979) Proc. Nat. Acad. Sci. 76 4335-4339.

Meyer D.J., Yancey S.B. and Revel J.P. (1981) J. Cell Biol. 91 505-523.

Moor R.M. and Smith M.W. (1980) Exp. Cell Res. 119 333-341.

Moor R.M., Smith M.W. and Dawson R.M.C. (1980) Exp. Cell. Res. 126 15-29.

Morell P., Wiggins R.C. and Gray M.J. (1975) Anal. Biochem. 68 148-154.

Murray A.W. and Fitzgerald D.J. (1979) Biochem. Biophys. Res. Commun. 91 395-401.

Newall D.R. and Edwards J.R.G. (1981 a) Teratology 23 115-124.

Newall D.R. and Edwards J.R.G. (1981 b) Teratology 23 125-130.

Newbold R.F. and Amos J. (1981) Carcinogenesis 2

Newbold R.F. (1982) In : "Functional Integration of Cells in Animal Tissues" (Pitts J.D. and Finbow M.E. eds.) pp 147-155 Cambridge University Press.

Nicholson B.J., Hunkapiller M.W., Grim L.B., Hood L.G. and Revel J.P. (1982) Proc. Nat. Acad. Sci. 78 (12) 7594-7598.

Oakley B.R., Kirsch D.R. and Morris N.R. (1980) Anal. Biochem. 105 361-363.

Oliveira-Castro G.M. and Loewenstein W.R. (1971) J. Membrane Biol. 5 51-77.

Otten J., Johnson G.S. and Pastan I. (1971) Biochem. Biophys. Res. Commun. 44 1192-1195.

Pappas G.D., Asada Y. and Bennett M.V.L. (1971) J. Cell Biol. 49 173-188.

Pennypacker J.P., Lewis C.A. and Hassell J.R. (1978) Arch. Biochem. Biophys 186 351-358.

Peracchia C. (1973 a) J. Cell Biol. 57 54-66.

Peracchia C. (1973 b) J. Cell Biol. 57 66-76.

Peracchia C. (1977) J. Cell Biol. 72 628-641.

Peracchia C. (1978) Nature 271 669-671.

Peracchia C. and Dulhunty A.F. (1976) J. Cell Biol. 70 419-439.

Peracchia C. and Peracchia L.L. (1980) J. Cell Biol. 87 719-727.

Peterson P.A. (1971) J. Biol. Chem. 246 44-49.

Piccolino M., Neyton J., Witkovsky P. and Gerschenfield H.M. (1982) Proc. Nat. Acad. Sci. 79 3671-3675.

Pitts J.D. (1971) In: "Ciba Foundation Symposium on Growth Control in Cell Cultures" (Wolstenholme G.E.W. and Knight J. eds.) pp 89-105 Churchill: London.

Pitts J.D. (1972) In : "Cell Interactions" (Silvestri L.G. ed.) pp 277-285 North Holland Publishing Company.

Pitts J.D. and Burk R.R. (1976) Nature 264 762-764.

Pitts J.D. and Finbow M.E. (1977) In : "Intercellular Communication" (de Mello W.C. ed.) pp 61-86 Plenum Press.

Pitts J.D. and Simms J.W. (1977) Exp. Cell Res. 104 153-163.

Pitts J.D. and Shaw K. (1980) Cell Biol. Int. Rep. 4 758.

Pitts J.D., Burk R.R.,Murphy J.P. (1981) Cell Biol. Int. Reports Vol. 5 Suppl. A. p 45.

Politoff A.L., Socolar S.J. and Loewenstein W.R. (1969) J. Gen. Physiol. 53 498-515.

Potter D.D., Furshpan E.J. and Lennox E.S. (1966) Proc. Nat. Acad. Sci. 55 328-336.

Porvaznik M., Johnson R.G. and Sheridan J.D. (1979) J. Supramol. Structure 10 13-30.

Prutkin L. (1973) Cancer Res. 33 128-133.

Prutkin L. (1975) Cancer Res. 35 364-369.

Quick D.C. and Johnson R.J. (1977) J. Ultrastruct. Res. 60 348-361.

Rao M.R.S., Vinayaka R., Prasad G.P. and Ganguly S. (1979) Biochem. J. 183 501-506.

Raviola E., Goodenough D.A. and Raviola G. (1978) J. Cell Biol. 79 229a.

Raviola E., Goodenough D.A. and Raviola G. (1981) J. Cell Biol. 87 273-279.

Revel J.P. and Karnovsky M.J. (1967) J. Cell Biol. 33 C7-C12.

Revel J.P., Yee A.G. and Hudspeth A.J. (1971) Proc. Nat. Acad. Sci. 68 2924-2929.

Revel J.P., Yip P. and Chang L.L. (1973) Dev. Biol. 35 302-317.

Revel J.P., Griepp E.B., Finbow M.E. and Johnson R. (1978) Zoon 6 139-144.

Revel J.P., Yancey S.B., Meyer D.J. and Nicholson B.J. (1980) In Vitro 16 1010-1017.

Rindler M.J., Chumar L.M., Shaffer L. and Saier M.W. (1979) J. Cell Biol. 81 635-648.

Roberts A.B. and De Luca H.F. (1967) Biochem. J. 102 600-605.

Roberts A.B., Lamb L.C. and Sporn M.B. (1980) Arch. Biochem. Biophys. 199 374-383.

Robertson J.D. (1963) J. Cell Biol. 19 201-221.

Rose B. (1971) J. Membrane Biol. 5 1-19.

Rose B. and Loewenstein W.R. (1971) J. Membrane Biol. 5 20-50.

Rose B. and Loewenstein W.R. (1975) Nature 254 250-252.

Rose B. and Loewenstein W.R. (1976) J. Membrane Biol. 28 87-119.

Rose B., Simpson I. and Loewenstein W.R. (1977) Nature 267 625-627.

Sani B.P. (1977) Biochem. Biophys. Res. Commun. 75 8-12.

Schenk P. (1980) Arch. Oto. Rhino. Laryngol. 228 51-56.

Schindler J., Matthaei K.I. and Sherman M.I. (1981) Proc. Nat. Acad. Sci. 78 (2) 1077-1080.

Seigenbeck van Heukelom J., Denier van Der Gon J.J. and Prop F.J.A. (1970) Biochem. Biophys. Acta. 211 98-101.

Shamberger R.J. (1971) J. Natl. Cancer Inst. 47 667-673.

Shapiro S.S. and Poon J.P. (1976) Arch. Biochem. Biophys. 174 74-81.

Sheridan J.D. (1968) J. Cell Biol. 37 650-659.

Sheridan J.D. (1970) J. Cell Biol. 45 91-99.

Sheridan J.D. (1971) J. Cell Biol. 50 795-803.

Sheridan J.D. (1978) In : "Intercellular Junctions and Synapses" (Feldman J., Pitts J.D. and Gilula N.B. eds.) pp 39-59 Chapman Hall.

Sheridan J.D. (1981) J. Cell Biol. 91 573-578.

Sheridan J.D., Finbow M.E. and Pitts J.D. (1979) Exp. Cell Res. 123 111-117.

Sheridan J.D. and Larson D.M. (1982) In : "Functional Integration of Cells in Animal Tissues" (Pitts J.D. and Finbow M.E. eds.) pp 130-139. Cambridge University Press.

Simpson I., Rose B. and Loewenstein W.R. (1977) Science 195 294-295.

Socolar S.J. (1973) Exp. Eye Res. 15 693-696.

Soiursh M. and Meier S. (1973) Calc. Tissue Res. 13 131-142.

Spray D.C., Harris A.L., Bennett M.V.L. and Model P.G. (1978) Soc. Neurosciences Abst. 4 238.

Spray D.C., Harris A.L. and Bennett M.V.L. (1981) Science 211 717-715.

Stepp W. (1909) Biochem. Z. 22 452.

Stepp W. (1911) Z. Biol. 57 135.

Stubbs G.W., Saari J.C. and Futterman S. (1979) J. Biol. Chem. 254 (17) 8529-8533.

Subak-Sharpe J.H., Burk R.R. and Pitts J.D. (1966) Heredity 21 342-343.

Subak-Sharpe J.H., Burk R.R. and Pitts J.D. (1969) J. Cell Sci. 4 353-367.

Tickle C., Alberts B., Wolpert L. and Lee J. (1982) Nature 296 564-565.

Todaro G.J. and Green H. (1963) J. Cell Biol. 17 299-313.

Traub O., Janssen-Timmen U., Druge P.M., Dermictzel R. and Willecke K. (1982) J. Supramol. Structure and Cell Biochem. 19 27-44.

Trosko J.E., Dawson B., Yotti L.P. and Chang C.C. (1980) Nature 285 109-110.

Turin L. (1977) J. Physiol. 269 6p-7p.

Turin L. and Warner A.E. (1977) Nature 270 56-57.

Unwin P.N.T. and Zampighi G. (1980) Nature 283 545-549.

Van Euler B., Van Euler H. and Hellström H. (1928) Biochem. Z. 203 370.

Van Jaarsveid P.P., Edelhoch H., Goodman D.W.S. and Robbins J. (1973) J. Biol. Chem. 248 4698-4705.

Walker N.A. (1976) In : "Intercellular Communication in Plants" (Gunning B. and Robards A.R.W. eds.) pp165-179 Berlin-Springer Verlag.

Warner A.E. and Lawrence P.A. (1982) Cell 28 243-252.

Willingham M.C., Jay G. and Pastan I. (1979) Cell 18 125-134.

Wolf K. and Quimby M.C. (1962) Science 135 1065-1066.

Wolpert L. (1969) J. Theoret. Biol. 25 1-48.

Wolpert L. (1978) In : "Intercellular Junctions and Synapses" (eds. Feldman J., Pitts J.D. and Gilula N.B.) pp 83-96. Chapman Hall.

Yancey S.B., Easter D. and Revel J.P. (1979) J. Ultrastruct. Res. 67 229-242.

Yancey S.B., Nicholson B.J. and Revel J.P. (1981) J. Supramol. Struct. and Cell Biochem. 16 221-232.

Yancey S.B., Edens J.E., Trosko J.E., Chang C.C. and Revel J.P. (1982) Exp. Cell Res. 239 329-340.

Yee A.G. and Revel J.P. (1978) J. Cell Biol. 78 554-564.

Yotti L.P., Chang C.C. and Trosko J.E. (1979) Science 206 1089-1091.

Yuspa S.H. and Harris C.C. (1974) Exp. Cell Res. 86 95-105.

Zampighi G. and Unwin P.N.T. (1979) J. Mol. Biol. 135 451-464.

Zamphigi G., Corless J.M. and Robertson J.D. (1980) J. Cell Biol. 86 190-195.