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Interactions of embryonic with neoplastic cells

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A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy.

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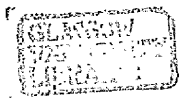
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This thesis is dedicated to my parents who supported me financially during my studies.

Summary

Adhesive interactions of embryonic with neoplastic cells have been studied in cultures of 7 day old chick embryo neural retina cells and embryo hamster, baby hamster cell lines and some of their neoplastic derivative cell lines. Two features of adhesion were studied, the alteration of neural retina cell adhesiveness by the conditioned media of the hamster cell lines and the mutual adhesiveness of the neural retina cells and the hamster cell lines.

Experiments to test for conditioned media were done using either the Couette Viscometers or the collecting cell lawn assay. The results of these experiments indicate the presence of factors which affect the adhesiveness of 7 day old chick embryo neural retina cells. The results varied depending upon which technique was used.

The mutual adhesiveness of the neural retina cells and the hamster cell lines was studied with the collecting cell lawn system and in two and three dimensional mixed cultures.

The collecting cell lawn assay indicates that the neural retina cells do not associate with the neoplastic cells as well as the normal cells and that the embryonic hamster cells associate better than the baby hamster cells. The results are discussed on the bases of a classification of the different cell types according to their origin as baby, embryonic or neoplastic cells.

The two dimensional mixed cultures show that the neural retina cells and the hamster cells do not make any important contacts and that the increase by growth of the number of the hamster cells force the neural retina cells to come off the culture dishes.

The three dimensional mixed cultures indicate that the neural retina cells associate to some extent with the normal baby and embryonic

hamster cells but not with the neoplastic ones.

In these mixed cultures, two and three dimensional ones, the sorting out pattern observed was discussed in relation to the secretion effects.

The results give support to the "morphogen theory" for the control of sorting out in mixed aggregates.

CONTENTS

| | Page |
|-------------------------------|------|
| Acknowledgments | (I) |
| Summary | (II) |
| Contents | (IV) |
| Introduction | 1 |
| Materials and methods | 23 |
| Tissue culture | 23 |
| Techniques | 26 |
| Results | 32 |
| Effects of cell secretions | 32 |
| Mutual adhesiveness | 38 |
| Cell positioning | 40 |
| Discussion | 45 |
| Conditioning effect | 45 |
| Monolayer collection | 50 |
| Two dimensional sorting out | 55 |
| Three dimensional sorting out | 59 |
| Conclusions | 62 |
| References | I |

INTRODUCTION

The organisation of cells into a living multicellular organism requires their assembly in a definite and exact relationship with one another. During the development of an animal, the relationships between the cells change extensively. As the animal matures these changing relations slowly settle down into the more constant characteristics of maturity. One of these relations is observed in the adhesion of cells to one another; without it there would be no multicellular animals.

The positions the cells hold in the organisms are critical for the life of the animals. Changes in these positions could cause serious or fatal abnormalities. The study of cell position in animals has for a long time been under experimental investigation, (Wilson 1907, Huxley 1921). One of the main systems that has been used is the formation of aggregates and segregation.

Huxley's (1921) finding that only the types of cells allowed to reaggregate were found in the resultant aggregates led to the general acceptance of stability of the cell type during the reaggregation and the occurrence of cell segregation according to cell type. Trinkaus and Groves (1955) observed that the differentiation of any particular tissue used in mixed aggregates will not occur unless the required number of cells of that tissue type are clustered and concluded that cell transformation may occur in the differentiation procedure. Townes and Holtfreter (1955) observed that not only the disaggregated cells segregated in the aggregates in groups of like cells, but also these groups took up defined positions in the aggregates. This study of Townes and Holtfreter led to further investigation of cell segregation and positioning of groups of like cells in aggregates of sponge, embryonic amphibian, chick and mouse material.

The most important procedure in these studies is the identification of the cell types used in the reaggregation. The difficulty of this procedure had been realised as early as 1907 by Wilson. Since then the search for reliable cell markers has continued so that the sorting out process can be more accurately examined. Histological markers, such as glycogen in embryonic chick heart cells (Steinberg 1962a), can not be used reliably in these experiments as they can be affected easily during disaggregation and reaggregation. Moscona (1957 and onwards) used the different staining properties and sizes of mouse and chick nuclei for his studies in mixed aggregates of chick and mouse embryonic tissues. This technique is of limited application and identification of individual cells is not always possible due to gradation of nuclear size, (Auerbach and Grobstein 1958). Trinkaus (1963) and Trinkaus and Lentz (1964) have used the granules of chick embryonic pigmented retinal cells to recognise this tissue in mixed aggregates. This marker is also of limited application as it only exists in pigmented retina cells and it is subjected to the degradation of the pigmented granules. Another natural marker used but of limited application is the natural colour of the different sponge species. This marker has the disadvantage that only a proportion of the sponge cells are stained. Artificial markers were widely used during recent years. Okada (1965) introduced fluorescent antibody labelling techniques to examine sorting out of embryonic mesonephros cells. Trinkaus and Gross (1961) introduced the use of radioactive compounds as markers for sorting out experiments. These two techniques have only been used very recently both for sorting out experiments and also for the study of other related phenomena such as cell adhesion.

The positions the cells hold in aggregates of different tissues may be a characteristic of their adhesive properties, (Moscona 1957, Steinberg 1962a). The mechanism controlling the cell positioning is

still unknown. Different theories have been proposed on the possible mechanism of positioning; the specific adhesion theory (Wilson 1907, Galtsoff 1925, Moscona 1962), differential adhesion theory (Steinberg 1962a, 1963a), timing hypothesis (Curtis 1961, 1962b) and morphogen theory (Curtis 1974).

These theories attempt to explain the positioning of cells from different normal tissues, but almost no work has been done on the positions cells hold in aggregates of normal and malignant tissues.

The aim of this work is to investigate this problem.

As early as 1894 Roux started the study of cell aggregation when he observed the aggregation of frog blastomeres. Wilson (1907) found that disaggregated sponge cells were capable of reconstituting a sponge when allowed to reaggregate in a glass dish. He tried to explain this reconstitution by two hypothesis: Either that the sponge cells redifferentiated according to their position in the aggregate, or that during the reaggregation the sponge cells of one type moved to a similar ^{position} in the aggregate as in the intact sponge.

A large variety of tissues have been tested to show the occurrence of cell sorting out in aggregates using different combinations. Two techniques have been used; (i) small pieces of tissues were fused together, and (ii) single cell suspensions, produced by various methods of dissociation, were aggregated under certain conditions.

Four main theories have been developed to explain the final positioning of cells in an aggregate (see page 3). First Wilson (1907), Galtsoff (1925) and Moscona (1962) proposed that cells show a considerable degree of specific adhesion such that either only like cells will stick together or that like types of cells tend to stick together more than unlike. The two next theories, Curtis (1961, 1962a) and Steinberg (1962a, 1963a), suggest that like cells will position themselves in a manner determined by the quantitative value

of the strength of the cell adhesion. A fourth theory was introduced by Curtis and Van de Vyver (1971) with the examination of the phenomenon of non-coalescence between different strain types of the sponge Ephydatia fluviatilis, first observed by Van de Vyver (1970). Curtis and his associates further studied this theory and Curtis (1974) introduced the morphogen theory which supposes that the cells produce factors that affect the adhesiveness of unlike cell types in a concentration dependent manner.

Steinberg (1963a) and Curtis (1961, 1967) criticised the specific adhesion theory of the Moscona group on the grounds that it does not provide any mechanism for patterning. This theory provides an explanation of the aggregation of like cells and in the extreme case of segregation where there is no intertype adhesion, (complete specificity), and it predicts that entirely separate bodies of the two cell types will form, but it does not provide any explanation for the positions that the different cell types occupy in mixed aggregates.

The second theory is divided in two parts - the one of Steinberg's group called "differential hypothesis" and the other due to Curtis called "the timing hypothesis".

Steinberg suggests that sorting out takes place so that the system reaches optimal thermodynamic conditions. These thermodynamic conditions refer to the surface energies of the individual constituents of the system. In this system the units adhere to one another rearranging themselves until the free surface energy of the system is reduced to a minimum. This minimum is achieved when the total work done through adhesion in the system is raised to a maximum, in other words, when all the individual units are mutually oriented in such a manner that they adhere to one another with the greatest average tenacity. At this point the thermodynamic equilibrium, the distribution of the two different types of units within the system, is a function of the work

of cohesion of each of the two units and of the work of adhesion between them. In order for the aggregate to sort out the cells have to move under the influence of the relative difference of the interfacial energies of the various adhesions. Steinberg considered a system of two cell types (a) and (b) which are cohesive and motile. Thus if the adhesion strength (W) of unlike cells is greater than the average adhesion strength of like cells, then the cell types will mix, i.e. $W_{ab} > \frac{W_a + W_b}{2}$. If the average adhesion strength of like cells is greater than or equal to the adhesion strength of unlike cells, the cell types will segregate forming separate aggregates, i.e. $W_{ab} \leq \frac{W_a + W_b}{2}$, or there will be partial enclosure, i.e. $W_{ab} \leq \frac{W_a + W_b}{2}$. Finally, if the average adhesion strength of like cells is greater than the adhesion strength of the unlike cells, where both of these are greater than the adhesion strength of the one cell type, the cells will then form concentric masses, i.e. $\frac{W_a + W_b}{2} > W_{ab} > W_b$, (b) cells surround (a) cells.

Steinberg's hypothesis depends on cells being freely motile within aggregates. It also suggests that sorting out may take place at early stages of aggregation as soon as a choice of adhesion is available. Supporting this are the observations of Trinkaus and Lentz (1964) by time lapse cinematography of living pelleted aggregates, suggesting that segregation in this system might begin after an hour in culture. As regards the motility of cells in aggregates there is little evidence for its occurrence. Weston and Abercrombie (1967) fused homonomic, i.e. identical, tissue fragments, with the cells of the one fragment labelled and further cultured these in shaker cultures. They showed that the cells of the two fragments were not all intermixed, so no free motion could be observed between the cells. Trinkaus and Lentz (1964) did not observe any movement of clusters of cells in living aggregates of embryonic chick heart and retinal pigmented cells. Contrasted to this was the finding of

De Haan (1964) who observed pericardial cells migrating in vivo on an endodermal substrate.

Curtis (1961) using disaggregated cells from amphibian mid-gastrulae found that if the endoderm was reaggregated for four hours before the ectoderm and mesoderm were added, the ectoderm associated with the endoderm though initially separate from it. He also showed that the longer the endoderm has been reaggregating before ectoderm and mesoderm were added the further inside the aggregate were the ectoderm and mesoderm. The first result shows that the specificity of sorting out is destroyed by confusing the timing of the reaggregation process; the second that the positions of the cell types in the reaggregate can be altered by changing the timing of the addition of the various types. Together these conclusions suggest that sorting out and positioning of the cell types in the reaggregate are controlled by timing processes. This control has been named by Curtis (1961) as "temporal specificity".

Steinberg (1964) suggested that by the timing theory a single internal mass of the one cell type will result, which does not always happen. Curtis (1967) suggested that the timing hypothesis explains the occurrence of many small discontinuous regions in the internally segregating types in some aggregates. Thus when the number of cells of each type are fairly similar, the internally segregating type will be found to move towards the centre of the aggregate during segregation. Slight variations in the distribution of the various cell types in the aggregate at the start of aggregation, or a variation in the time at which trapping starts in various parts of the aggregate, would lead to eccentric segregation of the internally segregating type, as has been found by Steinberg (1964, 1962b).

The observations of Townes and Holtfreter (1955) and Steinberg (1964) that tissue fragments, which have not been treated with

disaggregating agents, sort out, argue against the timing hypothesis to some extent. For the timing theory the adhesiveness of cells may be differentially affected by the disaggregation procedure or the medium in which reaggregation occurs. Trinkaus (1969) commented that there was no evidence that disaggregation procedure differentially affected cell types. Curtis (1970) showed that the adhesiveness of chick embryo neural retina and liver cells varied according to the disaggregation procedures applied. Components of the medium, such as the presence of serum, could conceivably alter the adhesiveness of tissues as well as suspensions (Curtis 1965).

Active cell motility during sorting out is essential for both the differential adhesion hypothesis and the timing hypothesis. Experiments with tissue fragments fused overnight on an agar substrate and then cultured on millipore filters or agar, supposed substrates of low adhesiveness, or in shaker cultures, which are believed to provide a substrate of higher adhesiveness in the presence of growth medium, have shown that: a) heteronomic tissues sorted out following the patterning of aggregates in the case of shaker cultures, but they did not show any patterning in the cases of agar or millipore filter cultures, (Steinberg 1964, Weston and Abercrombie 1967, Wiseman et al 1972); b) homonomic tissue fragments fused without the cells infiltrating the apposing tissue, (Weston and Abercrombie 1967).

The fourth theory has been recently advanced by Curtis (1974). This theory -the morphogen theory- a term used by Edelman (1970) for hypothetical factors that are supposed to control cell positions in aggregates by chemotactic action, was first introduced by Curtis and Van de Vyver (1971). They were testing the mechanism by which the non-coalescence phenomenon (Van de Vyver 1970) is developed in the sponge Ephydatia fluviatilis. When sponges of unlike strains were placed in contact they appeared to adhere temporarily but soon

afterwards separate, (Van de Vyver 1970). This observation was explained (Curtis and Van de Vyver 1971) by the assumption that specific diffusible substances were secreted from the sponges that diminished the adhesiveness of the opposite strain cells, resulting in the separation of the sponges. Curtis and Van de Vyver (1971) confirmed this by measuring cell adhesion in the presence of these factors.

Curtis (1974) and in press, found that embryonic neural retina and liver cells released substances into the medium during active growth that reduce the adhesiveness of the opposite cell type without stimulating the adhesion of the type from which they were derived. Similar action has been found for the mouse B and T lymphocyte system in vitro, (Curtis and De Sousa 1973, 1975) and a hypothesis was advanced that lymphocyte recirculation and thus the positioning is controlled by B-T interactions mediated by soluble factors.

In order to test one or more of these theories a great variety of experiments have been carried out using either dissociated cells from different tissues or whole tissue fragments from different species. Chick and mouse embryos are widely used in these experiments. However other animals have also been used for this study, i.e. sponges and amphibians. The selection of the different species and tissues is based mainly on the availability of these animals and on the facility of the disaggregation of the cell types from these tissues.

Attempts to distinguish differences in the morphogenetic processes of the various tissues led to the use of tissues of the same species in sorting out experiments. Simultaneously there were attempts to study if differences exist between the same tissue of different species and between different tissues of various species

in order to study possible changes on the positioning pattern of tissues between different species so that possible evolutionary processes could be further studied.

In the study of sorting out the following terminology has been applied: For the combinations of tissues of the same species, "homospecific" combinations, there are the 'homologous' aggregates and 'homonomic' fragments, where cell type is identical, and the 'heterologous' aggregates and 'heteronomic' fragments, where cell types are different. For the combinations of tissues from different species, "heterospecific, xenospecific" combinations, there are the 'isotypic' and 'heterotypic' aggregates when the aggregates are from dissociated cells either from the same or different tissues respectively.

Moscona and his associates and Burdick and Steinberg (references see below) prepared mixed aggregates of mouse and chick embryonic cells from either the same or different tissues. Thus the tissue specificity observed, that is the cellular self recognition, among tissues of the same animal was extended to species specificity. Moscona and his associates in a series of publications have reported that, for heterospecific isotypic combinations of limb precartilage, (Levak-Svajger and Moscona 1964, Moscona 1957, 1961a), liver, (Moscona 1957, 1961a), neural retina, (Moscona 1961b), embryonic kidney, (Moscona 1962), brain, (Garber and Moscona 1972), and skin, (Garber and Moscona 1964, Levak-Svajger and Moscona 1964, Moscona 1961c, 1964, Moscona and Garber 1968, Moscona M. and Moscona A 1965), the cells did not sort out from each other according to the two species types. These results suggested the generalisation that for any one embryonic cell type the properties responsible for cell sorting are indistinguishable among even very distantly related warm-blooded vertebrate species.

Contrasting results to those of Moscona's group are those of Steinberg's group. Burdick and Steinberg (1969) observed sorting out in heterospecific aggregates of myocardial cells and Burdick (1970) in limb mesoblast cells. While these observations of Steinberg's group contrast those of Moscona's group, Burdick (1972) carried out experiments and showed that mixtures of mouse and chick liver cells do not sort out and so he confirmed the results of Moscona's group. He also fused mouse and chick liver fragments and observed that they sorted out with the chick tissue enveloping the mouse tissue. Weston and Abercrombie (1967) and Gershman (1970) observed that in fusing chick liver fragments there was no significant envelopment of the one fragment by the other. From the above results it is apparent that the generalisation of Moscon'a group is no longer valid. The facts that chick embryonic liver fragments when fused do not envelop each other, but when fused with mouse liver they envelop it, and also that chick liver cells sort out from chick heart cells and that mouse liver and chick heart cells do not sort out in mixed aggregates, indicate that the morphogenetic properties of mouse liver cells are not the same as those of chick liver cells, (Burdick 1972). In all these experiments the different cell types were recognised by staining the aggregate sections with haematoxylin.

Aggregates prepared from different tissues from mouse and chick embryos showed tissue specific sorting out as stated by Moscona (1957) for mouse liver and chick chondrogenic cells, by Auerbach and Grobstein (1958) for mouse metanephrogenic mesenchymal cells and chick spinal cord cells, by Moscona (1961a) for limb bud mesoblast and liver cells in both combinations of mouse and chick embryos, and from unpublished results of Burdick referred in Burdick (1972) for mouse liver and chick neural retina cells. Meanwhile Burdick (1972) reported that the combination of mouse liver and chick heart ventricle cells did not

sort out and instead formed intermingled aggregates.

The grouping behaviour of embryonic cells and tumour cells was examined by Moscona and Kuroda, (references see below), as a result of the observations of Leighton et al (1960) and Wolff and Wolff (1961) and of others, that a variety of mammalian tumours were capable of invading chick embryonic tissues. An early attempt at this was made by Moscona (1957) who mixed chick embryo chondrogenic cells with mouse pigmented melanoma cells, S 91, and found that the aggregates derived from these cells consisted of a central mass of cartilage surrounded by S 91 cells. Moscona (1961a) repeated this experiment with liver cells and observed that the aggregates consisted of a central mass of S 91 cells surrounded by hepatic parenchyma. In both the experiments, upon a further culture, the clarity of the regional segregation of the two kinds of cells was progressively lost owing to the invasive activity of the neoplastic elements.

Further experiments on this embryonic-neoplastic cell behaviour were carried out by Kuroda (1968a). He combined chick liver, limb bud and skin cells with HeLa cells. On the first combination, chick liver-HeLa cells, there was a complete segregation of the two cell types. On the second, limb bud-HeLa cells, the aggregation of ^{esp. consists of} composite structures of chick mesoblasts and HeLa cells. On the third, skin-HeLa cells, chick epidermal cell masses were surrounded by a chimaeric tissue in which HeLa and chick dermal cells were interspersed.

It is also worth considering experiments carried out with cells with common germ layer origin within one species. Organisms with which work has been done are the sponges, amphibians, chick and mouse embryos.

The readhesion of aggregating sponge cells was shown to be preferentially species specific, (Wilson 1907, Galtsoff 1925), i.e. mixtures of cells from two species tend to adhere to homologous cells.

More recent work on the aggregation of sponge cells indicated the same conclusions of Wilson and Galtsoff. Humphreys (1970) using the sponges Microciona prolifera, Haliclona oculata and Halichondria panicea, observed complete segregation of the different sponge cells during reaggregation by time lapse photography. In these experiments the different cell types were not recognised precisely and on occasions as Humphreys reported the cells made 'mistakes'.

John et al (1971) studied the aggregation of the sponges Ophlitaspongia seriata and Halichondria panicea in mixed aggregates. They separated the archeocytes from the mucoid cells and they concluded that archeocytes must be present in dissociated monospecific cell populations for aggregation of all cells to occur. It seems that the archeocytes interact with the mucoid cells before the latter show any aggregative potential. Aggregated archeocytes from two species will not separate from one another unless the mucoid cells from one of the species are present. Meanwhile aggregated mucoid cells from both species will not separate from one another unless both their homologous archeocytes are present. Species specificity in the sorting out process is conferred by mucoid cells in interaction with the archeocytes. The term mucoid cells is not well understood. In this work the recognition of the cells in the aggregates formed was on the basis of colour only.

In addition to these cases where complete segregation of the different species occurs during reaggregation, Curtis (1962b) studied the reaggregation of the sponges Microciona sanguinea, Suberites ficus, Halichondria panicea and Hymeniacidon perleve. He found that sorting out patterns depended on the conditions of the experiment. By varying the conditions of the experiments, that is the time at which the different species were mixed, he could elicit different types of sorting out behaviour, namely (1) the aggregates

separated completely, (2) the aggregates formed chains, (3) the aggregates formed concentric coating masses and (4) the aggregates appeared intermingled. These observations support Curtis' timing hypothesis for sorting out. If one attempts to apply the differential adhesion hypothesis which presupposes that cells in aggregates are freely motile, to the previous observations, then it would be difficult to give any logical explanation on the different types of sorting out behaviour referred to earlier.

The specific adhesion theory may explain the sorting out pattern of sponge cells, and the findings of Turner and Burger (1973) and of Weinbaum and Burger (1973) can give further support to a particular explanation of this type of the species specific segregation of sponge cells. The macromolecules they have isolated appear to control sponge cell segregation.

The individual aggregative potential of chick embryonic tissues was studied before the study of the morphogenetic movements of the chick embryo. The effect of the dissociating agents, components of the media and other reagents involved in the reaggregating system were studied first.

The positioning of the different cell types in mixed aggregates may depend on the selectivity they show towards like and unlike cells. Studies on this selectivity were carried out by Roth and Weston (1967), who prepared aggregates of neural retina and liver cells and continued the aggregation in the presence of labelled suspensions of neural retina and liver cells. They concluded that isotypic associations are more stable than heterotypic ones as isotypic aggregates collected more labelled cells than heterotypic aggregates. Similar experiments were carried out by Roth (1968) and Roth et al (1971).

Bearing in mind this adhesive selectivity shown by cell aggregates we can now advance to the segregation patterning found in mixed

aggregates. Many combinations have been studied and Steinberg (1964) demonstrated a "hierarchy" of sorting out in some combinations of embryonic chick tissues within aggregates or tissue fragments systems, and Steinberg (1970) explains this hierarchy in terms of 'preference' of various tissues for the internal positions. The term preference is used because instances of reversal of positions have been observed. The tissues used were from different embryonic stages between the 36th hour and the 8th day of incubation. The hierarchy found is as follows: germinative layer of epidermis > limb bud precartilage > pigmented epithelium of the eye > myocardium of heart ventricle > neural tube > liver, where the observation is that every member of the series tends to envelop each member preceding it. This work of Steinberg agrees with the results of all other workers who used chick embryonic tissues for their studies.

Under certain conditions it is possible to alter this generally accepted hierarchy. Armstrong and Niederman (1972), Wiseman et al (1972), Steinberg (1970) and Wiseman (1970) have reported cases where the normal pattern of sorting out was reversed. This phenomenon was called 'position reversal', (Steinberg 1970). Wiseman et al (1972) concluded that the dissociation-reaggregation procedure decreased heart intercellular cohesiveness, that organ culture increased the cohesiveness of heart fragments and reaggregates, and that as a result of such changes a pair of combined cell populations could become reversed in relative cohesiveness during the course of an experiment, and that differences in phase ratio merely facilitated the detection of such time depended reversals in relative intercellular cohesiveness. Crosby (1967) suggested that the proportions in which two cell populations are mixed, which he termed "phase ratio" might also, under certain circumstances, influence their final arrangement. All the experiments in which reversal of positioning has been observed included

heart tissue. Therefore, Wiseman et al (1972) studied the heart ventricle-liver combination, and Armstrong and Niederman (1972) the combinations of heart ventricle-pigmented retina, and of heart ventricle-limb bud mesoderm cells.

Different factors could influence the pattern of sorting out. Such a factor is the drug cytochalasin B which inhibits active cell movement, (Carter 1967). Steinberg and Wiseman (1972) reported that when cytochalasin B was removed from the incubation medium of liver explants growing on plastic the inhibitory effect was reversed. The reversibility also exists in mixed aggregates, where in the presence of cytochalasin B heart and liver cell mixtures fail to sort out. Cytochalasin B also inhibits isotypic fusions of heart and liver fragments, but when removed the fragments recovered from the inhibition. Carter (1967, 1972) observed that the effect of cytochalasin B on cell motility was a reversible effect and Sanger and Holtzer (1972) reported the reversibility of the effect of cytochalasin B on cell adhesion and sorting out. Armstrong and Parenti (1972) also studied the effect of cytochalasin B on other combinations of chick embryonic tissues. They found that the sorting out of pigmented retina-heart cells was completely inhibited but the sorting of neural retina-pigmented retina aggregates was only slightly affected.

Other factors which possibly affect cell positioning are the secretions of cells. The latest experiments of Curtis' group show a control of cell positioning by such secretions, (Curtis and Van de Vyver 1971, Curtis and De Sousa 1973, 1975, Curtis 1974). Such secretions can even cause position reversal. When neural retina and liver cells from the chick embryo were reaggregated in liver conditioned medium intermingled aggregates resulted. If aggregated in neural retina conditioned medium a position reversal phenomenon was observed. If presorted aggregates were incubated in neural retina conditioned

medium no effect on sorting out was seen, but in liver conditioned medium randomisation occurred within the aggregates, (Curtis 1975 in press). These factors not only affect cell positioning significantly, but also affect the rate of cells adhering together before any positioning procedure commences, (Curtis and De Sousa 1973).

Armstrong and Parenti (1972) observed that the adhesion of neural retina cells to form aggregates was not inhibited by cytochalasin B, but limb bud cells formed smaller aggregates than in the controls. However Steinberg and Wiseman (1972) demonstrated the reverse, that limb bud cells were hardly affected by cytochalasin B, and neural retina and liver cells formed smaller aggregates than in the controls. In addition they demonstrated that heart cell reaggregation was enhanced by cytochalasin B. These results show that cytochalasin B has a variable effect on cell adhesiveness.

Jones and Partridge (1974) reported that cytochalasin B inhibited the aggregation of limpet haemocytes, (but did not disrupt preformed cellular contacts), whereas colchicine did not significantly affect their aggregation. Waddell et al (1974) showed that the aggregation of BHK fibroblasts was sensitive to the alkaloids colchicine and vinblastine. Prostaglandins and cyclic nucleotides have a varying effect on the adhesion of cells onto protein coated plastic. For example, Ehrlich ascites tumour cells responded differently to various prostaglandins, and dibutyryl-cyclic-AMP decreased the adhesiveness of these cells, (Weiss 1973). Grinnell et al (1973) reported that dibutyryl-cyclic-AMP did not affect the stickiness of BHK 21 C13 cells nor of the polyoma transformed derivative BHK-Py cells.

Another approach to the elucidation of the mechanism of sorting out has been to ask the question does specific adhesion exist and if so how is it mediated.

Moscona (1960, 1962) described a gel which he termed extracellular material found in association with reaggregating cells. Rosenberg (1960) reported that freshly trypsinized cells released a gel material which bound to glass. Steinberg (1963b) and Steinberg and Roth (1964) suggested that extracellular material was derived from cells lysed by trypsinisation, since they demonstrated that extracellular material could be lysed with DNA ase.

Moscona (1962, 1963) found that supernatants from actively metabolising suspensions of chick embryonic cells would increase the size of aggregates formed at 25°C. Moscona (1963) and Humphreys (1963) reported that cells from the marine sponges Haliclona oculata and Microciona prolifera would not aggregate at low temperatures if they had been dispersed in a medium lacking divalent cations, although mechanically disrupted cells would adhere at low temperatures. Fractions of these divalent cation free media in which the cells had been dispersed would cause aggregation of homologous cells, but not of heterologous cells. Thus the factors appear to be specific promoters of aggregation. Margoliash et al (1965) found that Haliclona oculata factor was a complex macromolecular mixture of high molecular weight. Humphreys (1965) reported slightly different properties for the same material.

Lilien and Moscona (1967) obtained a factor from the medium of neural retina cultures, exposed to serum free media for two days, which increased the diameter of aggregates of cells. Lilien (1968) showed that several other cell types could produce non specific adhesion factors when treated like the ones of Lilien and Moscona. Kuroda (1968b) obtained similar evidence, that the conditioned medium from liver cell cultures would stimulate aggregation of these cells. Takahashi and Okada (1970, 1971) fractioned a conditioned medium from chick embryonic fibroblasts and myoblasts to obtain two factors. One

promoted the aggregation of cells, the other was not effective by itself but only on cells treated with the other factor. Kondo and Sakai (1971) reported that a factor released during the dispersion of sea urchin embryos stimulated the aggregation of these cells. Kondo (1974) found in crude ovacquenin, a reaggregation promoting substance from sea urchin embryos, a factor which inhibits reaggregation of dissociated blastomeres. Pessac and Defendi (1972a) suggested that the factors reported by Lilien and others were hyaluronic acid. Mateyko and Kopac (1963) found that hyaluronidase was ineffective in cell dispersion, so the suggestion of Pessac and Defendi (1972a) seems improbable. Wasteson et al (1973) reported that feline lymphoma cell aggregation is depended on hyaluronic acid, but BHK 21 cell aggregation is not inhibited in the presence of 12.5 μ g/ml bovine testicular hyaluronidase, (Edwards et al 1975).

Pessac and Defendi (1972b) demonstrated that some mammalian cell lines produce factors that stimulate the aggregation of their own cells and of heterologous cell lines. Roth (1968) using undialysed conditioned media of the same type as Lilien, found that the factors from both retina and liver increased the collection of cells by isologous aggregates whether isotypic or heterotypic factors were used. Oppenheimer and Humphreys (1971) obtained a specific adhesion factor from the ascitic fluid in which teratoma cells were grown. This factor promoted the aggregation of mouse teratoma cells but not the aggregation of sarcoma 180 or 7 day old chick embryo neural retina cells. Curtis and Van de Vyver (1971) examined the adhesion of cells from different strains of the fresh water sponge Ephydatia fluviatilis. They discovered that these sponges produce soluble factors that diminish the adhesion of unlike cell types while increasing the adhesiveness of homologous cells.

Muller and Zahn (1973) studied a factor from the sponge Geodia

cydonium which was released after calcium and magnesium free sea water treatment, and which appears to be species specific with its functional group consisting of a protein with polar amino acids. Turner and Burger (1973) and Weinbaum and Burger (1973) studied the aggregation of cells of the sponge Microciona prolifera and described an aggregation factor which had a carbohydrate constituent, was calcium dependent, and was bound on the cell surface on a special receptor site which they isolated and called base plate.

The existence of factors in cell cultures affecting cell adhesion and positioning either positively or negatively is indubitable.

There are a group of factors which may or may not be identical with these described above which are present in sera, (Fisher et al 1958, 1959, Curtis and Greaves 1965, Orr and Roseman 1969, George et al 1971).

Divalent cations play a very important role in cell adhesion. Ringer (1880) found that calcium was necessary to preserve the normal intercellular contacts in tissues. Roux (1894) found that in calcium free media frog blastomeres were more easily separated than in complete salt solution. All workers studying the aggregation of sponge cells have shown the importance of calcium ions in sponge cell dissociation and adhesion, (Weiss 1960, Garvin 1961, Armstrong and Jones 1968, Takeihi and Okada 1972, Hornby 1973b, Deman et al 1974). Edwards et al (1975) also studied the effect of the divalent cations on cell to cell and cell to substrate adhesion. They found that the formation of adhesions of BHK cells does not require addition to the medium of divalent cations, although it is increased by divalent manganese and cobalt ions.

Because of the involvement of calcium in cell adhesion, chelating agents (such as ethylene-diamine-tetra-acetate, EDTA) are used in the disaggregation of chick and mammalian embryonic tissues, (Anderson

1953, Zwilling 1954). Townes and Holtfreter (1955) used alkaline pH, Rinaldini (1958), Wilmer (1945), Moscona (1952), Easty and Mutolo (1960) and others used enzymes to disaggregate embryonic tissues, such as trypsin, papain, e.t.c. Argument has been growing for years between scientists as to which method is best for disaggregation of embryonic tissues. Moscona A. and Moscona M. (1967) and Lilien (1969) claimed that EDTA separated cells do not show histogenetic aggregation and thus that the cells are so damaged that they display none of the adhesive properties exhibited by trypsinized and presumably normal cells. Glaeser et al (1968) compared the formation of aggregates of neural retina cells after EDTA or trypsin treatment and found that similar aggregates in size and shape formed after both treatments. Allen and Snow (1970) and Snow and Allen (1970) reported that BHK cells harvested with trypsin suffered more damages than harvested with EDTA. Crude trypsin released similar amounts of RNA as EDTA did, but higher amount of DNA than EDTA, meanwhile crude trypsin released more macromolecules containing amino sugars than EDTA did, (Snow and Allen 1970).

Tickle (1970) and Elton and Tickle (1971) showed that EDTA treated cells sorted out in aggregates. Glaeser et al (1968) reported that the adhesion of EDTA treated cells was not affected by puromycin, whereas trypsinized cells were inhibited by this antimetabolite. Kemp et al (1967) found that puromycin inhibited the aggregation of trypsinized chick embryonic muscle cells extensively, while the adhesion of EDTA treated cells was affected to a less extent. Curtis (1970) showed that the adhesiveness of chick embryonic neural retina and liver cells as measured by Couette Viscometry, (Curtis 1969), varied according to whether the cells were disaggregated with EDTA or trypsin and discovered the recovery phenomenon after trypsinization.

Three principle methods have been used for the preparation of aggregates. One method is to allow disaggregated cells to settle and reaggregate in cavity slides where cell movement and Brownian motion are presumed to form aggregates, (Wilson 1907). A variation of this method has been to allow cells to reaggregate on the chorioallantoic membrane of the chick embryo, (Weiss and Taylor 1960). In the other two methods, aggregation of cells is not dependent on cell locomotion. In the first of these methods disaggregated cells are pelleted with centrifugation and the pellets cultured on agar, (Trinkaus and Lentz 1964). In the other method cells are brought together to form aggregates in shaking flasks. This technique was introduced by Gerisch (1960) and has been widely used by many workers since then.

Several methods have been used for the study of cell adhesiveness. These methods are based on the measurement of the force required to break an adhesion or the rate at which adhesion form or on the final size of the aggregates. Dan (1936) counted the proportion of echinoderm eggs that remained adherent to a glass plate after it was inverted so that gravity tended to pull the eggs away from the plate. Coman (1944) and Malenkov et al (1963) measured the deformation of a microneedle which was used to pull two cells apart at the time of separation of the two cells. Easty et al (1960), Weiss (1961a,b) and Berwick and Coman (1962) measured the proportion of cells attached to a glass slide after it had been exposed to centrifugal force acting along the plain of the slide. Moscona (1961a) used the diameter of aggregates that were formed on shaking a cell suspension as a measure of cell adhesiveness, but Gershman (1970) could not find any relationship between adhesiveness measured from aggregate diameter and that measured by following aggregation rate.

Curtis and Greaves (1965) measured the rate of inclusion of

cells into aggregates by counting the population density of cells that were not incorporated in aggregates after a period of time. Curtis (1969) and Curtis and Hocking (1970) put this kinetic method on a fully quantitative basis and used it to measure adhesiveness. Edwards and Campbell (1971) suggested that the slope of the plot of the total particle number against time is proportional to the adhesiveness of the cells.

Roth and Weston (1967) used the collecting aggregate system to study the adhesiveness of chick embryonic cells. In this method the proportion of cells 'trapped' from suspensions by a cell aggregate is a measure of adhesion. Roth et al (1971) introduced the collecting cell lawn system which has been developed by Walther et al (1973). This method is a variation of the previous stated, ~~on~~ where instead of aggregates collecting cells, cell monolayers are used for the collection, (for further details see methods). Curtis (1969) used the Couette Viscometer to measure the collision efficiencies of various proportions of freshly disaggregated cells, as the collision efficiency has been shown to measure the adhesiveness of cells, (for further details see methods). These three last methods can be used for the study of the specificity of the adhesions as well as to measure cell adhesiveness.

MATERIALS & METHODS

For this study the following cell lines and tissues were used: BHK 21 C13, BHK Py C1, BHK Py C1i, HP, HP HSV2, T2, T3 and 7 day old chick embryo neural retina and liver cells.

Tissue Culture

A. Chick embryonic tissues.

1) Neural retina cells.

Hen eggs (Golden Comet, Hubbard hybrid) were incubated for 7 days, stage 30 of the development. The embryos were removed aseptically from the eggs and placed in ice cold Hanks H₂O BSS (HH). The eyes then were removed and kept in HH and with the use of fine forceps they were opened through the choroid fissure and the neural retinas were dissected and placed in HH. The retinas were next treated by either EDTA or trypsin.

a) EDTA disaggregation, (after Curtis and Greaves 1965).

The tissues were washed with CMF saline (pH 7.8) and next treated with 0.001 M EDTA in CMF saline (pH 7.8) for 10 min. at 2°C. After two further washings with CMF saline the tissues were mechanically disaggregated in HH and finally the single cells, derived after removing by centrifugation the clumps, were suspended in HH.

b) Trypsin disaggregation (after Roth and Weston 1967).

The tissues were washed with HH and treated with 10 ml of 0.25% trypsin (Difco 1:250, 1000 BAEE units/mg) in Tris saline for 15 min. at 37°C. The trypsinization was next stopped by adding 10 ml of fresh growth medium to the disaggregation medium. After discarding the disaggregation medium the tissues were mechanically disaggregated in fresh growth medium plus 1 µg of DNA ase. The single cells left after the clumps had been removed by centrifugation were suspended in fresh

growth medium. The growth medium consisted of 8 parts of Eagles MEM, (Glasgow modification), plus one part of calf serum, (Biocult Lab.), plus one part of tryptose phosphate broth, (Oxoid), (ECT). These cells were used for culture preparations, Fig. 1.

2) Liver cells.

Livers were dissected from stage 30 hen eggs and placed in HH. The blood was removed from the liver by squeezing the lobes with fine forceps and next by chopping the liver followed by a wash with HH and treated either with EDTA or trypsin with the same methods described before for neural retinas.

B. Syrian hamster embryonic cell cultures.

All the cell lines were kindly provided by Mrs. Macnab (Institute of Virology, Glasgow University).

1) Hamster embryo primary cell cultures, (HP). (After Duff and Rapp 1970).

The cells were kept in cultures of low passages in 120 cm² flat glass culture bottles. After 10 passages the cells were discarded and new ones were prepared by the same method. Cultures were prepared every 3 days, from previous subcultures by seeding 4×10^4 cells/cm² in the culture bottles. The growth medium in which the cells grew was: 8 parts of Eagles MEM, plus one part of foetal calf serum, (Biocult Lab.), plus one part of tryptose phosphate broth, (EFT). The cultures were buffered with 5% CO₂ and 95% air as the gas phase and the bottles were sealed and placed at 37°C, Fig. 2.

2) Herpes simplex type 2 virus transformed hamster embryo primary cell line, (HSV2). (After Duff and Rapp 1970).

The media in which cells were growing were kindly provided by Mrs. Macnab. The same culture conditions were used as for the hamster embryo cells.

3) Herpes simplex type 2 virus transformed hamster embryo primary

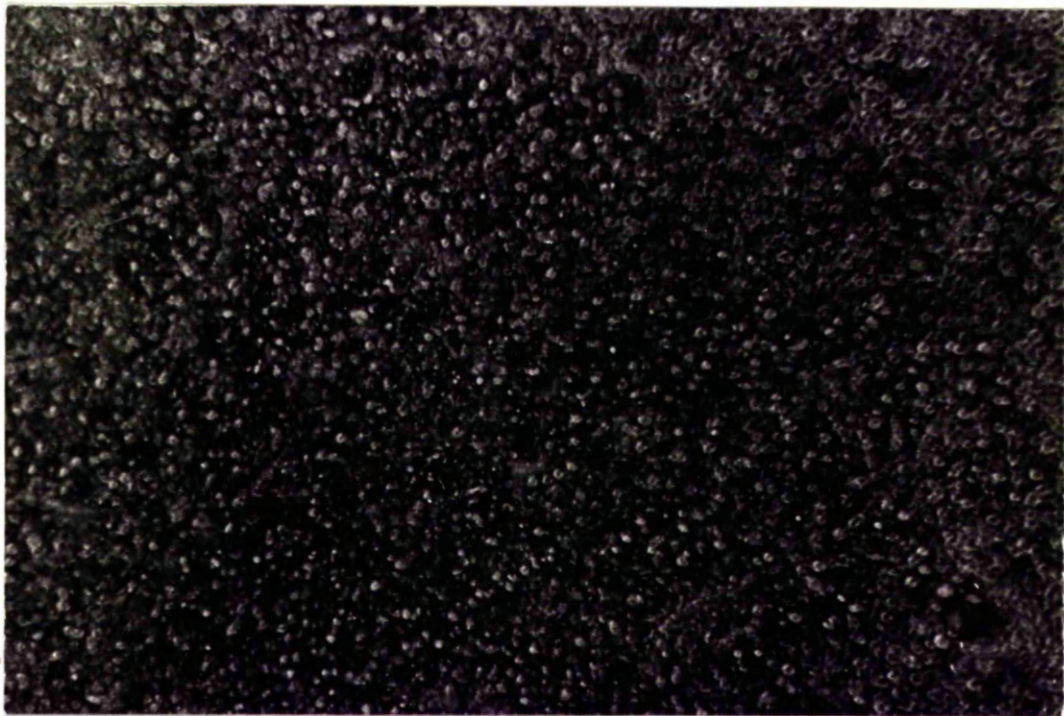


Fig. 1. NR cell culture. Magn. 1500

derived tumours, (T2, T3). (After Duff and Rapp 1971).

Stocks of these cells were prepared in 13 parts of Eagles MEM, plus one part of glycerol, plus 6 parts of foetal calf serum, and were kept in liquid nitrogen. The same culture conditions were used as for the hamster embryonic cells but 1×10^4 cells/cm² were seeded in the culture bottles, Fig. 3.

C. Syrian baby hamster cell cultures.

All the cell lines were kindly provided by Dr. J.G. Edwards, (Department of Cell Biology, Glasgow University).

1) Baby hamster kidney fibroblasts, (BHK 21 Cl3). (After Stoker and Macpherson 1964).

Stock of these cells was prepared in 13 parts of Eagles MEM, one part of glycerol, 6 parts of foetal calf serum, and kept in liquid nitrogen. The growth medium for these cultures was the ECT. The cells were seeded at the concentration of 2×10^4 cells/cm², under the previous described conditions, Fig. 4.

2) Polyoma transformed baby hamster kidney cells (BHK Py Cl). (After Macpherson and Montagnier 1964).

All conditions were identical as for the BHK 21 Cl3 cells but only 1×10^4 Cells/cm² were seeded per culture bottle.

3) Polyoma transformed baby hamster kidney cells, (BHK Py Cl1).

This cell line was cloned by Dr. J.G. Edwards from the BHK Py Cl1 cell line. All culture conditions were identical as for the BHK Py Cl1 cells, Fig. 5.

Dissociation of cell lines, (after Edwards and Campbell 1971).

The same procedure was used to obtain single cell suspensions of hamster embryo and baby hamster cell lines.

The cultures were washed with Tris saline and next treated with a mixture of 1:4 parts of 0.25% trypsin (Difco 1:250) in Tris saline: 0.55 mM EDTA in Phosphate buffer saline, for 30 sec. at room temperature.

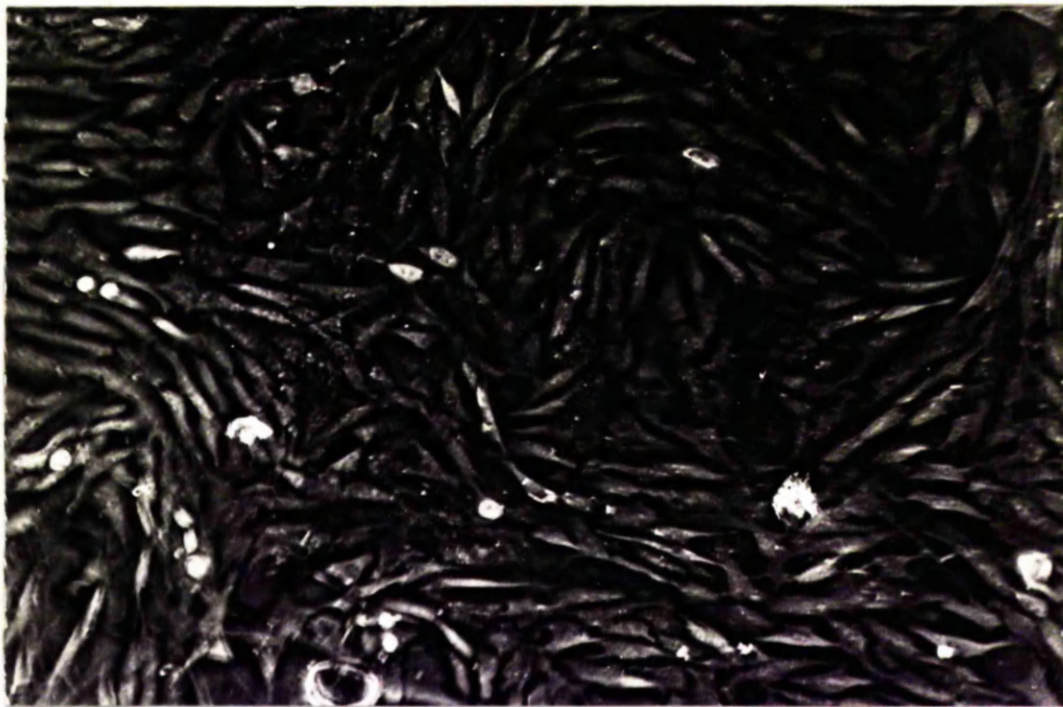


Fig. 2. HP cell culture. Magn. 1500

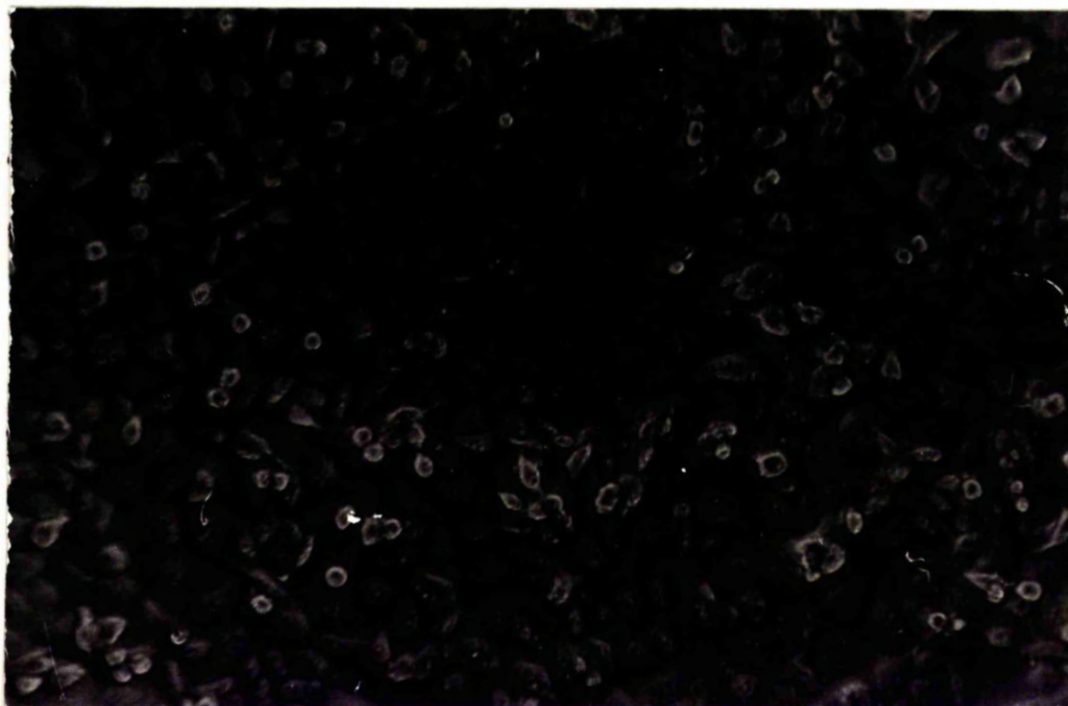


Fig. 3. T2 cell culture. 1500

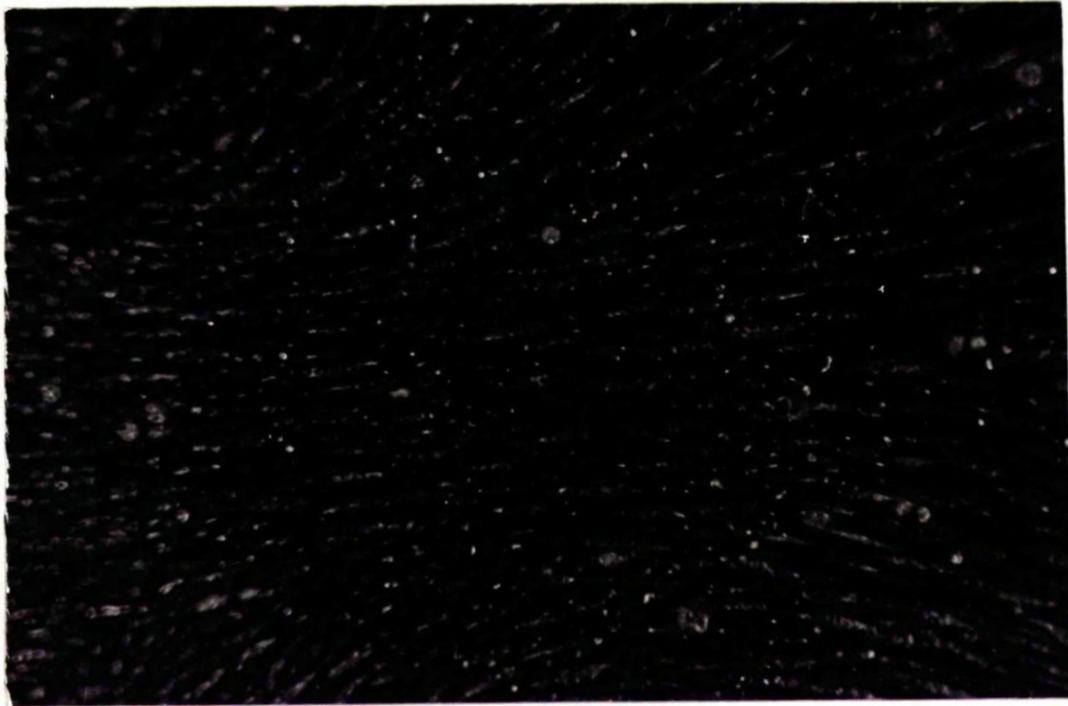


Fig. 4. BHK 21 C13 cell culture. Magn. 1500



Fig. 5. BHK Py C11 cell culture. Magn. 1500

The dissociating mixture was next discarded. When the cells started coming off the glass 10 ml of fresh growth medium was added per culture bottle and the cells were ^{broken} ~~scraped~~ smoothly off the glass. After mechanical dissociation of the clumped cells, a single cell suspension was obtained.

Techniques

The study of the embryonic and neoplastic cell interaction was carried out using the following techniques:

Measurement of cell adhesion by:

Couette Viscometry.

The effect of the different conditioned media upon neural retina cell adhesion was investigated using the Couette Viscometers, (Curtis 1969).

Couette Viscometers consist of a pair of concentric cylinders of radial dimensions such that when the smaller is suspended freely inside the larger a narrow gap exists between the two cylinders and laminar flow conditions obtain when one cylinder is rotated with respect to the other. The suspension of cells whose adhesiveness is to be measured is placed in the gap between the concentric cylinders and the one of the cylinders is rotated resulting in a laminar shear flow. The shear rate G is determined by the rate of rotation of the cylinder and the radial dimensions of the cylinders. A Couette Viscometer constructed by Barholm Tool and Gauge Co. Ltd., Glasgow, was used to measure the adhesiveness of the neural retina cells. The cylinders were treated with 1% silicone fluid DC 1107 (Hopkin and Williams Ltd.) in ethylacetate followed by overnight U.V. irradiation to polymerise the silicone and make a non adhesive surface on top of the stainless steel surfaces of the cylinders. The cells make collisions under the influence of the laminar shear flow. The probability that a collision between two particles results in their adhesion, collision efficiency,

is a measure of adhesiveness in this system. The collision efficiency is calculated from the formula that Swift and Friedlander (1964) developed:

$$\ln \frac{N_{\infty t}}{N_{\infty 0}} = - \frac{4G\phi\alpha t}{\pi}$$

where $N_{\infty 0}$ and $N_{\infty t}$ are the total number of particles of all classes at the start and at the time t of aggregation respectively, G is the shear rate, ϕ is the fractional volume occupied by particles and α the collision efficiency. This relationship ceases to apply when the aggregates begin to approach their equilibrium size, (Curtis 1973).

Approximately one ml of cell suspension was added per Couette. The cells were reaggregated for 28 min. and samples were taken every 7 min. and counted on a modified Fuchs-Rosenthal (BS 748) haemocytometer with the use of a Vickers Patholux microscope. The adhesiveness of the cells was measured as the mean value of the collision efficiencies. The shear rate developed during the rotation of the Couettes was of the value 10.25 sec^{-1} .

Cell lawn system, (Walther et al 1973).

Cell monolayers growing on a substrate, either glass or plastic, collect ~~eggs~~ ^{cells} from a suspension placed on top of the monolayers. In this system the adhesiveness of cells is measured as the proportion of cells collected from the original cell suspension by the monolayer. In this system the collected cells are labelled with either ^{32}P , ^{14}C or ^3H and they are counted in a liquid scintillation system. With this assay we measure cell adhesiveness and the specific adhesion of cells. In the present work this assay will be used to study the effect of the conditioned media on the neural retina cell adhesiveness and the adhesive relationships the different cell types develop between themselves.

Monolayers of cells were cultured in 16mm diameter plastic wells

(Linbro Chemical Co. Inc.) to confluency. The monolayers were washed with HH twice and next the appropriate medium was added on top of the monolayers. The assay was carried out at 37°C using radioactively labelled cell suspension. The appropriate number of labelled cells were next placed on top of the monolayers and were left for the appropriate time to interact, more details see during the experiments description. The medium next with the non attached cells was drained off and the monolayers were washed twice with warm HH. One ml of 1 N NH_4OH was added per monolayer to dissolve it. The dissolved monolayers were then transferred in scintillation counting vials and 10 ml of scintillation fluid was added per vial. The radioactivity was counted in a Beckman LS 200B liquid scintillation system. The Dioxan cocktail 100 gm Naphthaline (scintillation grade BDH), 5 gm PPO (Koch-Light Lab.), made up to one litre with Dioxan (Nuclear Enterprises Ltd.) , or the Aquasol (New England Nuclear) were used as scintillation fluids.

Radioactive labelling.

^3H leucine (1.0 curie/mmol) and ^{32}P (every free P is a ^{32}P), from the Radiochemical centre Amersham, were used as markers.

Cells were cultured in small glass bottles, 35 cm^2 , in the presence of the markers. In the presence of ^{32}P the growth medium in which the cells were cultured did not contain tryptose phosphate broth. 24 hours after the addition of the marker the culture medium was discarded and fresh complete medium was added to the cultures which were kept for another 24 hours. The cells according to the experiment were recovered either with EDTA or trypsin or trypsin-versine. The dose of radioactivity given was $5\ \mu\text{Ci}$ of ^3H leucine and $50\ \mu\text{Ci}$ of ^{32}P .

Conditioned media preparation.

The conditioned media to be tested were prepared as follows: Cells growing in glass or plastic culture bottles for 24 hours were washed with Tris saline and fresh growth medium was added. The cells

then were left to reach the confluent state for another 24 hours when the medium was collected, passed through a 0.22 μ m filter (Millipore filter corporation USA.) and stored either in deep freeze or at 4°C up to 14 days when it was discarded and new one was prepared. The final concentration of the cells in the culture before the conditioned media were collected was approximately 0.6×10^6 cells/ml of medium.

Flask shaker system, (Moscona 1961a).

Cells were mixed in the desired proportion in siliconed 25 ml conical flasks (Quickfit). The flasks stoppered with silicone bungs usually contained a minimum of 0.8×10^6 cells/ml in 4 ml solution. The gas phase was 5% CO₂ and 95% air. The cells were reaggregated for 48 hours in the flasks in a reciprocating shaker, (Gallenkamp) at 80 strokes/min. at 37°C, (Curtis and Greaves 1965).

Histology.

The aggregates which were formed after 48 hours in shaking flasks were transferred in conical tubes and washed twice in HH and fixed with 4% formaline in HH (pH 8.0) for 5 min. The high pH was used to prevent the cells from developing autofluorescence. After fixation the aggregates were frozen onto Cambridge microtome block holders and stored in a deep freeze. A Cambridge microtome placed in a Slee cryostat (South London Electrical Co.) was used for preparing sections. The 6 μ m thick sections were placed on 'subbed' slides. Sulfuric/nitric acid washed slides were dipped into a filtered 5% w/v gelatin and 0.1% w/v chrom alum solution at room temperature and allowed to dry in dust free conditions. The 'subbing' of the slides acts as an adhesive for the sections, (Rogers 1967).

Antibody preparation.

Neural retina antibody was prepared in a sheep, (by kind permission of Professor Hemingway, Veterinary School, Glasgow University).

In three successive weeks 2×10^7 neural retina cells in HH were

injected into the left jugular vein of the sheep. A week after the last injection blood was collected in sterile bottles without using anti-coagulant. The blood clot was broken up with a sterile glass rod. A 2500 rpm centrifugation for 15 min. separated the serum from the plasma. The serum was next collected 5 min. separated the ^{complement} as deactivated at 56°C for 30 min. Finally a small amount of sodium azide ^{and the complement} was added in the serum as a preservative.

The serum was tested for specificity towards the neural retina and the C13 cells. After 10 times of absorption with approximately 5×10^8 C13 cells each time the serum was tested for specificity and gave a faint fluorescence for the C13 cells and a bright fluorescence for the neural retina cells after counter stained with fluorescent rabbit antisheep immunoglobulin (The Wellcome Foundation Ltd.).

The different cell types did not develop autofluorescence when seen under illumination to detect fluorescence, and the fluorescent rabbit antisheep immunoglobulin did not conjugate with the cells.

For the detection of fluorescence ploom incident illumination was used. The light passes through a series of filters. Two systems of filters were used: a) excitation filters, there were successively two BG 12 1mm thick Vickers filters, one FITC excitation filter and one dichotic filter reflecting blue and transmitting green light. b) barrier filter was used a 530 nm filter and placed just before the ocular lenses. Under this blue light illumination the fluorescent cells were seen as bright green.

Staining.

The sections of the aggregates were treated for 30 min. with the sheep neural retina antibody. They were next washed with HH to remove non bound serum and counter stained for another 30 min with fluorescent rabbit antisheep immunoglobulin. Finally the sections were washed thoroughly with HH to remove any excess of fluorescent

antibody that would cause disturbing fluorescence to the sections.

The sections were observed with the use of a Vickers Photoplan M

41 microscope.

-22-

RESULTS

The adhesiveness of the fibroblastic cells, BHK 21 C13, BHK Py C11, HP and T2 was examined. Tests were carried out with the use of a shaking bath controlled at 37°C with a speed of 90 strokes/min. 4 ml of cell suspension at the concentration of approximately 1×10^6 cells/ml was placed in 10 ml siliconed conical flasks. Samples were taken every 5 min. and counted with a Coulter Counter, model Zb, (aperture 200 μ , 1/apert.cur. 0.354, 1/ampl. 1/4, lower thres. 20 and upper thres. max).

The results obtained for BHK C13 cells agreed with those of Edwards and Campbell (1971), while the results for the BHK Py C11, cloned BHK Py C1 cells, agreed with those of Edwards et al (1971). In the short term aggregation tests, up to one hour, the BHK C13 cells aggregated rapidly, while the BHK Py C11 cells did not aggregate at all. In the long term aggregation tests, overnight, both BHK C13 and BHK Py C11 cells formed aggregates, but the BHK Py C11 aggregates were very loose and after being shaken harder than normal they break up, (Edwards personal communication).

The HP and T2 cells were tested for their aggregative potential with the short term aggregation test. Tests were carried out over several months and at no time was aggregation seen at all after 2 hours.

When the BHK C13, BHK Py C11, HP and T2 cells were coaggregated with neural retina cells from 7 day old chick embryos they formed aggregates, (their structure is discussed later), which were of fairly large size for the BHK C13, HP and T2 cells but of very small size for the BHK Py C11 cells.

Effects of cell secretions.

The effect ^{on} cell adhesiveness of cellular secretions was studied with the Couette Viscometer and the collecting cell lawn assay.

For the study of the secretions, the media in which cells were growing for a certain period of time, (cultures grown up to confluency), conditioned media, were collected. These media were filtered to remove dead and detached cells from the cultures and stored at 4°C before being tested.

Couette Viscometry.

Neural retina cells were dissociated with EDTA and suspended in HH and mixed with the conditioned media to be tested to a final concentration 1.0 to 1.5×10^6 cells/ml. The cell suspension was subjected to a standard shear rate of the value 10.25 sec^{-1} in a Couette Viscometer. Under this shear rate the cells are brought into collisions. The rate of the formation of contacts by the cells depended on the nature of the media tested.

The conditioned media tested were from the following cell cultures: BHK 21 C13's (C13), BHK Py C1's (C1), BHK Py C1i's (C1i), HP's (HP), HP HSV2's (HSV2), T2's (T2) and T3's (T3) cells. Controls for these conditioned media were set up using the H+199 as a reference medium and also fresh ECT and EFT growth media, (used for setting up cultures).

The value of neural retina cell adhesiveness, (mean value for collision efficiencies), in the presence of these conditioned media is given in table I. The tests for the measurement of the adhesiveness were carried out over a period of 28 min. Samples were taken every 7 min. and counted with a haemocytometer. Statistical analysis for the collision efficiency as a function of time, from which the significance of the regression coefficient was studied, is given in table Ia. This analysis was done to check if adhesion had or had not changed during a set of measurements. There is no change in adhesion during the course of a set of measurement for neural retina cells aggregated in the following media: ECT, C1, C1i and HSV2. Regression analysis

TABLE I

Effect of the conditioned media on the adhesiveness of NR cells from 7 day old chick embryos. Adhesiveness measured as collision efficiency.

| Conditioning | α (%) | sd | n |
|--------------|--------------|------|-----|
| None H+199 | 8.96 | 2.91 | 248 |
| None ECT | 6.04 | 2.35 | 88 |
| C13 | 6.04 | 1.59 | 76 |
| C1 | 3.65 | 1.55 | 76 |
| C1i | 5.77 | 1.58 | 80 |
| None EFT | 6.66 | 1.30 | 128 |
| HP | 7.88 | 1.48 | 56 |
| HSV2 | 5.13 | 1.57 | 40 |
| T2 | 6.68 | 1.49 | 152 |

α collision efficiency, sd standard deviation, n number of repeats.

TABLE Ia

Regression analysis of the data of table I.

| Conditioning | b | sb | t | df | p |
|--------------|--------|------|------|-----|-------|
| None H+199 | -0.013 | 0.01 | 7.95 | 246 | <0.01 |
| None ECT | -0.03 | 0.03 | 1.09 | 86 | >0.05 |
| C13 | -0.06 | 0.02 | 2.76 | 74 | <0.01 |
| C1 | 0.02 | 0.02 | 1.13 | 74 | >0.05 |
| C11 | -0.01 | 0.02 | 0.65 | 78 | >0.05 |
| None EFT | -0.06 | 0.01 | 4.77 | 126 | <0.01 |
| HP | -0.07 | 0.02 | 3.41 | 54 | <0.01 |
| HSV2 | -0.004 | 0.03 | 0.14 | 38 | >0.05 |
| T2 | -0.07 | 0.01 | 5.12 | 150 | <0.01 |

b regression coefficient, sb standard deviation, t t test of b- $t=b/sb$,
df degrees of freedom, p probability.

shows that the regression of adhesion in time has a slope not significantly different from zero. On the other hand it is clearly seen that for H+199, C13, EFT, HP and T2 media the adhesiveness of neural retina cells changed during the set of measurement, 7-14-21-28 min. From the same analysis it is obvious that the collision efficiency as a function of time for the above media during this set of measurement fits a line $y=a+bx$. Thus the adhesiveness of neural retina cells under these treatments is reduced steadily at this time set of observations.

The data of table I are divided into three groups according to the culture media used in which adhesion was measured. The first group contains only the H+199 which does not contain any serum and it is used as a reference for a later discussion. The second group contains the conditioned media derived from the cell cultures growing in ECT growth medium. The third group contains the conditioned media derived from the cell cultures growing in EFT growth medium.

Statistical analysis of the neural retina cell adhesiveness based on the conditioned media used is given in table II. The different conditioned media in table II have been classified according to the conditioning to ECT or EFT derived and to normal or malignant condition. Thus from table II it is obvious that ECT, EFT and H+199 affect the adhesiveness of neural retina cells differently. ECT and EFT contain 10% of serum, thus their effects might be due to the serum they contain, i.e. calf and foetal calf serum respectively. From table Ia it is also seen that ECT and EFT affect the adhesiveness of neural retina cells differently at the time course studied. In general, conditioning of the growth media from non conditioning, (fresh growth media tested), had different effect on the adhesiveness of neural retina cells. The above means that the different cell types which conditioned the growth media either removed, added or activated some

TABLE II

Grouped analysis of the data of table I.

| Conditioning | M.S.am. | M.S.with. | F | df | p |
|------------------------------------|---------|-----------|--------|-------|-------|
| ECT-EFT-H+199 | 388.60 | 6.08 | 63.90 | 2-462 | <0.02 |
| ECT-EFT | 19.87 | 3.26 | 6.09 | 1-214 | <0.02 |
| ECT+EFT-H+199 | 757.37 | 6.11 | 123.90 | 1-462 | <0.02 |
| C13+C1+C1i+HP+ +HSV2+T2-ECT+EFT | 30.72 | 3.71 | 8.26 | 1-694 | <0.02 |
| C13-C1+C1i-ECT | 71.33 | 3.83 | 18.61 | 2-312 | <0.02 |
| HP-HSV2+T2-EFT | 49.46 | 3.70 | 21.66 | 2-374 | <0.02 |

M.S.am. mean square among classes, M.S.with. mean square within classes,
F F ratio ($F=M.S.am./M.S.with.$).

components of these media. In addition, normal conditioning has different effect from malignant conditioning. From the results of table I it is seen that Py C1 and HSV2 cells condition their growth medium more than Py C1i and T2 cells. Actually the Py C1 and HSV2 cells are of very low passage after transformation while the Py C1i and T2 cells have been selected from the Py C1 and HSV2 cells respectively. It is possible during cloning and tumour progression to select such a clone that does not appear to condition its culture medium too much with respect to the adhesion of certain cell types. The T2 cells do not appear to condition their medium with respect to the adhesiveness of neural retina cells. The T2 cells have been defined as tumours because their ability to produce tumours in young hamsters very frequently. These cells are derived and selected from secondary tumours, that is, after the growth of tumours in young hamsters with a low percentage of success, the tumours obtained were reinjected in other young hamsters and all produced tumours from which the T2 cells have been collected. So the non conditioning of their growth medium with respect to neural retina cell adhesiveness could be because they are tumourogenic.

The dose response curve of Py C1 conditioned medium against H+199 was studied to test the effect of a possible inhibitory factor for neural retina cell adhesiveness. Table III gives the results of this test and table IIIa the regression analysis of them. From the results it is obvious that Py C1 cells condition their medium with ~~no~~ inhibiting factor for neural retina cell adhesiveness, whose effect is concentration dependent.

The T2 conditioned medium against H+199 was also studied for the possible existence of factor(s) on a dose response curve. As a control to this test the fresh growth medium (EFT) in which the T2 cells are growing was studied. Table IV gives the results of these

TABLE III

Dose response effect of Py C1 conditioned medium against H+199 on the adhesiveness of 7 day old chick embryo NR cells.

| Py C1/H+199 | | α (%) | sd |
|-------------|-----|--------------|------|
| 100 | 0 | 3.15 | 0.36 |
| 90 | 10 | 4.50 | 1.02 |
| 80 | 20 | 5.02 | 0.85 |
| 70 | 30 | 5.22 | 1.17 |
| 60 | 40 | 5.15 | 0.79 |
| 50 | 50 | 5.45 | 0.87 |
| 40 | 60 | 5.82 | 0.90 |
| 30 | 70 | 6.28 | 0.67 |
| 20 | 80 | 7.01 | 1.40 |
| 10 | 90 | 7.76 | 1.12 |
| 0 | 100 | 8.91 | 1.60 |

TABLE IIIa

Regression analysis of the data of table III.

| Conditioning | b | sb | t | df | p |
|--------------|--------|-------|------|----|-------|
| Py C1/H+199 | -0.040 | 0.004 | 9.38 | 8 | <0.01 |
| H+199/Py C1 | 0.043 | 0.005 | 8.10 | 8 | <0.01 |

TABLE IV

Dose response effect of EFT and T2 media against H+199 on the adhesiveness of 7 day old chick embryo neural retina cells.

| EFT/H+199 | | α (%) | sd | T2/H+199 | | α (%) | sd |
|-----------|-----|--------------|------|----------|-----|--------------|------|
| 100 | 0 | 6.40 | 1.68 | 100 | 0 | 7.58 | 2.02 |
| 90 | 10 | 6.63 | 1.07 | 90 | 10 | 7.46 | 0.70 |
| 80 | 20 | 7.39 | 0.83 | 80 | 20 | 7.67 | 1.25 |
| 70 | 30 | 6.10 | 0.89 | 70 | 30 | 5.77 | 1.18 |
| 60 | 40 | 6.33 | 1.08 | 60 | 40 | 6.37 | 0.34 |
| 50 | 50 | 6.67 | 1.23 | 50 | 50 | 6.79 | 0.02 |
| 40 | 60 | 6.16 | 1.01 | 40 | 60 | 7.56 | 1.18 |
| 30 | 70 | 6.63 | 0.46 | 30 | 70 | 6.96 | 0.46 |
| 20 | 80 | 7.45 | 0.84 | 20 | 80 | 7.94 | 0.96 |
| 10 | 90 | 6.34 | 1.41 | 10 | 90 | 6.49 | 0.21 |
| 0 | 100 | 7.44 | 1.76 | 0 | 100 | 7.44 | 1.76 |

Grouped analysis

| Conditioning | M.S.am. | M.S.with | F | df | p |
|--------------|---------|----------|------|------|-------|
| EFT/H+199- | | | | | |
| -T2/H+199 | 3.68 | 1.33 | 2.75 | 1-86 | >0.05 |

tests and their variance analysis. From these it is seen that T2 and EFT media do not differ. Thus the previously stated from table I that the T2 cells do not condition their growth medium with respect to neural retina cell adhesiveness is supported from the results of table IV.

Another tumour cell line (T3) tested for conditioning appears to affect neural retina cell adhesiveness very considerably. The effect of this conditioned medium is given in table V. In table Va the variance analysis of table V is given. From the tables V and Va it is obvious that T3 cells condition their medium at different periods differently with respect to neural retina cell adhesiveness. T3a and T3b are conditioned media collected at different periods from the T3 cultures. Effects like these could possibly occur if we consider that in the conditioned media, i.e. T3a, T3b may contain more than one factor with contrasting effects but of complementary reaction. The variation in the effect can be explained if one of the factors occasionally is either inactivated or secreted in excess.

The effect of the tumour conditioned media on the adhesiveness of liver cells from 7 day old chick embryos dissociated with EDTA and suspended in HH is given in table VI. In table VIa is given the variance analysis of the data of table VI. From the data of tables VI and VIa it is obvious that both tumour cells (T2, T3) condition their medium with respect to liver cell adhesiveness. Comparing the results from the tables I, V and VI for the tumour conditioned media effect we see that the media have been conditioned differently with respect to the adhesiveness of neural retina and liver cells of the chick embryo.

In all the experiments testing for conditioned media, the cells were suspended in HH and 0.5 ml of the suspension were added to 1.5 ml of the conditioned media to be tested, to give a final concentration of about 1.0 to 1.5×10^6 cells/ml.

TABLE V

Effect of the T3 conditioned medium on the adhesiveness of neural retina cells from 7 day old chick embryos.

| Conditioning | a (%) | sd | n |
|--------------|-------|------|-----|
| None EFT | 6.66 | 1.30 | 128 |
| T3a | 8.52 | 2.22 | 84 |
| T3b | 5.12 | 0.72 | 48 |
| Pool. T3 | 7.28 | 2.47 | 132 |

TABLE Va

Grouped analysis of the data of table V.

| Conditioning | M.S.am. | M.S.with. | F | df | p |
|--------------|---------|-----------|-------|-------|-------|
| EFT-T3a-T3b | 189.86 | 2.56 | 73.89 | 2-258 | <0.02 |
| EFT-T3a+T3b | 25.52 | 3.93 | 6.49 | 1-258 | <0.02 |

TABLE VI

Effect of the tumour conditioned media on the adhesiveness of liver cells from 7 day old chick embryos.

| Conditioning | α (%) | sd | n |
|--------------|--------------|------|----|
| None EFT | 6.02 | 1.24 | 36 |
| T2 | 5.50 | 0.59 | 36 |
| T3 | 5.25 | 0.50 | 36 |

TABLE VIa

Grouped analysis of the data of table VI.

| Conditioning | M.S.am. | M.S.with. | F | df | p |
|--------------|---------|-----------|-------|-------|-------|
| EFT-T2-T3 | 5.64 | 0.71 | 7.87 | 2-106 | <0.01 |
| EFT-T2+T3 | 10.11 | 0.72 | 14.01 | 1-106 | <0.01 |

-21-

Collecting cell lawn assay.

Neural retina cell monolayers were prepared in Linbro plastic wells as described previously. The assay was followed in two steps. At the beginning the monolayers were washed twice with cold HH at room temperature and one ml of fresh HH was added per well. They were next transferred at 37°C and left for 15 min. to equilibrate the temperature and to allow the monolayers to recover. The HH was next removed and one ml of the media to be tested was added per well. On top of these monolayers 2×10^5 ^3H leucine labelled cells were placed and left for 50 min. to interact with the monolayers and to make contacts. The subsequent treatments were those described in methods (page 28).

The following conditioned media were tested on this system: HH, EFT, T2 and T3. The effect of these media was tested on the collection of 7 day old chick embryo neural retina and liver cells and of T2 cells by the neural retina (NR) monolayers.

In table VII and histogram I the results of these experiments are given as the percentage of the collected cells from the added ones. Statistical analysis of these results is given in table VIII. The data of table VIII indicate that the collection of trypsinised neural retina cells is affected by the presence of the conditioned media. The collection of the EDTA treated cells is affected only by the T2 conditioned medium, but the T2 and liver cell collections are not affected by the conditioned media tested.

The contradictory results between the Couette Viscometer and the monolayer collection could be explained as the consequence of the use of two different techniques. In addition to this, different times used for the assays, 28 min. for the Couette Viscometry and 50 min. for the monolayer collection. In the Couette Viscometer the cells are in a continuous state of motion, while in the collecting assay the

TABLE VII

Effect of the tumour conditioned media on the collection of NR, Liver and T2 ^3H labelled cells by NR monolayers. Collection measured as the percentage of the collected cells.

| Collected cells | | conditioning | collection (%) | sd | n |
|----------------------|------|--------------|----------------|------|----|
| NR tryps. | None | EFT | 36.2 | 8.3 | 14 |
| | | T2 | 47.9 | 10.7 | 14 |
| | | T3 | 44.9 | 10.1 | 14 |
| NR EDTA treat. | None | HH | 59.1 | 11.2 | 14 |
| | None | EFT | 41.3 | 7.8 | 14 |
| | | T2 | 49.0 | 8.1 | 14 |
| Liver | | T3 | 45.3 | 8.4 | 14 |
| | None | HH | 48.5 | 7.0 | 14 |
| | None | EFT | 34.5 | 10.6 | 6 |
| T2 | | T2 | 42.4 | 15.8 | 6 |
| | | T3 | 36.2 | 12.1 | 6 |
| | None | HH | 49.1 | 13.1 | 6 |
| T2 | None | EFT | 34.6 | 5.8 | 6 |
| | | T2 | 31.1 | 7.3 | 6 |
| | | T3 | 31.3 | 4.6 | 6 |
| | None | HH | 38.4 | 9.0 | 6 |

HISTOGRAM I

Effect of the tumour conditioned media on the collection of NR, Liver and T2 cells by

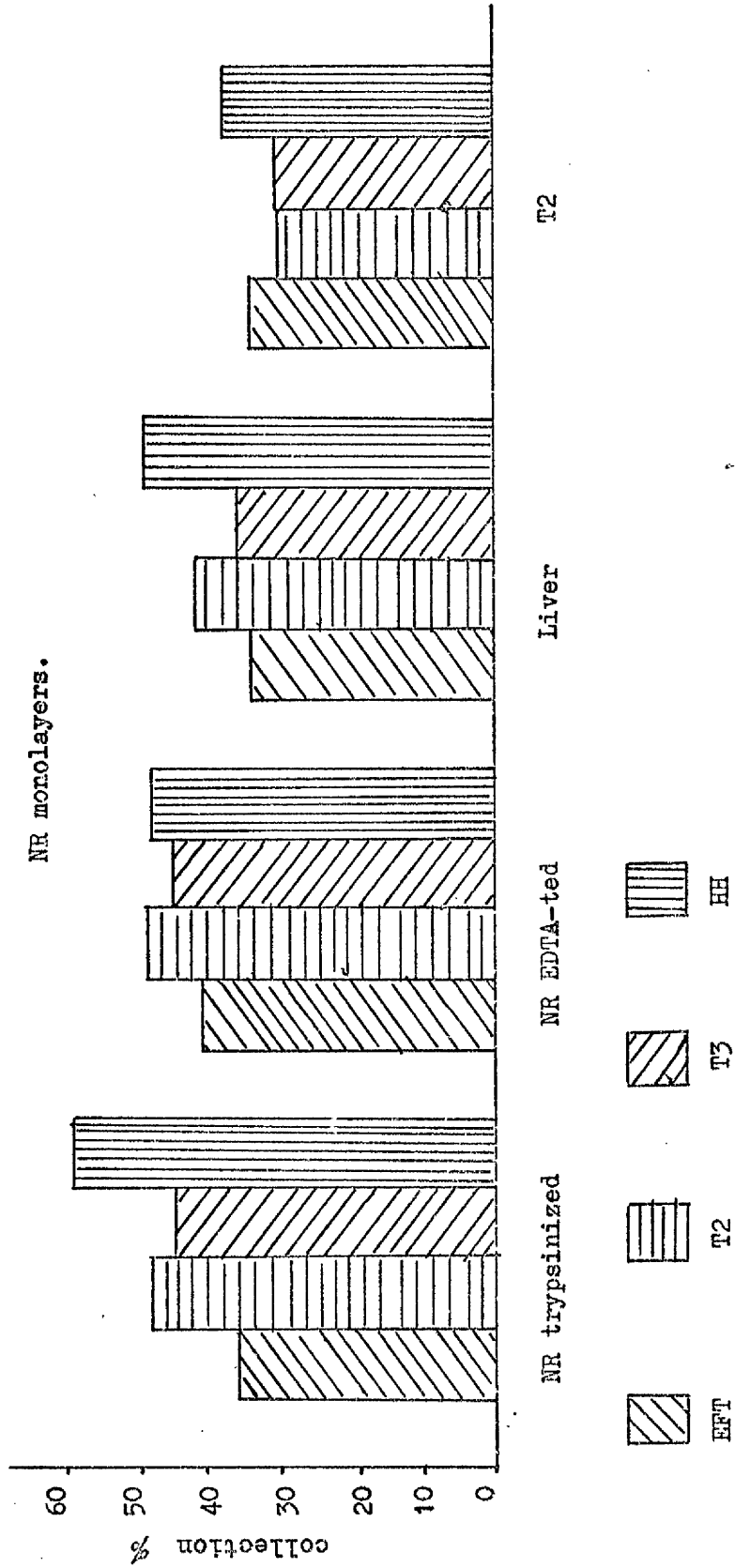


TABLE VIII

Grouped analysis of the conditioned media effect on the collection of NR,
Liver and T2 cells by NR monolayers.

| Collected cells | conditioning | t | df | p |
|----------------------|--------------|-----|----|-------|
| NR tryps. | EFT - T2 | 3.2 | 26 | <0.02 |
| | EFT - T3 | 2.4 | 26 | <0.02 |
| | EFT - HH | 6.1 | 26 | <0.02 |
| | HH - T2 | 2.7 | 26 | <0.02 |
| | HH - T3 | 3.4 | 26 | <0.02 |
| NR EDTA treat. | EFT - T2 | 2.5 | 26 | <0.02 |
| | EFT - T3 | 1.3 | 26 | >0.1 |
| | EFT - HH | 2.5 | 26 | <0.02 |
| | HH - T2 | 0.1 | 26 | >0.1 |
| | HH - T3 | 1.1 | 26 | >0.1 |
| Liver | EFT - T2 | 1.0 | 10 | >0.1 |
| | EFT - T3 | 0.2 | 10 | >0.1 |
| | EFT - HH | 2.1 | 10 | >0.1 |
| | HH - T2 | 0.7 | 10 | >0.1 |
| | HH - T3 | 1.7 | 10 | >0.1 |
| T2 | EFT - T2 | 0.9 | 10 | >0.1 |
| | EFT - T3 | 1.0 | 10 | >0.1 |
| | EFT - HH | 0.8 | 10 | >0.1 |
| | HH - T2 | 1.5 | 10 | >0.1 |
| | HH - T3 | 1.7 | 10 | >0.1 |

cells since they sediment on the monolayer they remain relatively immobilised. In these two systems the cells are subjected to different treatments which may explain the different results.

In these experiments the cells suspended in HH were resuspended in the conditioned media to a final concentration of 0.1 ml of the suspension in one ml of the media to be tested. In the case of the Couette Viscometry the concentration of the conditioned media was approximately 75% and in the case of the monolayer assay was approximately 99%. This also might account for the different results obtained.

The previously presented results show that neoplastic cells are capable of secreting factors affecting either positively or negatively the adhesiveness of embryonic chick tissues. In addition to these factors the different sera used appear containing inhibiting factors. Such factors can not be detected with every method used, and their detection is dependent on the accuracy of the method used and on the effectiveness of the factors.

Mutual adhesiveness (adhesive relationship).

The adhesive relationships of the different cell types involved in this work was studied with the collecting cell lawn assay.

The cells tested were the BHK C13's (C13), BHK Py C11's (C11), HP's (HP), T2's (T2) and neural retina (NR) from 7 day old chick embryos. The assay was carried out at 37°C. Cultures of the above cells were prepared in glass culture bottles, 35 cm² culture area, and labelled with ³²P (see page 28). The cells dissociated with the routine method were suspended in HH to a final concentration of 5x10⁵ cells/ml. After the monolayers were washed free of serum with one ml of fresh HH was added per monolayer and on top of these were placed 5x10⁴ radioactively labelled cells. The cells were left to interact with the monolayer for 30 min. The subsequent steps of the assay are

described in methods (page 28)

In table IX and histogram II the results of these experiments are given. The difference in the amount of collection of trypsin treated NR cells in HH recorded in the tables VII and IX can be explained by the different times of the assays, 50 min. and 30 min. respectively. Another important factor for this difference in results is that the washing technique after the cell interaction was more vigorous during the later experiments so that only the strongly attached cells remained on the monolayers. A third factor is that during the later experiments four times less cells were plated on top of the monolayers, than during the earlier experiments.

On table X is given the statistical analysis of table IX on the base of the collected cells per monolayer. From the data recorded on tables IX and XI it is obvious that: NR monolayers collect more NR cells than the other monolayers, and so NR cells express a greater adhesivity between themselves than among the other cell types. The results of these tables to the other cell types suggest a preference of certain fibroblastic cell types towards other ones. A quantitative representation of these results can be given based on the collection of the different cell suspensions by the different monolayers.

Monolayers

Cell suspensions

| | | | |
|-----|-----------|---|---------|
| C13 | C1i=C13 | » | HP=T2 |
| C1i | C1i=C13 | » | T2 > HP |
| HP | C1i=C13 | » | T2 » HP |
| T2 | C1i > C13 | » | T2=HP |

In general we can represent the results as C1i=C13 » T2 » HP in terms of collecting ability of these monolayers for all these cells. The above is based on the results of table XI.

A possible explanation of the C1i monolayers collecting almost the same or more cells than the C13 monolayers is given in discussion

TABLE IX

Monolayer collection of the different cell types. Collection measured as the percentage of the collected cells.

| Monolayer | collected cells | collection (%) | sd | n |
|-----------|-----------------|----------------|------|---|
| NR | C13 | 39.8 | 7.0 | 6 |
| | C11 | 25.3 | 4.8 | 6 |
| | HP | 42.6 | 4.2 | 6 |
| | T2 | 40.8 | 6.9 | 6 |
| | NR | 21.5 | 3.0 | 6 |
| C13 | C13 | 39.0 | 11.8 | 6 |
| | C11 | 26.1 | 5.5 | 6 |
| | HP | 39.5 | 4.7 | 6 |
| | T2 | 35.1 | 8.3 | 6 |
| | NR | 13.1 | 1.4 | 6 |
| C11 | C13 | 44.8 | 8.6 | 5 |
| | C11 | 28.6 | 6.8 | 5 |
| | HP | 46.0 | 8.3 | 5 |
| | T2 | 46.8 | 3.1 | 5 |
| | NR | 9.6 | 2.6 | 5 |
| HP | C13 | 33.5 | 4.6 | 4 |
| | C11 | 12.5 | 3.5 | 4 |
| | HP | 27.2 | 8.4 | 4 |
| | T2 | 28.5 | 6.0 | 4 |
| | NR | 5.7 | 0.9 | 4 |
| T2 | C13 | 32.6 | 6.4 | 5 |
| | C11 | 20.4 | 4.1 | 5 |
| | HP | 36.2 | 4.6 | 5 |
| | T2 | 26.6 | 5.5 | 5 |
| | NR | 14.8 | 4.3 | 5 |

HISTOGRAM II
Monolayer collection

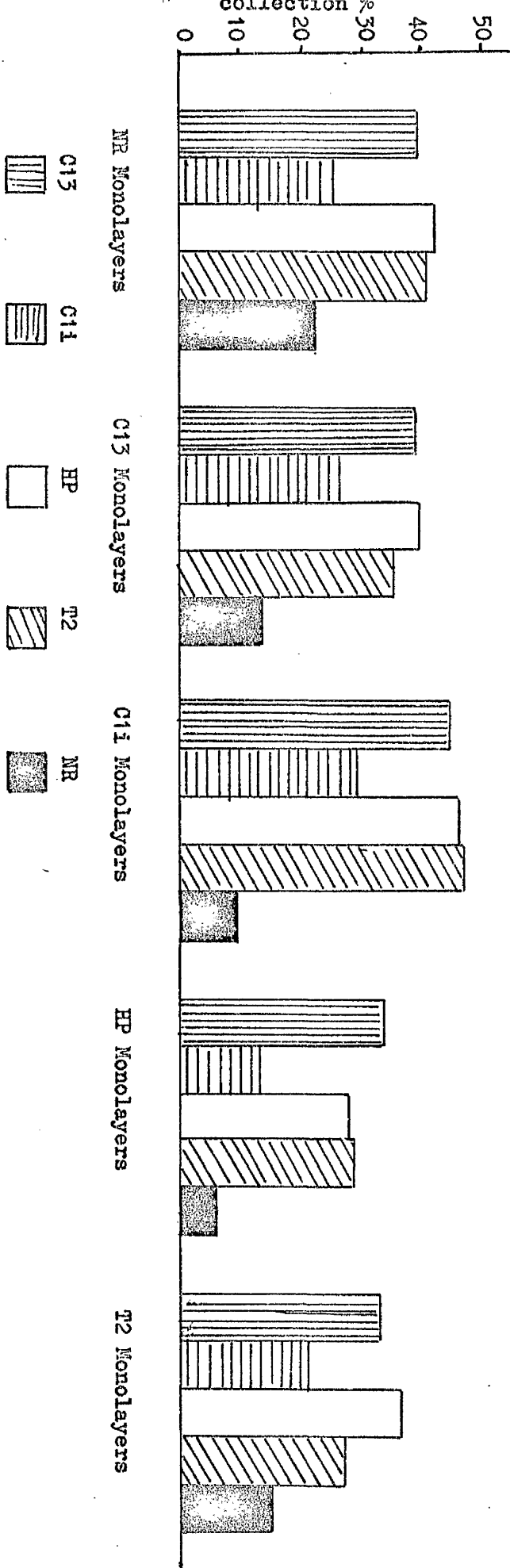


TABLE X

Grouped analysis of the monolayer collection results.

| Collected cells | corresp.monol. | compar.monol. | t | df | p |
|-----------------|----------------|---------------|-----|----|--------|
| | | C13 | 5.9 | 10 | < 0.05 |
| NR | NR | C1i | 6.8 | 9 | < 0.05 |
| | | HP | 9.7 | 8 | < 0.05 |
| | | T2 | 3.0 | 9 | < 0.05 |
| | | C1i | 0.9 | 9 | > 0.05 |
| C13 | C13 | HP | 0.8 | 8 | > 0.05 |
| | | T2 | 1.0 | 9 | > 0.05 |
| | | NR | 0.1 | 10 | > 0.05 |
| | | C13 | 0.6 | 9 | > 0.05 |
| C1i | C1i | HP | 4.2 | 7 | < 0.05 |
| | | T2 | 2.2 | 8 | > 0.05 |
| | | NR | 0.9 | 9 | > 0.05 |
| | | C13 | 2.9 | 8 | < 0.05 |
| HP | HP | C1i | 3.5 | 7 | < 0.05 |
| | | T2 | 2.0 | 7 | > 0.05 |
| | | NR | 3.8 | 8 | < 0.05 |
| | | C13 | 1.9 | 9 | > 0.05 |
| T2 | T2 | C1i | 7.0 | 8 | < 0.05 |
| | | HP | 0.4 | 7 | > 0.05 |
| | | NR | 3.7 | 9 | < 0.05 |

TABLE XI

Monolayer collection. Numbers representing
the percentage of collected cells.

Suspensions in HH

| Monolayers | | | | | |
|------------|------|------|------|------|------|
| NR | 21.5 | 39.8 | 25.3 | 42.6 | 40.8 |
| C13 | 13.1 | 39.0 | 26.1 | 39.5 | 35.1 |
| C11 | 9.6 | 44.8 | 26.8 | 46.0 | 46.8 |
| HP | 5.7 | 33.5 | 12.5 | 27.2 | 28.2 |
| T2 | 14.8 | 32.6 | 20.4 | 36.2 | 26.6 |

TABLE XII

Grouped analysis of the different monolayers collecting the different cell suspensions.

| Coll.cells | comp.monol. | t | df | p |
|------------|-------------|------|----|------------------|
| C13 | C11 - C13 | 0.90 | 9 | $\gg 0.05$ |
| | C11 - HP | 2.33 | 7 | $0.1 > p > 0.05$ |
| | C11 - T2 | 2.52 | 8 | < 0.05 |
| | C13 - HP | 0.87 | 8 | $\gg 0.05$ |
| | C13 - T2 | 1.07 | 9 | $\gg 0.05$ |
| | HP - T2 | 0.23 | 7 | $\gg 0.05$ |
| C11 | C11 - C13 | 0.65 | 9 | $\gg 0.05$ |
| | C11 - HP | 4.26 | 7 | < 0.05 |
| | C11 - T2 | 2.29 | 8 | $0.1 > p > 0.05$ |
| | C13 - HP | 4.43 | 8 | < 0.05 |
| | C13 - T2 | 1.91 | 9 | $0.1 > p > 0.05$ |
| | HP - T2 | 3.02 | 7 | < 0.05 |
| HP | C11 - C13 | 1.62 | 9 | $\gg 0.05$ |
| | C11 - HP | 3.33 | 7 | < 0.05 |
| | C11 - T2 | 2.29 | 8 | ≈ 0.05 |
| | C13 - HP | 2.97 | 8 | < 0.05 |
| | C13 - T2 | 1.15 | 9 | $\gg 0.05$ |
| | HP - T2 | 2.03 | 7 | $0.1 > p > 0.05$ |
| T2 | C11 - C13 | 2.52 | 9 | < 0.05 |
| | C11 - HP | 6.02 | 7 | < 0.05 |
| | C11 - T2 | 7.09 | 8 | < 0.05 |
| | C13 - HP | 1.41 | 8 | $\gg 0.05$ |
| | C13 - T2 | 1.95 | 9 | $0.1 > p > 0.05$ |
| | HP - T2 | 0.42 | 7 | $\gg 0.05$ |

These experiments were carried out because of the results of the earlier monolayer experiments testing for conditioning, where it was found that the collection of the T2 cells by the NR monolayers is ^{not} dependent on medium conditioning. The effect of EFT, ECT or HH on the other cell types (C13, C1i and T2) has not been tested.

For the rest of this work the collection of the NR cells will be taken into account as the following comparisons were made between the NR cells and other cell types.

Cell positioning.

The sorting out of the NR cells and the C13, C1i, HP and T2 cells was studied in two and three dimensional cultures. The two dimensional sorting out was studied in mixed cultures prepared in 60mm diameter falconised plastic petri dishes. The three dimensional sorting out was studied in sections of aggregates.

Two dimensional sorting out.

Mixed cultures of NR and of C13, C1i, HP and T2 cells were prepared and observed after 24 and 48 hours. Cells were plated on the following proportions: 60/1 for the combinations of NR/C1i or NR/T2, 30/1 for the combination of NR/C13 and 20/1 for the combination NR/HP. The reason for using these proportions is first that the NR cells have not been observed to divide in culture while the other cell types divide so that more NR cells must be present initially to establish a large number at the time of the fixation, and second that the NR cells and the other cell types differ in size resulting in the requirement of a larger number of NR cells than of the other cell types for the preparation of the same area size of monolayers. The cultures were studied for possible patterns of cell arrangement developed after 24 hours culture. Fig. 6 to 9 show the pattern developed after 24 hours in culture for the combinations NR/C13, NR/C1i, NR/HP and NR/T2 respectively. All cultures appear with isolated NR islands surrounded

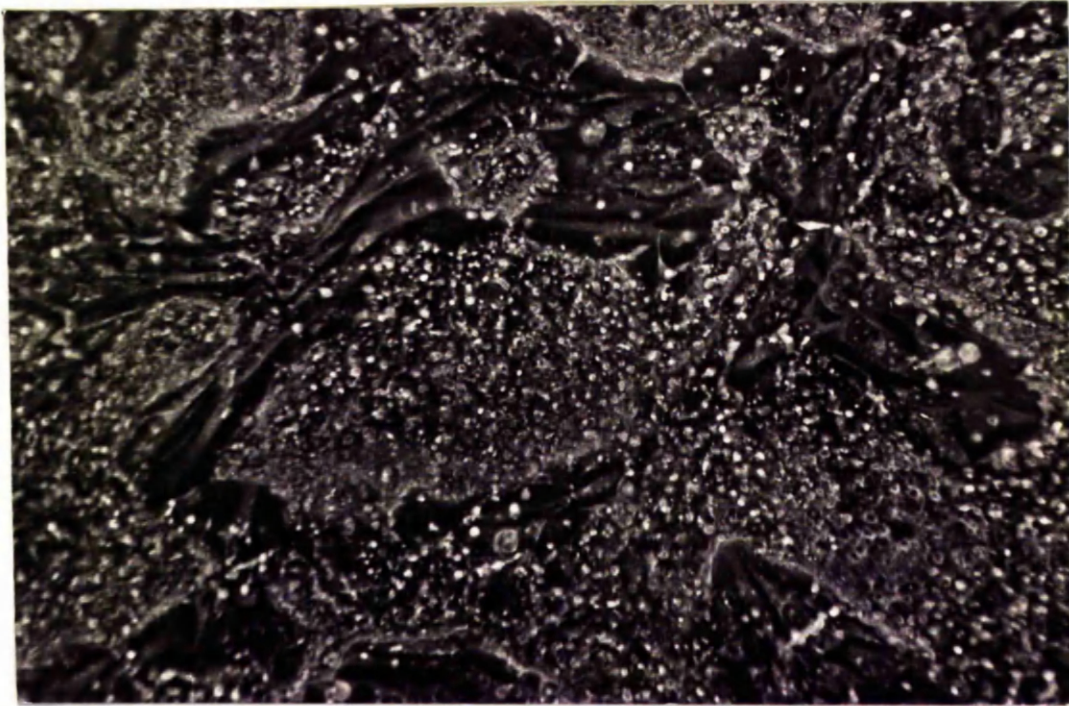


Fig. 6. NR/C13 mixed culture after 24 hours. Magn. 1500

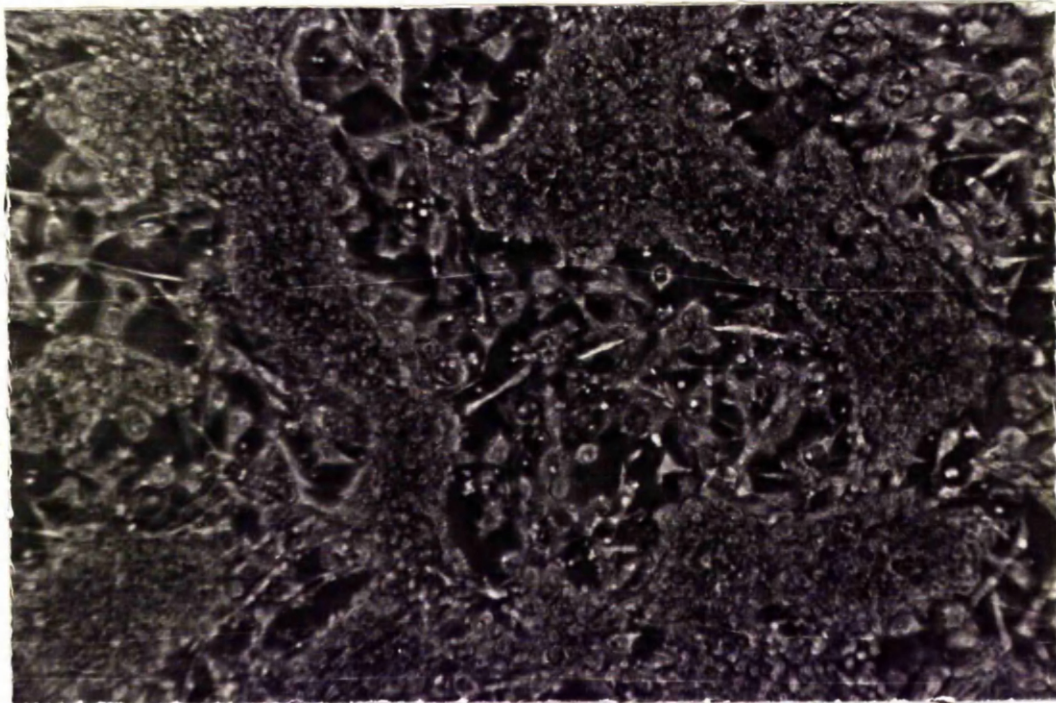


Fig. 7. NR/C11 mixed culture after 24 hours. Magn. 1500

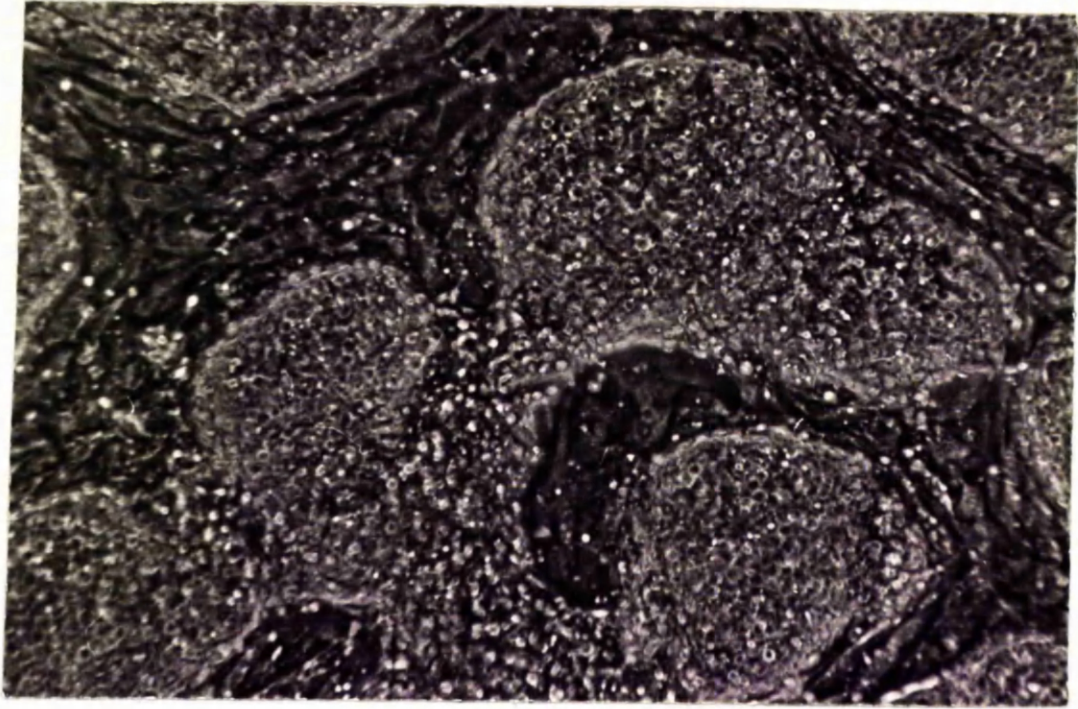


Fig. 8. NR/HP mixed culture after 24 hours. Magn. 1500

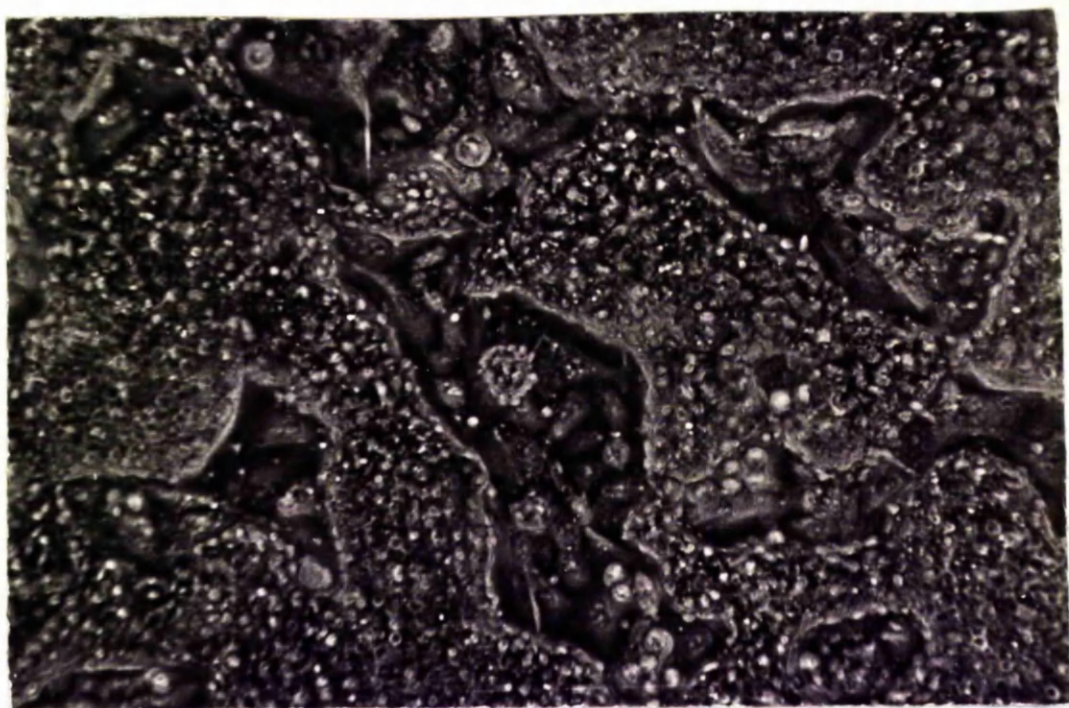


Fig. 9. NR/T2 mixed culture after 24 hours. Magn. 1500

by the fibroblastic cell types. These observations agree with the results of the NR collection, where NR cells appear to stick better to themselves than to the other cell types. The low association of the NR cells with the other cell types studied, as seen from the monolayer collection results, leads to the formation of the NR cell islands. The ability of the other cell types to divide in culture and their mobility may lead to the isolation of the NR islands in the location at which they were formed. In these mixed cultures the fibroblastic cells, C13 and HP retain their fibroblastic orientation. The C1i and T2 cells also retain their culture appearance in these mixed cultures. The relevance of the unaltered appearance of these cell types is that maintenance of orientation may cause the NR cells to separate into islands. This tendency to cross underneath the NR cell islands is also obvious in the figures. After a further culture for 24 hours, that is after 48 hours from plating the cells, the cultures were again observed for the progression of the sorting out pattern. At this stage the cultures consisted mainly of a fibroblastic substrate with a very limited number of the NR cell islands still attached on the plastic petri dishes and with most of the islands floating in the medium and a lot of NR cells floating in the medium. It has been observed that the islands detach from the substrate, as some of the islands still attached on the plastic surface were partially detached.

Three dimensional sorting out.

Mixed aggregates of NR and C13, C1i, HP and T2 cells were prepared in shaking cultures for 48 hours. Cells were mixed at the following concentrations: 2/1 for the combinations of NR/C13 and NR/HP and 10/1 for the combinations of NR/C1i and NR/T2, according to their ability to divide in suspension cultures. The Py C1i and T2 cells can be grown in suspension cultures. If these cells had been mixed with the NR

cells at higher concentrations this could lead to such dense populations of Py Cli and T2 cells, judged by their ability to grow in suspension, that the pattern formed could possibly be altered.

After 48 hours of aggregation the flasks contained aggregates and multilayered cell sheets attached to the glass walls (which have been siliconed). This was found for the combinations of NR/C13 and NR/HP and also for NR/C11 and NR/T2; in addition, the latter also had single cells and cell clumps floating in the medium which were exclusively of Py Cli and T2 cells respectively.

The aggregates had the following general structure: The C13 and HP aggregates were mainly of large size, ranging between 0.5 and 1.0mm in diameter, and fairly compact. The C11 and T2 aggregates were smaller, ranging from large clumps up to 0.3mm in diameter, and very loose. It was very easy to disrupt these aggregates while removing the aggregates from the culture flasks into test tubes for fixation.

The multilayered sheets on the flask walls could have arisen either from small aggregates which were stuck onto the glass and from which cells had spread, or from single cells which adhered to the glass surface and which then trapped small aggregates. Such cases where aggregates were trapped onto spread cells on the glass surface were observed many times. I attempted to eliminate the number of cells forming sheets onto the glass surface by reducing the total number of cells plated per flask, and by siliconing the flasks more than once. I have observed that when the total number of cells was reduced the cell sheets on the glass were not very extensive.

These aggregates were treated as described earlier and then were observed under the appropriate illumination. Fig.10 to 13 give the appearance of the aggregate sections under fluorescent excitation. Fig.10a to 13a give the appearance of the aggregate sections under

normal illumination.

After staining with the sheep NR antibody and counterstaining with the rabbit anti sheep fluorescent immunoglobulin the different cell types had the following appearance under fluorescent excitation illumination. The C13, C1i, HP and T2 cells were stained faintly because the antibody was not completely specific, while the NR cells were much more brightly stained. The sorting out patterning in the mixed aggregates was studied using this criterion for the identification of the cells.

Fig.10 and 10a give the appearance of the NR/C13 mixed aggregates. Under fluorescence excitation illumination among the faint C13 cells the bright NR cells are distinguished, either in very small clumps or singly distributed throughout the C13 network. The C13 cells appeared spread in the aggregates. This is more clear in less compact aggregate sections or in aggregate sections damaged during sectioning.

Fig.11 and 11a give the appearance of the NR/C1i mixed aggregates. Under fluorescence excitation illumination the sections appeared faintly stained. No sign of bright fluorescence, that is of NR cells, appeared in all sections studied. The C1i cells were spread in the aggregates as clearly seen in the normal illumination photograph.

Fig.12 and 12a give the appearance of the NR/HP mixed aggregates. Under fluorescent excitation illumination among the faintly stained HP cells NR cells are distinguished. The NR cells are distributed throughout the HP network. The HP cells are also spread in the aggregates.

Fig.13 and 13a give the appearance of the NR/T2 mixed aggregates. Under fluorescence excitation illumination the sections appeared faintly stained. Occasionally in several aggregate sections scattered bright spots were seen, which because they were very few can not be

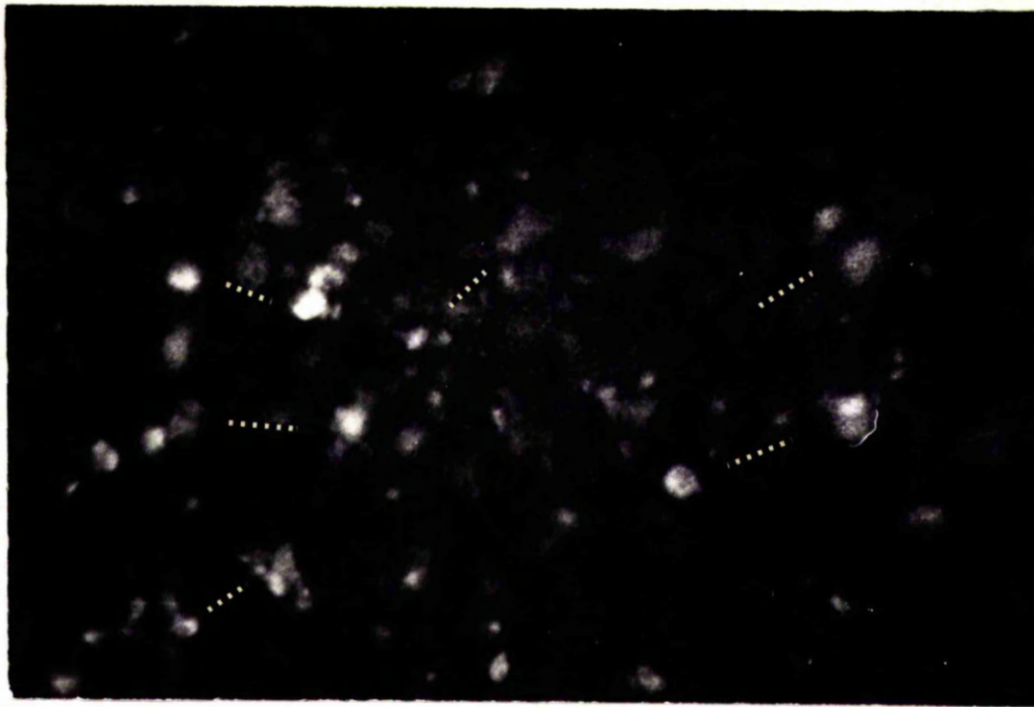


Fig. 10. NR/C13 mixed aggregate after 48 hours. Magn. 1500
Fluorescence excitation illumination. NR cells (arrows).

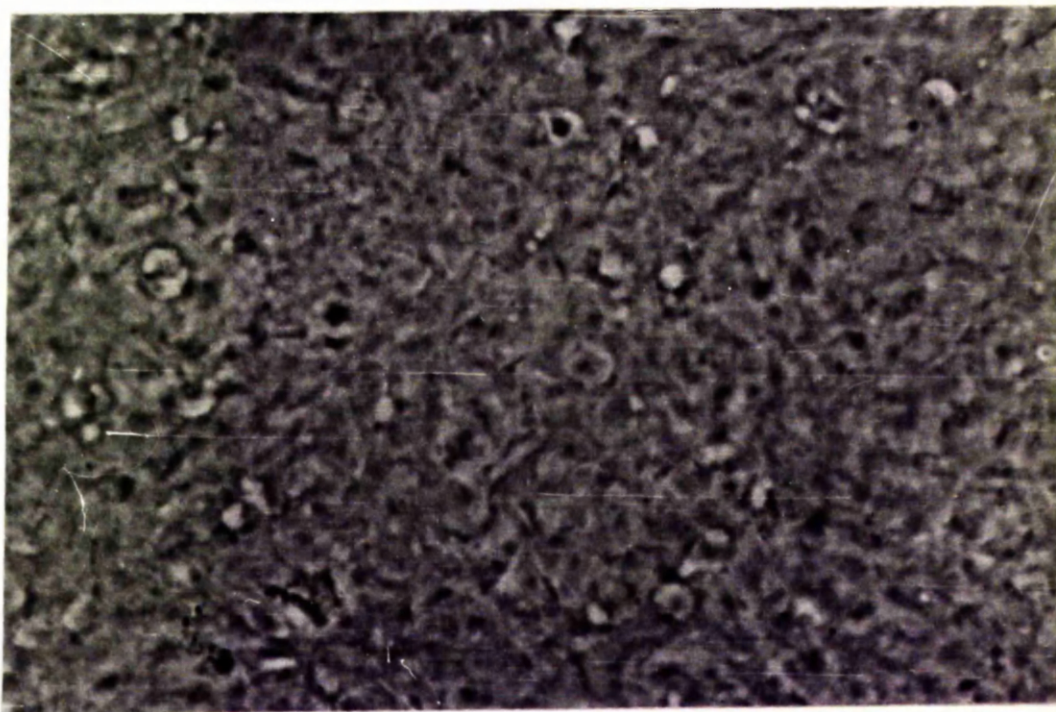


Fig. 10a. NR/C13 mixed aggregate after 48 hours. Magn. 1500
Bright field illumination.



Fig. 11. NR/C1i mixed aggregate after 48 hours. Magn. 1500
Fluorescence excitation illumination. Possible NR cells (arrows).

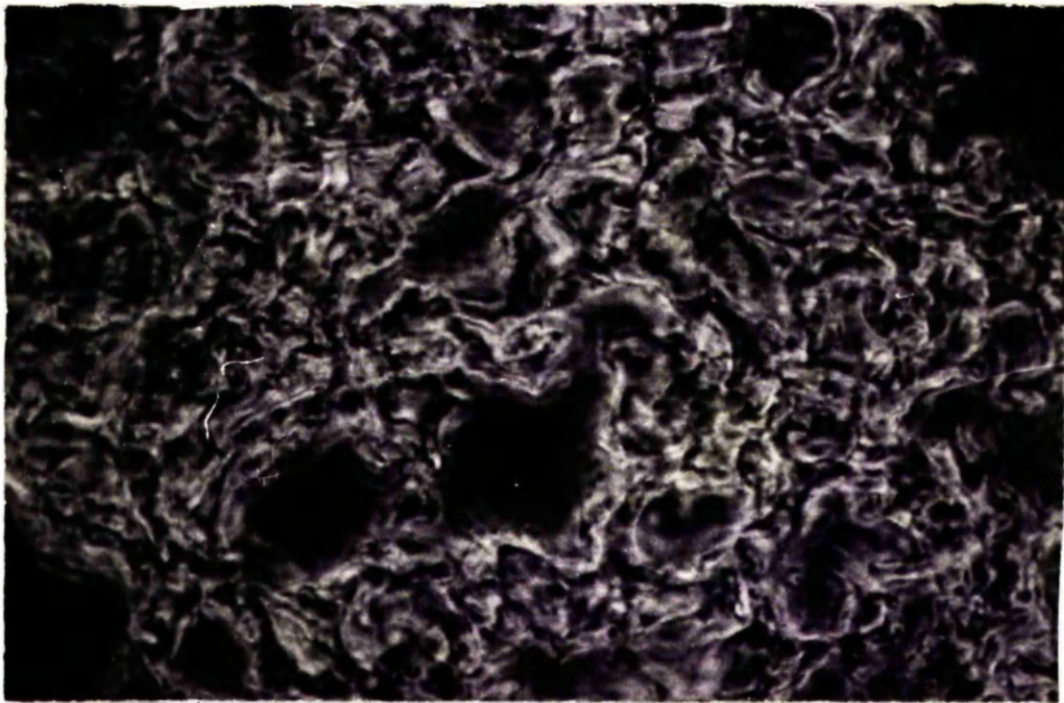


Fig. 11a. NR/C1i mixed aggregate after 48 hours. Magn. 1500
Bright field illumination.

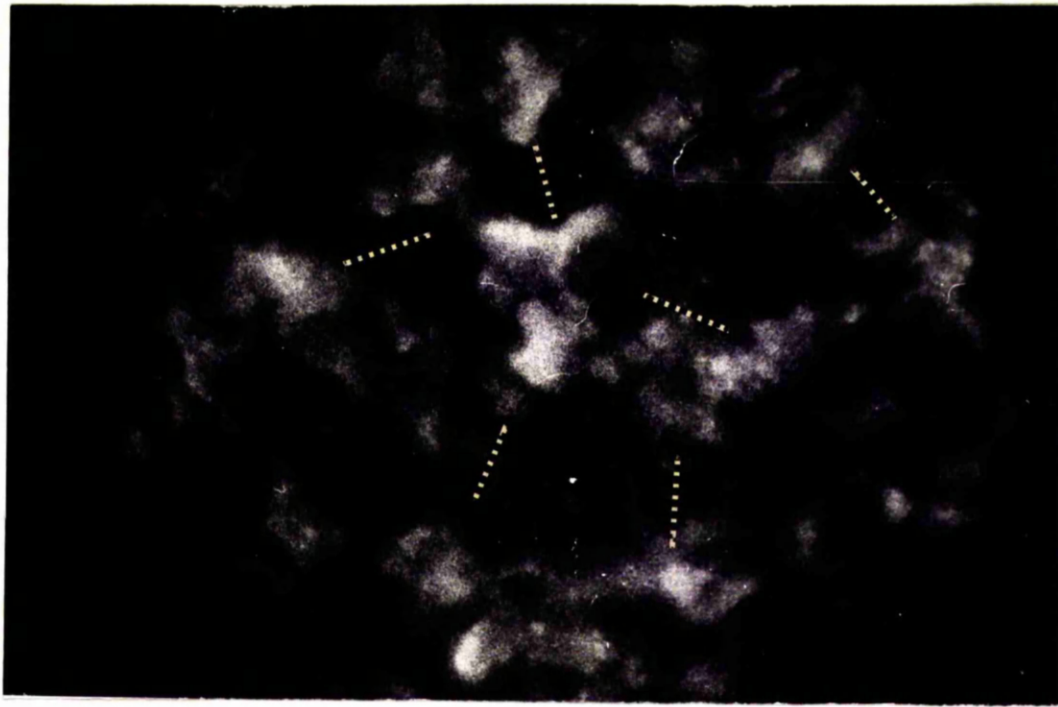


Fig. 12. NR/HP mixed aggregate after 48 hours. Magn. 1500.
Fluorescence excitation illumination. NR cells (arrows).

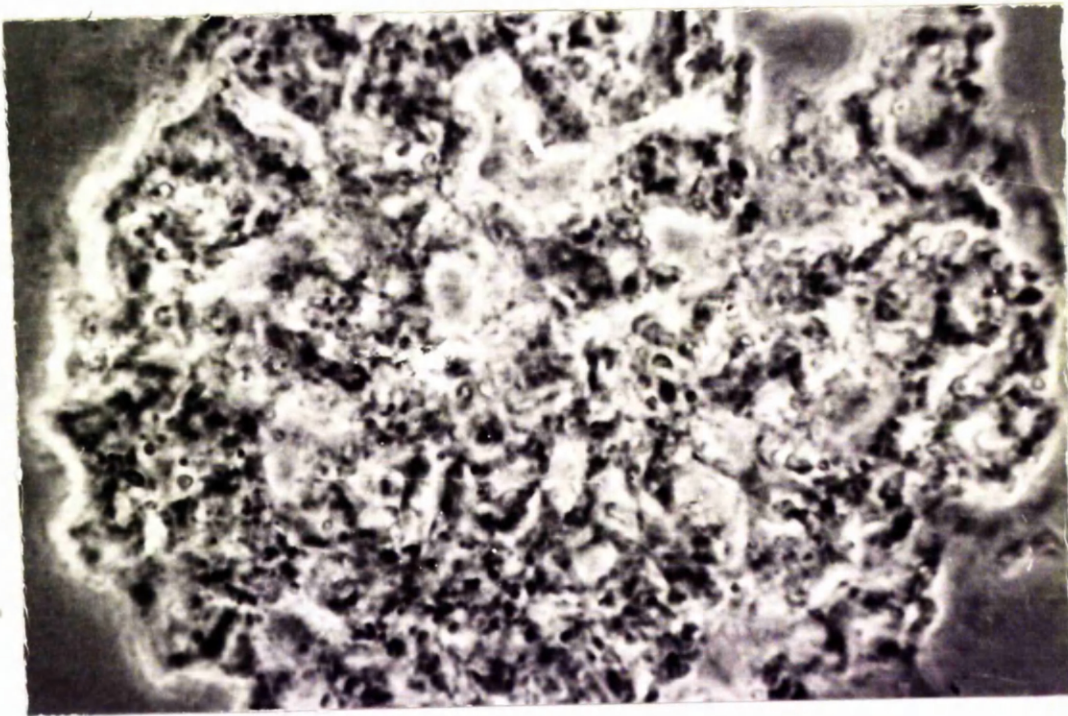


Fig. 12a. NR/HP mixed aggregate after 48 hours. Magn. 1500
Bright field illumination.



Fig. 13. NR/T2 mixed aggregate after 48 hours. Magn. 1500
Fluorescence excitation illumination. Possible NR cells (arrows).

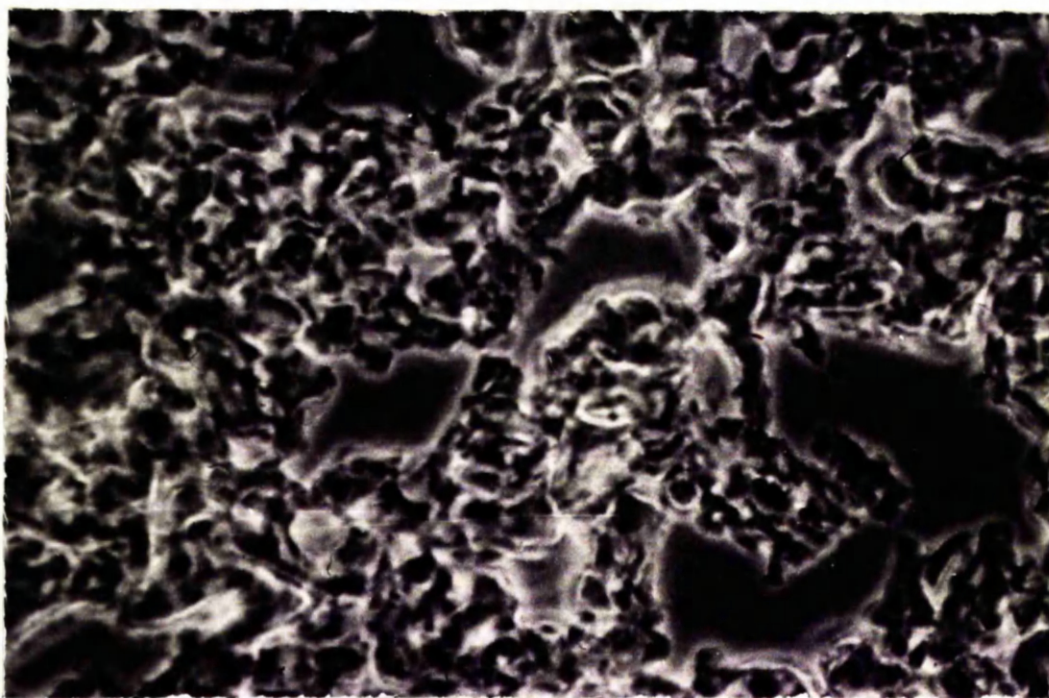


Fig. 13a. NR/T2 mixed aggregate after 48 hours. Magn. 1500
Bright field illumination.

considered of any importance. In these aggregates also there was a lack of NR cells, and the T2 cells were also spread.

In general it is seen that the normal origin cells, that is the C13 and HP cells, associated with the NR cells in mixed aggregates while the neoplastic cells, Py C1i and T2 cells, did not associate with the NR cells in mixed aggregates.

From the above it is seen that while the normal cells failed to segregate, the neoplastic cells were found not to associate with the NR cells in mixed aggregates. In the cases of the Py C1i and T2 cells where no NR cells were found in the aggregates, the medium of the cultures was observed and found not to contain any NR cells, while the cell sheets on the flask walls contained almost exclusively NR cells. In the cases of C13 and HP cells the cell sheets on the flask walls contained both NR C13 or HP cells mixed respectively.

DISCUSSION

In the investigations presented here three aspects of cell interactions have been studied on normal and neoplastic cells and in interacting mixtures of normal and neoplastic cells; first the effect of the cell products on cell adhesion, second the adhesive relationships and third the positional relationships of the different cell types used.

Conditioning Effect.

(a) Serum effect

The control tests for conditioning using fresh growth media showed that the adhesiveness of NR cells was affected by the presence of the sera used. Comparing the adhesiveness of the NR cells in the presence of H+199, ECT and EFT (see table I) and the collection of the NR cells by the NR monolayers in the presence of HH and EFT (see table VII) we observe that in the culture media containing sera there appear to be factors diminishing the adhesiveness and the rate of collection of the NR cells.

There have been contrasting reports on the effect of sera on the adhesiveness and the attachment of different cell types onto plastic and glass surfaces. Unhjem and Prydz (1974) observed that HeLa 71 cells attached rapidly on plastic culture vessels in the presence or absence of serum but in the presence of serum the attachment was temperature dependent. Curtis and Greaves (1965) separated a serum protein that inhibited the aggregation of embryonic chick and quail limb bud, heart and liver cells at low temperatures. Curtis et al (1975) reported that the aggregation inhibiting factor found in horse serum, (Curtis and Greaves 1965), is in fact phospholipase A₂. Witkowski and Brighton (1972) reported that serum retarded the rate

of spreading of MRC-5 cells on a glass surface. Martin and Rubin (1974) reported that differences observed on the rate of adhesion of chick embryonic fibroblasts onto bacteriological dishes were due to serum. These differences were not detectable on tissue culture dishes. Gail and Boone (1972) reported that BALB/3T3 fibroblasts adhered to pyrex and cellulose acetate better than did BALB/SV3T3 transformants in the presence of foetal calf serum. Keppel et al (1974) found that trypsin bound on serum coated plastic inhibited the adhesion of BHK cells onto the coated plastic. Revel et al (1974) observed that calf serum absorbed to culture dishes formed a coating to which cells attached. It is possible that they were looking at cell spreading rather than cell adhesion. The above indicate that not only serum plays an important role on cell attachment to different surfaces but also the cell surface plays a very important role.

(b) Factors effect

The variance analysis of table I showed that the unconditioned and the conditioned media, and different types of conditioning (none-normal-malignant) had different effects on NR cell adhesiveness. From table I we see that the primarily transformed cells, (PyC1 and HSV2), condition their growth medium with inhibiting factor(s) activity more than the secondary transformed cells, (Py C1i and T2), which have been selected from the primarily transformed ones. From the secondary transformed cells the Py C1i appear to condition their growth medium with detectable inhibiting factor, while the T2 cells appear not to condition their growth medium with any detectable factor with respect to NR cell adhesiveness. Meanwhile table VI indicates that the T2 cells condition their medium with inhibiting factor activity with respect to liver cell adhesiveness. This means that either T2 cells condition their growth medium with respect to NR cell adhesiveness with

some factor which I failed to detect with the system I used or that the conditioning was ineffective to NR and only effective to liver cell adhesiveness. Meanwhile T3 cells appear to condition their growth medium with inhibitory factor with respect to liver cell adhesiveness and with mainly promoting factor, but occasionally with inhibitory factor with respect to NR cell adhesiveness. For the normal cells, C13 and HP, table I indicates that C13 conditioned medium appears inactive while the HP medium appears to be conditioned with promoting factor with respect to NR cell adhesiveness. The variance analysis showed that the normal conditioning, (C13 and HP) has a different effect from the fresh growth media, (ECT and EFT). From the regression analysis it is also obvious that C13 conditioned medium and ECT growth medium affect the time course of NR cell adhesiveness differently, which means that the C13 cells condition their growth medium but the conditioning was not detectable with the system used.

From the above it is seen that different cell lines condition their growth media with respect to the adhesiveness of other cell types. It is also seen that the normal cells either did not condition their medium with respect to NR cell adhesiveness or, if they did, condition it with promoting factor(s), while the neoplastic cells usually produce inhibiting factors with respect to NR and liver cell adhesiveness. Another consideration is that the neoplastic cells instead of conditioning their media with inhibitory factors they may have removed promoting factors if present in the growth media. The negative value of the regression coefficient, of the value of collision efficiency with increase in time supports the above.

Further experiments which might have been done leading to further information on the nature of these factors are the following: A thorough purification of the different factors for better comparison

of their effect. Mixed factors or mixed factors with the reference medium Couette runs or monolayer assays for the differentiation of promoting^{or} inhibitory factors. Tests for the effect of these factors onto other cell types studying their specificity. Biochemical assays for comparison of their nature with other known factors. Invivo assays for studying their invivo effect. Complex monolayer assays for studying their diffusibility. Such experiments could have been to distinguish if the above-mentioned factors were inhibitors of cell aggregation or if promoters have been removed from the growth media leading to the decreased adhesiveness. To find out their nature and their molecular weight and to test for possible effect on other cell types.

Using the collecting cell lawn system different results were obtained with the tumour conditioned media. Both T2 and T3 media promoted the extent of attachment of trypsin treated NR cells in comparison with the extent of attachment in the presence of fresh growth medium. The extent of attachment of EDTA treated NR cells was promoted in the presence of T2 medium but in the presence of T3 medium was slightly increased. Meanwhile T2 medium promoted the collection of the chick embryonic liver cells by the NR monolayers but the T3 medium had no effect. However, both T2 and T3 media either had no effect or, if they had, it appeared to be inhibiting the collection of T2 cells by the NR monolayers. In general as seen on table VII both T2 and T3 media had inhibitory activity if they were compared with Hanks Hepes.

Balsamo and Lilien (1974), Daday and Graeser (1970), Lilien (1968) and Lilien and Moscona (1967) studied the NR aggregation factor derived from NR cell cultures. Takahashi and Okada (1970, 1971) described an aggregation promoting factor from leg muscle, lung, kidney and dorsal skin cells of 11 day old chick embryos. Kuroda (1968b

described a promoting factor for chick liver cells.

Inhibiting factors have also been described for NR and liver cells of 7 day old chick embryos by Curtis (1974) and for lymphoid cells by Curtis and De Sousa (1973, 1975). These inhibiting factors were ineffective on the cell types from which they derived but were effective on at least a range of other tissues.

Daniel (1967) studied a conditioned medium from L-M mouse cells which were adapted to grow in serum free medium. He found that not only did it enhance the attachment of these cells onto glass bottles (French), but it also increased the viability of these cells after mechanical removal from plastic surfaces which were treated with this conditioned medium. Pessac and Defendi (1972b) and Pessac and Mayet (1968) studied factors enhancing the aggregation of several malignant cell lines. Modjanova and Malenkov (1973) studied a factor which could restore the adhesive stability of hepatic cells during the progression of hepatomas. Oppenheimer and Humphreys (1971) isolated a macromolecule which was required for the adhesion of mouse tumour cells. Maslow and Weiss (1972) studied the adhesiveness of Ehrlich ascites cells to different substrata in relation to cell exudation. From the above it is obvious that neoplastic cells like embryonic cells secrete factors which affect their adhesion and are also affected by factors from different sera. The existence of the factors studied in this work seems indubitable. The factors studied are of the type that Curtis and Van de Vyver (1971) and Curtis and De Sousa (1973, 1975) have described. Their effect as described here can vary according to which method one uses for the tests. Whether the above factors were derived from the different cell lines, or existed in the sera used in the culture media and were unmasked by the cultured cells, is difficult to certify. Experiments carried out at the earlier stages of this work treating the different media in different

ways, (temperature inactivation, positive pressure filtration, incubation without any cells at the appropriate temperature, etc.), showed that these factors were probably secreted by the different cell types and were molecules of high molecular weight and were probably contained protein. No effect of these media could be detected upon the aggregation kinetics of their own cell types. The inability to detect any effect of these factors on their own cell types might be dependent on the system used for their study.

The cell types used for the preparation of the conditioned media can be classified depending on the developmental stage they were at when isolated as either neonatal, or embryonic cell types. Neoplastic cells are subjected to a degree of differentiation and as differentiated cells have the appearance of an embryonic or quasi-embryonic state, (Brown and Bertke 1969), the classification of the neoplastic cells next to the embryonic is not far from a real developmental position. Thus the cells tested for conditioning are as follows: Neonatal cells BHK 21 C13, embryonic cells HP and neoplastic cells BHK Py C1, BHK Py C1i, HSV2, T2 and T3. From the results obtained during these experiments one can see that neonatal cells, BHK C13, do not secrete any detectable factor, while embryonic and neoplastic cells do secrete detectable factors.

Monolayer Collection.

An important observation is that the collection of T2 cells by the NR monolayers (see table VII) is not affected by the presence of the conditioned media, while in suspension. This may indicate that the collection of the different cell types by the different monolayers is probably not equivalent to collecting adhesion in suspension. Thus the adhesive relationship the cell may develop may be dependent only on the ability of the one cell type to make contacts with the others.

From the results of table IX it is obvious that the NR cells appear to develop a preference towards their own type cells as they are collected at a higher rate by the NR monolayers than by the other monolayers. Roth and Weston (1967) and Roth et al (1971) with the collecting aggregate system observed a specificity of chick embryonic NR cells towards other chick embryonic cell types. Similarly Walther et al (1973) using the collecting cell lawn assay observed the specificity of NR cells towards heart cells. These studies were comparing NR cells with homospecific fibroblastic cells. My results show that NR cells exhibit a specificity towards hetero-specific fibroblastic cells. It is possible to generalise that the NR cells exhibit a specificity towards fibroblastic cells.

From the results of tables IX and XI it is obvious that the BHK C13 and Py C1i are more adhesive than the HP and T2 monolayers as the C13 and C1i monolayers collect higher percentages of all cell types than the HP and T2 monolayers. For a more systematic study of the monolayer collection results, there will be a comparison first between groups of cells with similar origins and then within different groups. Under this grouping the cells are classified to the baby hamster and to the embryo hamster derived cells.

Both C13 and C1i monolayers seem to be more adhesive towards the C13 than C1i cells as they collect more C13 than C1i cells. Because of the time limit of the assay, the collected cells do not have time to spread on the monolayer, or possibly the upper cell surface of the monolayer does not support the spreading of the cells (DiPasquale and Bell 1974) and so the reaction of the cells with the monolayer will be mainly by adhesive sites projected towards the monolayer. The above means that the C13 cells in suspension (rounded cells) are more adhesive than the C1i cells may be by exposing to the surrounding environment a larger adhesive surface. Meanwhile the slightly

higher collection of the C13 cells by the C1i monolayers than by the C13 monolayers indicates that the C1i monolayers are more adhesive than the C13 monolayers towards the C13 cells. This maybe means that if the C13 cells project adhesive sites towards the monolayers, then the C1i monolayers either might support the spreading and the adhesion of the other cells, suggestion contrast in the findings of DePasquale and Bell 1974, or that the C1i monolayer is not a real monolayer but either a multilayer or a monolayer leaving empty spaces between its cells that trap the suspended cells.

In contrast to the C13 and C1i monolayers, being very adhesive towards the C13 cells, the HP and T2 monolayers appear not to be so adhesive towards the HP cells. HP monolayers do not express any preference towards the HP or T2 cells but the T2 monolayers collect more HP than T2 cells. Since the heterotypic collection of the HP cells by the T2 monolayers is higher than the other combinations of homotypic or heterotypic collection, this means that the HP cells in suspension (rounded cells) are more adhesive than either HP cells spread (monolayer) or T2 cells in their spread and suspension states.

It is obvious from the results that all monolayers collect neoplastic cells at a very low level as both C13 and C1i monolayers collect less C1i than C13 cells and both HP and T2 monolayers collect very low percentages of T2 cells. The collection of the HP cells by the HP monolayers is lower than by the T2 monolayers. This might be due to differential adhesiveness of the different cell types but also of possible conditioning of the collecting medium with inhibitory factors.

Using a classification of the different cell types in the order neonatal, embryo and neoplastic, we see that neonatal cells and their neoplastic derivatives collect more neonatal than neoplastic cells, while embryonic cells collect very low levels of embryonic cells and

their neoplastic derivatives, and that the embryonic derived neoplastic cells collect higher levels of embryonic but lower levels of neoplastic cells.

From the study of the collection among the two groups as earlier defined we see that: The Cli monolayers collect to the same extent HP and T2 cells without distinguishing the two cell types, that is the embryonic from the neoplastic. The same happens with the C13 monolayers. The difference between the two types of monolayers is that the C13 monolayers collect lower percentages of both HP and T2 cells than the Cli monolayers do. Meanwhile the HP and T2 monolayers exhibit a preference for the C13 cells as they collect more C13 than Cli cells. It is remarkable that the Cli monolayers collect more T2 cells than the C13 monolayers and the T2 monolayers collect more Cli cells than the HP monolayers.

The above results could also be explained if we consider as earlier that the different cell types have different adhesive properties. That the Cli monolayers collect more cells than the other monolayers give rise to the questions how a monolayer is constructed and do added cells penetrate gaps if they exist? Light microscopy does not provide any great information on the structure of a monolayer. From Fig. 4 and 5 it is clearly distinguished that the C13 and Cli cultures differ in structure and that the Cli cultures leave large gaps between their cells. If added cells can penetrate gaps in the monolayer only electron microscopy could provide reasonable data. That the Cli monolayers collect more cells than the C13 monolayers could support the idea of the cells penetrating gaps in the monolayers. From the above rises the problem if the added cells penetrating the gaps in the monolayer make contacts only with the cells of the monolayer or and with the substratum. To this point also electron microscopy can only give an explanation. If cells make

contacts with the cells of the monolayers then in the gaps do they make contacts with the sides of the cells or with the upper cell surface? Then is collection controlled by adhesion or by spreading? DePasquale and Bell (1974) demonstrated that the upper cell surface of cells does not support cell spreading. However, Middleton (1973) reported that the dorsal surface of pigmented retina epithelial cells does not provide a suitable substrate for cell locomotion but he observed that pigmented epithelial cells formed aggregates on top of pigmented epithelial monolayers. The above also raises the question if cell collection is a property of the cells by itself or a phenomenon controlled by different cell properties. The above questions require very long study to be solved.

Gail and Boone (1972) reported that BALB/3T3 fibroblasts adhered to pyrex and cellulose acetate better than BALB/SV3T3 transformants did. Similarly, in my results we see that BHK C13 fibroblasts adhered better to all monolayers than BHK Py C1i transformants did. Walther et al (1973) reported that BHK C13, polyoma transformed BHK and 3T3 cells do not show any specificity as they attach to homologous and heterologous monolayers at the same rate. My results are consistent with those of Walther et al (1973) for the C13 and C1i cells collected by the C13 and C1i monolayers but it differs on the combinations of C13 and C1i cells collected by the HF and T2 monolayers. Walther et al measured the rate of collection and I measured the extent of collection, that could account for the difference of my results and Walther et al results.

The preference of the C13 and C1i cells is expressed between the two groups, the cells of neonatal and the embryonic origin cells. The term specificity of adhesion applies to cells or tissue types with higher affinity for one type of adhesion than another where this cannot be explained in terms of a quantitative property graded over a range of

cells. In the present experiments in the combination of C13-Cli cell suspensions against the C13-Cli and HP-T2 monolayers specificity might explain the results. In the combination of HP-T2 cell suspensions against the HP-T2 and C13-Cli monolayers any specificity that exists is reversed. The HP-T2 cell suspensions prefer the C13-Cli monolayers than their homologous HP-T2 monolayers. All this argues that specificity of adhesion does not exist.

As a result of these experiments it is obvious that in this case of hamster cells we must speak of higher or lower adhesivity between the different developmental stages and not of specificity. So from these results it is obvious that the earlier the developmental stage the lower the adhesivity of the cells. The previously stated hierarchy of developmental stages from neonatal to embryo to neoplastic also corresponds with the adhesivity of the tissues at the different stages.

From aggregation kinetic experiments it was shown that the C13 cells were highly adhesive while the other cell types were not. Similar results were obtained from the monolayer collection experiments. The collection of the C13 cells onto the C13 monolayers is higher than the collection of the Cli, HP and T2 cells onto the Cli, HP and T2 monolayers respectively. So within this small sample ^{of the Hamster tissues the proposed hierarchy seems correct} Hornby (1973a) has shown that between chick embryonic tissues of different developmental stage the adhesiveness of the tissues changes greatly. Therefore the proposed hierarchy has to be interpreted carefully bearing in mind that adhesiveness of permanent cell lines can vary depending on the culture density, (Edwards and Campbell 1971 for BHK cells).

Two Dimensional Sorting Out.

Earlier in this text it was stated that NR cells express a greater preference for NR cells than for the other types. The results from mixed cultures of NR and of the other cell types in falconised

petri dishes show that the NR cells really express this preference. The formation of the NR cell islands has two possible explanations. First the cells expressing higher adhesivity towards themselves gather together leading to the formation of a main NR cell mass.

The presence of the fibroblastic cells which are considered as highly motile, and of considerably high growth rate and their tendency to develop their characteristic appearance separate the initially formed NR masses at their locations and prevent them from forming a central NR cell mass. In general if NR cells are plated on a plastic petri dish in a lower concentration than that of confluency then they form a discontinuous monolayer of NR cell islands. If this is what happened in this case then what was considered as sorting out is just an artefact of the inability of the NR cells to move in culture.

A second explanation is suggested by the contact inhibition of cell movement. Garrod and Steinberg (1973) and Steinberg and Garrod (1975) explained the formation of chick embryo liver islands surrounded by limb bud cells in monolayer cultures as a result of discouragement of overlapping due to adhesive phenomena. Chick embryo liver cells are a population of two different types of cells. The one type is epithelial and the other is fibroblastic. It is doubtful how Garrod and Steinberg could distinguish the liver fibroblasts from the limb bud ones. When liver cells are plated in culture bottles then they form a network of fibroblastic cells surrounding the epithelial cells. However, if contact inhibition directs the formation of the NR islands in these mixed cultures then it is difficult to explain the tendency of the fibroblastic cells to cross underneath the NR islands as is clearly seen in Fig.6 to 9. In the work of Garrod and Steinberg (1973) and Steinberg and Garrod (1975) it is possible the sorting out pattern, if the distinction of the cell types is undoubtful, ~~could~~^{to} be due to contact inhibition or discouragement of

overlapping as they observed no crossing of the two cell types.

The crossing of the fibroblastic cells underneath the NR cell islands shows that there is some interaction between the NR cells and the fibroblastic cell types which is not as strong as the one between the NR cells. DiPasquale and Bell (1974) described a case where fibroblastic cells can not move over the upper surface of epithelial cells but they can move underneath the epithelial sheet in a concave region of the epithelial margin not in contact with the substratum. They do not report if contacts were made between the epithelial and the fibroblastic cells crossing underneath the epithelial cells. It is very important to know about the existence of such contacts. In my cultures the fibroblastic cells crossing underneath the NR islands seem not to make any important contacts as the NR islands can be detached from the underlying fibroblasts by a gentle shaking.

The observation that the islands after a further 24 hours in culture spontaneously come off the plastic surface, could be explained by the movement of the fibroblastic cells into the gaps the NR islands leave. This means that the NR cells do not make with the substrate very strong contacts and that the NR cells leave large spaces between their contacts with the substratum. The fibroblastic cells may take advantage of these spaces so they do not cease their locomotion and cross underneath the NR islands. If we consider that the upper surface of the fibroblastic cells is not active as stated by DiPasquale and Bell (1974), then the progressing fibroblastic cells could remove the NR cells from their substrate. Steinberg and Garrod (1975) have shown that the liver islands move throughout the culture to form larger islands in their mixed cultures with limb bud cells. However, in my system NR islands in their attempt to increase their size by joining two or more islands together after active or passive movement, come off the plastic because of weak contacts with the substrate and the

movement of the fibroblastic cells.

Another possible explanation is that the NR cells can be subjected to factors diminishing their adhesiveness which are secreted by the fibroblastic cells and vice versa. This explanation is not in contrast to the results of the secretion effects on NR cell adhesiveness. If we consider the treatment of the NR cells for the secretion effects tests and the treatment of the NR cells for these mixed cultures and during the culture period, it is possible for the above explanation to be correct. By such an assumption the presence of the single cells in the medium is explained by reduced adhesiveness of the NR cells and by disruption of their contacts. Edelstein (1970) studied theoretically the sorting out of mixed cell aggregates and suggested that the final pattern will be dependent on the effect of specific chemicals secreted by the cells involved. Curtis (1974) produced some evidence for the theory of Edelstein by testing the effect of conditioned NR and liver culture media, onto presorted mixed NR and liver aggregates from 7 day old chick embryo.

Moscona (1957, 1961a) reported experiments using mixed aggregates of chondrogenic and hepatic cells with S91 melanoma cells. After prolonged culture the melanoma cells infiltrated the cartilage and hepatic parenchyma. If we accept that neoplastic cells in general secrete factors affecting the adhesiveness of embryonic cells as it was reported earlier in this work then the observations of Moscona could be explained by reduced adhesiveness of the embryonic cells which enables the infiltration of the neoplastic cells. Meanwhile Kuroda (1968a) failed to observe infiltration of the embryonic cells by the neoplastic possibly because he studied the aggregates at early stages up to 48 hours. The cases of chick limb bud and dermal cells which he observed to have formed intermixed associations with the HeLa cells can not be explained by the assumption of reduced adhesiveness because they have

been studied only 24 hours after the start of aggregation, whereas the combination of liver and epidermal cells with the HeLa cells at the same time have not formed intermixed associations.

In my results it is possible that up to 24 hours in culture the cells in the monolayers keep their positions but possibly after this time the neoplastic cells appear to remove the NR cells from the plastic surface.

However, it is well known from the work that Wolff and her associates carried out that embryonic cells associate with the neoplastic ones. Wolff and Wolff (1961) studied the association of chick mesonephros and human tumours. Wolff and Schneider (1957) studied the associations of S180 cells and the following chick embryo organs: mesonephros, metanephros, dermis of the skin, intestinal teguments, liver, lungs and conjunctive tissues of the limbs. Sigot-Luizard (1974) and Lakshmi and Sherbet (1974) also studied the associations of embryonic and neoplastic cells and they found that the neoplastic cells invaded the embryonic tissues. Sigot-Luizard (1974) indicated that the neoplastic cells can not penetrate the intestinal epithelium and the epidermis as was shown by Leighton et al (1965).

From the above we see that the embryonic tissues and the neoplastic cells associate together after the latter ones penetrate the former ones. To my knowledge the association of the NR cells and the neoplastic cells has not been studied previously.

From what has been said above the question still remains: What is the structure of the sorting out pattern of the NR and the fibroblastic cells in mixed cultures in two dimensions? The pattern observed after 24 hours in culture could easily be an artefact and not a real pattern.

Three Dimensional Sorting Out.

From the mixed aggregate results it is seen that the normal

cells (C13 and HP) show a degree of association with the NR cells by forming intermixed aggregates. After that time, 48 hours reaggregation, if the cells could ~~aggregate~~^{segregate} in the aggregates they would have done so, considering that the C13 and the HP cells appeared spread in the aggregates, that is active cell movement could be in progress. The observation that in the monolayer cultures the NR cells came off the plastic surface as the C13 and HP cells increased in number, gives some support to the hypothesis that in the mixed aggregates the intermingled appearance of the cells is a result of the medium being conditioned by factors diminishing the NR cell adhesiveness.

The neoplastic cells do not associate with the NR cells and possibly segregate completely. These neoplastic cells made loose aggregates or remained in single cell suspension or small clumps form and the NR cells collected on the formed cell sheets on the glass surface of the flasks. This complete separation of the neoplastic and NR cells can also be explained by the factors hypothesis assuming that the neoplastic cells do not support at all the adhesiveness of the NR cells onto themselves. If we consider that the neoplastic cells, that is C1i and T2, do not support their own aggregation, (an observation from the short term aggregation experiments), because they form easily disrupted contacts, then the non-stickiness of the NR cells to the neoplastic is easily understood.

The results indicate that cell to cell and cell to substrate adhesions are different since NR cells do not adhere to the neoplastic cells but do adhere to the glass surface of the flasks even in the presence of the neoplastic cells. We can conclude from this that if factors are involved they affect the different types of adhesion in different ways. The surface of the flasks was siliconised and therefore should not support the formation of adhesion between the glass and the cells.

Moscona (1957, 1961a) demonstrated that the S91 melanoma cells sorted out to a concentric pattern in mixed aggregates with limb bud and liver cells from chick embryos but later the melanoma cells infiltrated the surrounding or surrounded tissues resulting in an intermingled association. Kuroda (1968a) demonstrated that HeLa cells associated with limb bud mesoblasts and skin cells, but formed separate aggregates when mixed with liver cells. He reported that when HeLa cells were cultured by rotation for 24 hours they produced aggregates. He did not report whether the cells divided or not during the culture period. The Py Cli and T2 cells since they grow in suspension and do not form any aggregates can easily condition their culture medium and affect the ability of the NR cells to form aggregates or to associate with them, resulting in the complete absence of NR aggregates in these mixed cultures.

The work of Moscona's group and of Burdick and Steinberg reported earlier in the introduction, (see page 10), shows that in several cases embryonic chick and mouse tissues in mixed aggregates of dissociated cells sorted out and in several other cases failed to sort out according to the two species.

Moscona (1961c) reported for mouse and chick embryonic neural retina cells that failed to sort out according to species. The same happened when mesonephros cells were mixed, (Moscona 1962). Burdick (1972) reported that mouse liver cells sorted out from chick neural retina cells but he did not report their pattern. From my results it is seen that chick neural retina cells and hamster fibroblasts, (BHK C13 and Hamster embryo primary cells), failed to sort out in mixed aggregates.

From chick embryo liver cell cultures it is known that liver fibroblastic cells surround liver epithelial cells in two dimensional cultures. If mouse embryo liver cell cultures have the same structure,

then it is possible the chick embryo neural retina cells have associated better with the mouse liver epithelial cells than the fibroblastic ones, so sorted out in the aggregates referred by Burdick (1972). If it is so then, in my experiments as I have pure fibroblastic cells would not sort out in the mixed aggregates.

In general it is seen that the embryonic cells (NR) associated with the normal cells (C13 and HP) in mixed aggregates, while they did not associate with the transformed and the tumour cells (C1i and T2 respectively).

These results of mine contrast with those of Moscona (1957, 1961a) and of Kuroda (1968a) for neoplastic cells. The most obvious difference in these experiments is that their experiments were carried out with only fibroblastic cells and mine with fibroblastic and neural cells which presumably have different adhesive properties.

Conclusions.

From the work I carried out on the association of the embryonic and neoplastic cells I can conclude that this association might be governed by factors secreted by the different cell types. The proposed classification of the different cell types to neonatal, embryonic and neoplastic fits with all the results obtained during this work.

The neonatal cells, (C13), do not secrete any detectable factor promoting or inhibiting the adhesiveness of the NR cells from 7 day old chick embryos.

The embryonic cells, (HP), appeared to secrete a factor with promoting activity with respect to NR cell adhesiveness. This finding agrees with the results of other scientists that embryonic cells secrete factors affecting the adhesiveness of other cell types and not of their own.

The neoplastic cells, (PyC1, PyC1i, HSV2, T2 and T3) appeared to secrete factors with inhibitory activity with respect to NR cell adhesiveness. This also agrees with the work of other scientists that different cell types have been found to secrete factors affecting the adhesiveness of other cell types and not of their own. The activity of these factors is dependent on whether the factor has been prepared from primarily transformed cells, (PyC1 and HSV2), or from secondarily transformed cells, (Py C1i, T2 and T3).

The ability to detect the activity of these factors is dependent on the system used for the study. So it is seen that according to the developmental stage at which the cells have been classified the effect of the factor either is inhibitory, promotory or there is no factor.

The cells of embryonic and neoplastic origin, (HP, Py C1i and T2), are not aggregating while the neonatal ones, (C13), are. However, the collecting monolayers of neoplastic origin appear collecting cells to a larger extent than the corresponding monolayers of normal origin, but the collected cells responded in the same way as the aggregating cells. So aggregation and collection are two different cell properties related to adhesion but controlled by possibly different mechanisms. A possible mechanism is the structure and configuration of the cell membrane. These seem to differ not only between normal and neoplastic cells but also between rounded and spread cells of either normal or neoplastic origin.

The embryonic cells segregated from the C13, C1i, HP and T2 cells in two dimensional cultures forming a multiisland appearance in a fibroblastic network, possibly as a result of weak contacts with the substratum and the locomotion of the fibroblastic cells, while the disruption pattern after 48 hours in culture appeared to be dependent on the locomotion of the fibroblastic cells in combination of the possible conditioning of the culture medium by the fibroblastic cells

with factors diminishing the adhesiveness of the NR cells.

Whether the multiisland appearance in these cultures is a type of segregation equivalent to that in three dimensions or not is not well known. This appearance could be a result of possible preaggregation of the NR cells in suspension and then of the settlement of these aggregates or of the fast rate of multiplication of the fibroblastic cells. In three dimensional cultures, i.e. formation of aggregates, the NR cells associated with the normal cells, (C13 and HP), and did not associate with the neoplastic cells, (C1i and T2). The non segregation of the normal cells from the NR and the non association of the neoplastic cells with the NR cells might possibly be controlled by factors secreted by the normal and neoplastic cells.

Experiments with antibodies against the possible factors involved in these sorting out experiments and the effect of the conditioned media onto presorted aggregates could give further support to the idea that factors may control the above types of sorting out. Two dimensional sorting out experiments where the cells would be plated onto filters and the conditioned media would flow underneath the filters (or above the plated cells separated from the flowing medium by another filter), may give some idea on the diffusibility of the factors.

From the above work it is obvious that the normal and neoplastic cells differ from each other, possibly in the structure and configuration of their membranes as the normal cells aggregate more extensively than the neoplastic ones but collect less cells than the neoplastic.

My work also gives support to the morphogen theory for the control of cell positioning in aggregates.

It will be very useful to find out if the effect of the studied

factors does not apply only to the studied system but also to other systems in vitro. If the factors act in in vitro situations then it may be important to find out whether they act in in vivo situations. Such tests in vivo can be carried out in animals where tumours may have already grown or during tumourogenesis. In such cases it is possible to sort out whether such factors control malignancy or not. It will also be useful to study whether such factors can control not only malignant tumours but also benign ones. A preparation of antibodies against the tumour cell factors and the study of their effect onto the tumour cell development in vitro and in vivo might lead to new methods for the control of tumour metastasis.

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