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An Hypothesis on the Role of Cellular Colloid Osmotic Pressure in Determining Behavior of Cells *in vitro* including Anchorage Dependency and Maintenance of the Differentiated State

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The osmotic problems involved when cells are isolated from tissues are analyzed. Evidence is considered which indicates that *in vivo* the Na pump is operating at maximal or near maximal rates and that this depends on low leak rates for salts and water due to various aspects of the tissues structure. Dispersion of the tissue results in breakdown of these barriers on free diffusion and the isolated cell is subjected to an enormous increase in passive influx due to colloid osmotic pressure without being able to increase its pumping rate to the extent needed to maintain volume control.

It is proposed that the primary problem the cell faces *in vitro* is to compensate for the effective increase in its colloid pressure, e.g. the colloid osmotic pressure excess, emerging with the breakdown of the tissue structure. The finding that most normal cells have to adhere to a surface in order to grow or "anchorage dependency" is analyzed in terms of the way adhesion and spreading result in changes in ion and water movements into cells enabling them to achieve fluid balance in the face of the colloid pressure excess.

It is also proposed that the differentiated state is more dependent on colloid osmotic balance than proliferation. The failure of conditions used in tissue culture to compensate adequately for the colloid pressure excess results in limiting the amount of protein which can be synthesized, dissipation of cellular energy, and changes in orientation of cellular components which contribute directly to the loss of differentiation which occurs during growth *in vitro*.

Introduction

It is now recognized that in adult tissue the Na pump plays a major role in regulating physiology of the cell. It maintains the ion gradients which power transport of nutrients and provides the batteries for current flow in excitable cells. The Na pump is also the mechanism for regulating cell volume. By extruding Na and water it prevents dilution of cytoplasmic

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constituents and maintains the normal hydration state of ultrastructure in face of the constant drive of water into the cells because of colloid osmotic pressure. Tosteson (1964) quite early concluded that "regulation of cell volume is the most fundamental and primitive cellular function of active Na and K transport".

The major problem in tissue culture is that most differentiated cells don't survive long *in vitro*. Only about 5% of all cells whose explantation has been attempted have been found to survive more than 1–2 weeks. The few that do survive longer undergo rapid changes in morphology and lose most of their differentiated features. This is especially true of cells in the established lines which have very little similarity to the tissue of origin.

There has been no attempt at analyzing the physiological problems involved when cells are isolated from the tissue structure. We propose here that the primary problem is osmotic and due to the fact that the Na pump cannot maintain volume control in the totally isolated cell. We will attempt to show that the osmotic problems arising as a result of isolation can account in large part for the types of cells which survive, the way they behave and the changes they undergo in culture.

Effect of Isolation on Mechanisms for Volume Regulation of Cells

There are two aspects of the pump-leak system operating in tissues for maintaining volume control which are particularly relevant to understanding the changes involved when cells are isolated. As discussed by Whittam (1964), Tosteson (1964), and others (MacKnight & Leaf, 1977), the pump is energetically a very expensive mechanism for controlling cell volume. As much as 20–40% of the ATP produced by respiration may be expended just on extruding Na and water from the cells. These findings gave rise quite early to the suggestion that metabolic needs of the cells had to be budgeted in terms of the ATP available and that the pump as it operates *in vivo* may restrict rapid growth and division.

The second feature of the Na pump which has to be analyzed in evaluating the changes incurred when cells are isolated is that it appears to be so easily damaged by slight changes in the physiological or physical state of the tissues. *In vitro* manipulations of tissues using procedures very mild compared to those involved in making cell suspensions result almost immediately in osmotic distress. Studies with tissue slices show that there is a rapid loss of K, decline in membrane potential, decrease in the incorporation of amino acids, fall in ATP and swelling of cells. The loss of K and swelling can be retarded significantly by adding albumin or other non-permeant molecules (Armstrong & Knoeble, 1969; Robinson, 1975; MacKnight & Leaf, 1977).

This indicates that swelling and loss of K are due to colloid osmotic pressure of the cells. In the studies with tissue blocks, it was found that as much as 70% of the total protein of cells could be lost during the first 5 minutes (Chayen, 1978). This is also prevented by using high molecular materials in the solutions used during isolation (Chayen, 1978). Robinson (1975) has reviewed the extensive data on swelling in excised tissues and concluded that it is due to an impairment of the Na pump under which conditions colloid osmotic pressure becomes paramount. He attributed this to anoxia or membrane damage incurred during isolation. Herz & Schousboe (1975) have concluded that swelling of cells in tissues slices can be completely understood in terms of the "osmotic, electrical and Donnan equilibrium".

It seems well established that volume control in the highly differentiated cells in adult tissues is an expensive operation consuming a significant fraction of the cells ATP. Since the cell cannot do everything with a limited capability to synthesize ATP, the pump may well limit some growth processes. This can be understood as the price exacted for maintaining specialized functions. However, it is not clear why control of cell volume should be contingent on a pump mechanism requiring a very close, if not perfect, gearing between respiration and K transport. Both of these processes are very rapid, involve component systems, and depend on transport between cellular compartments. Since volume control is fundamental to all other organized cellular activities, it is difficult to understand why it would depend on a system which, as indicated by the *in vitro* studies, is so vulnerable to damage due to fluctuations in the physiological or physical state of the cells.

In considering this problem, the possibility arises immediately that tissue structure provides features which stabilize cells against volume changes and that these features break down when the cells are isolated.

There are several ways in which the pump-leak system could depend on tissue structure. The high rate of production of ATP or gearing of the pump *in vivo* could be due to the presence of special factors or hormones not available after isolation. However, no such factors have as yet been found. There are, however, many reasons to consider that the tissue structure determines the rate at which Na and water leak into the cells.

Cells are organized in tissues as the result of both morphological and physico-chemical specialized features. The different types of junctional complexes bring adjacent cells into very close apposition at certain areas and also provide preferential routes for permeation of salts and water. In addition, as schematized by Farquahr & Palade (1966) occlusion through junctions with high permeability for K, means that some domains of the cell's cytoplasm are in direct contact with the cytoplasm of other cells, rather than with the extracellular fluid. This would reduce the problems

involved in osmotic equilibration of cells in tissues. Osmotic studies have also shown that unstirred layers result in significant decrease in diffusion of ion and water through membranes. It has been estimated that the unstirred layers around cells in tissues may be as much as 500–800 nm thick whereas they may be only 10 nm thick in isolated cells (Dietsky, 1978). In addition to these features which reduce the free diffusion of salts and water into the cells, the proteins and other non-permanent macromolecules in the immediate vicinity of the cells would counter the non-diffusible molecules in the cells and decrease the effective colloid osmotic pressure. These mechanisms all provide a way of setting the leak rates of cells in tissues at relatively low levels.

Isolation of cells results in the breakdown of all of these tissue specialized features which counter the cells' colloid osmotic pressure or which impede influx into cells. The isolated cell is faced with having to maintain its normal volume against a greatly increased rate of influx. The problem is exacerbated further by the fact that on isolation it is also deprived of the control on the hydrostatic pressure of extracellular fluids maintained by the circulatory system (Granger, 1981; Comper & Laurent, 1978). The isolated cell would have to greatly increase its pumping rate in order to maintain normal volume. The deleterious osmotic changes exhibited by cells in tissue slices indicate, however, that this is not possible. They are found to swell rapidly following a manipulation which is much less severe than isolation. This indicates that the pump *in vivo* is already operating at maximal or near maximal rates and cannot increase its rate to meet any very great increase in passive influx. Furthermore, the fact that cells in most excised tissues do swell indicates that normal adult tissues have no adequate alternative mechanism for extruding Na and water which can come into play to maintain normal cell volume when the pump is impaired. This is completely consistent with the results from physiological studies which show that other mechanisms play only a minor role in volume regulation of cells in normal adult differentiated tissues.

According to this analysis the primary problem the cell faces on isolation is due to a component of colloid osmotic pressure which is no longer compensated by features inherent in the tissue structure. This analysis of swelling of cells in excised tissues differs from Robinson's in that it considers the primary driving force to be uncompensated colloid osmotic pressure rather than a failure of the Na pump. The increased influx would result in damage, probably almost immediately, to the pump. However, the distinction between the two hypotheses is important when trying to understand the problems the cell faces *in vitro*. Attributing swelling primarily to injury to the pump implies that when the injury is repaired, the pump is adequate

to maintain osmotic balance in the isolated cells. In contrast, if swelling is due to colloid osmotic pressure excess even when the pump is completely repaired, it would not be able to control the volume of the cell *in vitro*. The distinction focuses on what we propose to be the primary problem of cells in culture—how to compensate for colloid pressure excess.

Changes Cells Undergo to Compensate for Colloid Pressure Excess

The distinctive feature of the hypothesis we are proposing is not that tissue structure plays a role in osmotic balance of cells but that the influx of salts and water arising with the breakdown of the tissue structure exceeds the capacity of the cellular pumps. Differentiated cells isolated from normal adult tissues are in an osmotic predicament. They have developed to depend on the Na pump and have no alternative mechanism for controlling their volume when the pump defaults. Their very survival *in vitro* would depend on developing ways to cope with the influx so as to maintain some degree of morphological and physiological integrity.

There are four principal ways this could be done. The cells could lose protein so as to reduce their colloid pressure. They could simplify their ultrastructure so that the physiology would not be so susceptible to damage as a result of fluctuations in water movements. They could adopt a more permissive mechanism for osmo-regulation so that volume control did not depend on a perfect gearing between two rapidly firing systems, e.g. respiration through the mitochondria and transport of K at the cell surface, as required by the Na pump. Finally, they could adopt a mode of behavior which would enable them to compensate for the colloid pressure excess and achieve a new state of fluid balance.

Inspection of the way cells behave in primary culture indicates that these mechanisms are all used during the course of adaptation to *in vitro* life.

Studies with smooth muscle cells have shown that they lose more than 60% of their total cellular protein within the first week in culture (Chalmley-Campbell, Campbell & Ross, 1979). The earlier studies of Chayen and his collaborators with tissue blocks suggest that the loss of enzymes, and other proteins so frequently observed in primary culture are the result of the cell's effort to bail itself out from the flood due to uncontrollable influx of salts and water.

Cells also undergo ultrastructural changes during adaptation to culture. These include enlarged nuclei (Willmer, 1965), loss of membrane receptor sites, of respiratory enzymes and of mitochondria (White & Nand, 1977). The general decrease in respiratory capacity (Turner, 1962; Vail & Glinos, 1974) would be associated with a reversion to a more primitive system for

volume regulation than that provided by the pump. A mechanism where a greater fraction of energy was derived from glycolysis, similar to that used in the embryo before the development of the pump, would provide a more relaxed mechanism which could function over a wider range of structural alterations than the pump. Thus, because of the osmotic difficulties arising from the colloid pressure excess, cells which survive in culture would have a decreased complement of protein, a simplified ultrastructure and higher glycolytic activity than cells in the tissue of origin. These are in fact, the predominant characteristics of fibroblastic type cells which grow out so readily in culture and particularly of cells in the established cell lines.

Adhesion and Spreading—A Mechanism for Reducing Colloid Pressure Excess

Even growth of non-differentiated cells would depend on compensating for the colloid pressure excess. One method would be to add protein or other membrane compatible impermeant macromolecules to the medium so as to increase the oncotic index and normalize fluid balance. Serum would be the most readily available source of protein which would be compatible with cellular membranes. This may account, in part, for the fact that a serum supplement is always required for primary culture. Recent studies have shown that serum carries many hormones and that with cells from some of the established cell lines, it can be replaced by a suitable hormone supplement (Sato & Reid, 1980). This important development has tended to obscure the critical role that extracellular proteins play in osmotic balance of cells. This was indicated by results from earlier studies showing that addition of albumin (Todaro & Green, 1964) or of methylcellulose, hyaluronic acid, etc. with or without serum increase both the number of cells surviving in culture and their growth rate (Merchant *et al.*, 1962; Trible & Haguchi, 1960). The use of serum or of high molecular materials as colloid stabilizers both have limitations. Serum contains enzymes and other toxic materials and usually cannot be added at concentrations above 20–30%, thus limiting the amount of extracellular proteins which can be provided. The concentration of macromolecules which can be used is limited by the effects on cell membranes and miscibility with other high molecular materials in the medium. Thus, the colloid pressure excess of cells *in-vitro* is probably never compensated for by the liquid overlay medium used.

The *in vitro* system provides, however, a truly impermeant macromolecule in the form of the glass or plastic surfaces of the culture vessels. Cells adhere readily and spread on these surfaces. In most cases, with cells from normal tissue, this is required before growth can take place. It is a peculiar phenomenon in which cells that are only 10–15 μm in diameter in suspension

may spread to cover many 1000 μm^2 of substratum. Inspection of the physical changes involved shows that this represents a behavioral modification which enables the cells to attain osmotic equilibrium in the face of the colloid pressure excess.

Effect of Adhesion and Spreading on Fluid Balance

Adhesion and spreading result in physical and ultrastructural changes in the cell which would have profound effects on ion and water movements.

Electron microscopic studies show that cells are aligned on the substratum so that at certain points they approach within 10–20 nm while the remaining area is at distance of 200–300 nm (Heath & Dunn, 1978). This would result in a considerable structuring of the interfacial solution particularly in view of the extended regions of carbohydrates in the glycocalyx (Weiss, 1969). In addition, adhesion may be mediated by factors, frequently glycoproteins (Grinell, 1978, 1983), absorbed on the surface. Glycoproteins have exceptionally high osmotic coefficients (Comper & Laurent, 1978) which increase sharply with concentration. There is also evidence that they may be closely packed (Culp, 1983). Direct measurements on the interfacial fluid cannot be made because of the extremely small volumes involved. However, these conditions indicate that there would be a marked decrease in diffusion of salts and water. This has been shown by two different studies. Leighton *et al.* (1970) showed that epithelial cells blister as the result of sub-cellular accumulation of fluid. Indirect measurements of the pH of the cell-substratum interface during growth on glass using three different cells showed that it was several units below the pH of the nutrient medium (Rappaport, 1960). In addition, the close apposition of the cell with the fixed charge system on the substratum would result in changes in the normal profile of ions at the cell surface. In particular, if the cell is adhering to a predominately negatively charged surface there would be binding of some cations and a decrease in concentration of Cl due to co-ion exclusion effects.

These physical changes would result in a decrease in influx of salts and water into cells at the interface. This is an area which involves one-half of the total cell surface. The changes would be expected to have immediate effects on membrane mechanisms. Hypertonicity *per se* has been shown to decrease influx of salts and water into cells (Diamond, 1966). This has been ascribed to osmotic shrinking of membrane channels. It has also been shown that changes in shape rapidly affect volume control mechanisms (Kregenow, 1973, 1981; Roti Roti & Rothstein, 1973) and that stress in lipid bilayers cause conformational changes which determine permeability to water (Disalvio & de Geir, 1983). In view of the co-operative nature of membrane

interactions, it seems likely that the physical changes incident on adhesion and amplified by spreading would not be limited to the interface but would involve even larger areas of the surface and profoundly alter water and salt movements into cells.

Adhesion of cells involving multiple interactions with charged groups on the substratum would result in stabilization of the cell surface. This becomes significant in view of the fact that the osmotic gradient cells can maintain is limited by the very low hydrostatic pressure which their membranes can withstand. Thus, adhesion of cells and spreading on a solid surface would increase the resistance of the cell to swelling otherwise resulting from its colloid osmotic pressure. Cells in culture appear to have and to tolerate a relatively high internal hydrostatic pressure. This is suggested by findings which show that the blebbing of membranes and extension of pseudopodia can be rapidly and reversibly inhibited by adding hypertonic concentrations of an impermeable anion to the medium (Harris, 1973; Dipasquale, 1975).

The effects of adhesion and spreading on ion and water movements also depend on intracellular changes. Within a few minutes of adhesion there is an elaboration of a three-dimensional contractile network composed of actin and myosin-like micro-filaments (Goldman & Knipe, 1973; Pollard, 1976; Vasiliev & Gelfand, 1977). This has been extensively investigated in relation to motility and shape changes of cells. However, this network also provides an elastic component to the cells' ultrastructure. This would increase further the capacity of the cell to withstand a relatively high internal hydrostatic pressure. It may also bring into play new mechanisms for control and modulation of water movements.

As discussed by Clegg (1979) the elaboration of this macromolecular structure results in an enormous increase in surface area in the cell increasing the amount of bound water or water which would be held by capillarity. This would have important physiological consequences which, however, cannot be quantitatively evaluated at this time. There is now no information on the changes in the relative amount of free water, nor on the amount of cellular cations which would become associated with charged sites on the network. However, these changes could provide a mechano-chemical system for modulating hydrostatic pressure in cells—one which would also be responsive to changes in shape. In the lammelipodia, for example, where the array of microfilaments is the only internal structure, the mechanical expansion of the network as the cells spread across the substratum would result in significant decrease in the intracellular free fluid pressure.

Studies with cells in tissues have not provided a good system for understanding the role of hydraulic movements in osmoregulation. With cells in culture, the critical relationship between ultrastructural changes and fluid

balance is more readily apparent. The amount, charge and elastic modulus of the ultrastructural components would play an important role in modulating movements of both ions and water.

This discussion indicates the profound changes induced by adhesion and spreading on a solid substratum. These would be associated with and depend on biochemical changes. However, the physical changes would be primary. These include a decrease in influx of water and salts at the interface, alterations in passive membrane permeability, stabilization of the cell surface against an increased internal hydrostatic pressure and elaboration of ultrastructural frame work which would play a role in modulating free fluid pressure in cells. These mechanisms would act co-ordinately to enable the cell to achieve fluid balance in the fact of its colloid pressure excess.

Anchorage Dependency

The requirement that most normal cells from solid tissues have to adhere in order to grow has been termed "anchorage dependency". Stoker, O'Neill & Waxaman (1969) observed that the volume of cells held in suspension increased two-fold in 24 hours and the cells did not grow. When allowed to adhere, growth resumed. Since synthesis of all macromolecules declines sharply when cells are suspended (Benecke, Ben-Ze'ev & Penman, 1978) the increase in volume is not due to increase in mass but would be due to swelling.

An osmotic basis for anchorage dependency has not previously been proposed. Several other mechanisms have been advanced. Castor (1970) suggested that spreading is required so as to increase the number of sites available for uptake of mitogenic materials. According to others, adhesion is required to stabilize a particular Ca transport mechanism required for growth (Whitfield *et al.*, 1983), or to maintain a critical structuring of the cell (Heath & Dunn, 1978; Rees, Lloyd & Thom, 1977). Folkman & Greenspan (1975) have proposed that the shape of cells, in particular the degree of flatness, is the critical factor. They suggest that this results in changes in surface tension which in some way determine growth. Finally, Maroudas (1973) proposed that changes in shape provide a mechanochemical stimulus to growth.

The argument here does not exclude the important role that these various surface dependent mechanisms may be playing in growth. It focuses rather on the primary osmotic problem cells face *in vitro*—how to compensate for the colloid pressure excess. According to this analysis, adhesion and spreading results in both physical and physiological changes which enable the cell

to achieve fluid balance. This would be required before growth could take place.

Several features of the mechanisms involved are similar to those proposed by Folkman and by Maroudas. The significance of surface tension *per se*, however, is difficult to assess in cells *in vitro* which have considerable elastic component and which are also bonded to a substratum. As discussed by Kleinzeller (1972) the more relevant parameter may be the "stiffness" of the cortical layer since this determines the internal hydrostatic pressure which the cell can withstand. This would be affected by a number of factors, including the strength of adhesion and the elastic modulus of the components of the contractile network. The number and internal arrangements of the filaments would also be important. When anchored directly to the membrane, these internal filaments might also alter the effective radius of curvature thus providing a sensitive mechanism for modulating surface tension and the osmotic gradient cells could withstand. This analysis suggests that there is necessarily a very close relationship between interaction of cells with surfaces, changes in shape and osmotic mechanisms. This had already been suggested by Willmer (1962) and others (as reviewed by Kleinzeller, 1972). Wilmer considered that any change in morphology of cells represents a difference in the way the cells handle their ion and water balance.

Cells Behave so as to Minimize the Colloid Pressure Excess

According to the hypothesis being considered the very survival of a cell *in vitro* is jeopardized at every moment because the conditions do not compensate for its colloid osmotic pressure. This accounts not only for anchorage dependency but for the selectivity cells exhibit towards the surfaces to which they will adhere and grow. Carter proposed (1967) that cells move across surfaces according to adhesive gradients. The strength of the adhesion bond would be only one of the factors operating to establish fluid balance in the cells. As already discussed this would also depend on many other factors including the osmotic coefficients of the materials mediating adhesion, the number of close contacts and the intracellular free fluid pressure set up as a result of the traction or "grip" cells exert on the surface (Rees *et al.*, 1977; Vasiliev & Gelfand, 1977).

Recognizing the osmotic basis of adhesion and spreading can provide a more unified approach towards analyzing the way cells move across surfaces than provided by either the strictly physico-chemical or the morphological mechanisms which have been suggested. According to the physico-chemical viewpoint, the cell moves across a surface as the result of sequence of

preferred contacts it makes with sites available on the surface. According to the morphological viewpoint, the direction of movements is determined by the orientation of micro-filaments in the cellular cortex (Heath & Dunn, 1978) and the cell moves in a direction which puts least mechanical stress on this ultrastructural array. If, however, adhesion and spreading determines the fluid balance in the cells, it would depend on the osmotic situation set up by the entire interfacial region. This would depend on both the structural features of the substratum and on the capacity of the cells to accommodate mechanically. Thus, according to the osmotic hypothesis a cell behaves towards a surface, within the limitations imposed by its structure and deformability, in a way which minimizes its colloid osmotic pressure excess.

Two studies on adhesion can be cited as examples illustrating this point of view. O'Hara & Buck (1979) analyzed the movement of a fibroblast and epithelial cell on collagen surfaces which had been grooved so as to provide "contact guidance". Adhesion of these cells involves specialized regions of the cell surface having bundles of micro-filaments in the cortex which make exceptionally close or "focal" contacts with the substratum. Their analysis showed that the cells oriented readily on the grooves but also readily spanned the grooves, if these were not too far apart, so as to grow across the substratum in all directions. They concluded that the findings were most consistent with the hypothesis that cells move more or less in a direction parallel to the orientation of microfilaments but so as to maximize the number of focal contacts. This configuration would also maximize the amount of cell surface occluded at the interface and stabilization of the cells against internal hydrostatic pressure.

It has also been found that there is a critical relationship between the amount cells spread and their rate of growth (Cherny, Vasilieu & Gelfand, 1973; Maroudas, 1973; Folkman & Greenspan, 1975; Folkman & Moscona, 1978; Zetterberg & Auer, 1970). In the studies by Folkman and his colleagues, polymer hydrogels were cast on glass slides so as to provide a series of gel substrata varying in thickness from 0·0035 to 0·35 μm. Using several different cell lines they showed that the cells spread better the thinner the gels. For each cell type the cells had to spread to a certain critical degree before they could grow.

These findings are consistent with osmotic hypothesis on the role of adhesion since spreading would be correlated with the degree to which the colloid osmotic pressure was reduced and determine the point at which fluid balance could be attained. This would depend on the characteristics of the cell including the amount of protein, the complexity of its structure etc., since these would determine the control on movements of salts and water needed to maintain fluid balance. The fact that malignant or

transformed cells did not have to spread as much as their normal counterparts may be related to the fact that these cells are generally considered to be more hydrated and to have a more permissive physiology than normal cells.

The osmotic balance of cells adhering to gels would also be affected by the osmotic characteristics of the gels themselves. Differences in thickness of gels caste on glass slides would result in significant differences in surface rigidity, degree of cross-linking, and inter-gel free fluid pressure which would affect the movements of water in the interfacial region.

Limitations of Adhesion and Spreading as a Mechanism to Compensate for Colloid Pressure Excess

Adhesion and spreading as a way of achieving fluid balance entails many adverse changes in cells. It is available only to those cells which can survive great distention of their membranes and rearrangements of internal organelles. In addition, the limited diffusion at the substratum interface means that one-half of the cell is functioning in a micro-environment with fluctuating pH and anomalous concentration of ions. In view of the very small volumes involved these could result in osmotic and electrical effects which could play a role in many aspects of membrane dynamics. The cell *in vitro* is probably never—or only momentarily—in a state of fluid balance. Survival would depend on constant modulation of the osmotic mechanism and continuous dissipation of energy. These would not affect growth rates since the cells have been selected to grow under these conditions. However, they would limit the expression of many differentiated functions which depend on a higher level of physiological integration. Thus, the uncompensated colloid osmotic pressure emerging when cells are isolated not ony prevents prolonged survival of more highly differentiated cells in culture but provides a constant selection pressure against retention of specific markers in cells which have been adapted to culture.

A major problem in maintaining the differentiated state would be that the amount of protein which could be synthesized would be limited. According to the hypothesis this could not exceed that which could be accommodated osmotically by adhesion and spreading. The data already cited indicates that even for the highly selected cells which proliferate rapidly in culture, this is restricted. The synthesis of the much greater load of proteins, enzymes, and structural components characteristic of highly differentiated cells would be osmotically prohibited. Smooth muscle cells, for example, lose more than 70% of their content of myosin during the early phase of adaptation to culture during which they adhere and start spreading (Chalm-

ley-Campbell *et al.*, 1979). Although they can proliferate rapidly they never regain the capacity to synthesize normal levels of myosin, nor do they regain the capacity to contract when stimulated. It would appear that, in general, the proteins which can be maintained during growth *in vitro* are those required only for survival and proliferation. The fact that most cells taken from normal adult tissues do not adapt and cannot survive long in culture may indicate that even this relatively small fraction of proteins is more than can be accommodated osmotically by adhesion and spreading.

Maintenance of the Differentiated State—A Problem in Colloid Osmotic Balance

These considerations can account for the many findings indicating that expression of differentiated function in cells *in vitro* is critically dependent on the colloid osmotic properties of the conditions used for culture. In the light of the discussion here, these can be taken to include both the physical characteristics of the medium and the types and number of contacts the cells make with surfaces. Only a few of the very many observations made will be cited in support of the argument that both of these affect the expression of differentiated function as a result of altering fluid balance.

The most striking observations are those which show that the morphology of cells varies widely depending on the physical properties of their environment. Cells which grow as phase dark, flattened cells spread out on solid surfaces—and cannot grow except in this anchorage dependent configuration—grow as phase bright spindle shaped cells in plasma clots (Wiess & Garber, 1952; Elsdale & Bard, 1972). Weiss & Garber showed that this depended on the density of the clot. They pointed out the more normal morphology was favored by the denser clots where the water would be held by capillarity. This may account for the findings that cells grown on millipore filters where most of the water would also be held by capillarity were found to maintain a markedly improved state of differentiation compared to those grown on solid substrata (Savage & Bonney, 1978).

Collagen-coated surfaces have been found to enhance outgrowth in primary culture particularly of more differentiated types of cells. Reiske (1968) showed that using collagen surfaces not only stimulated growth but improved the morphological condition of the cells. In particular, the enlargement of nuclei generally characteristic of cells in culture was prevented and fine structural details maintained. More recently Godpodarowicz & Tauber (1980) have shown that the morphology of endothelial cells is determined by the substratum. They propose that the role of the substratum is "permissive" in that cells can respond to mitogens when adhering to a suitable

surface whereas these mitogens have no effect on cells adhering to less suitable surfaces. This would be consistent with our analysis of the osmotic role of adhesion since cells would have to be in fluid balance before they could respond to mitogens and grow. However, even on optimal surfaces many normal processes cannot take place. This is indicated by the studies of Leighton and his colleagues (Nicosia, Tehao & Leighton, 1982) where growth of endothelial cells was compared on solid surfaces and in plasma clots. Whereas cells grew as confluent monolayers with no tendency to aggregate or exhibit histotypic organization on a solid surface they grew as fenestrated structures in the plasma clots.

The stabilization of differentiated features which have been observed when media are supplemented with high molecular materials can also be analyzed as the result of better control on passive ion and water movements.

Patterson & Mains (1975) and Kaike & Pfeiffer (1979) found that adding 1% carboxymethyl cellulose to cultures of neo-natal nerve cells growing in a serum supplemented medium stimulated the production of neurites. They attributed this to an increase in viscosity. An alternative explanation is that this material with its high osmotic coefficient decreased the osmotic limitation on the amount of protein which could be synthesized and permitted expression of this differentiated function. This interpretation is supported by the finding (Hawrot, 1980) that when the cells were plated on a surface to which they adhered more tightly, they did not require the carboxymethyl cellulose in order to produce neurites. Studies with leukemic cells have shown that differentiation requires a higher concentration of protein in the medium than proliferation (Honna *et al.*, 1979). These cells could adhere and grow in a serum free medium but they required a supplement of bovine serum albumin in order to differentiate when challenged with an inducing agent.

Conclusion

Analysis of the osmotic changes involved when cells are isolated from tissues has led us to propose that the primary problem the cell faces *in vitro* is compensating for its colloid osmotic pressure. *In vivo* this is balanced to a considerable degree by the physico-chemical and morphological features of the tissue structure. The Na pump maintains volume control by virtue of low setting for leak rates. Isolation results in a breakdown of these constraints on movements of salts and water. The crux of the hypothesis is that the increased influx arising on isolation cannot be met by the cellular pumps. Survival of cells *in vitro* would depend either on providing physical conditions which curtail influx or on the cells adopting modes of behavior which compensate. Adhesion and spreading on a substratum has been

analyzed in terms of the way in which physical and ultrastructural changes would alter fluid movements. It is proposed that these changes act co-ordinately to enable the cell to achieve fluid balance in the face of its colloid pressure excess. However, the dependence of cells on adhesion to surfaces for fluid balance alters their normal structure and physiology. In particular, the amount of protein which could be synthesized would be restricted. This results in a loss of differentiated characteristics.

The de-differentiation which cells undergo in culture has frequently been ascribed to changes incurred when cells go from a three-dimensional to a two-dimensional state. The changes are usually unspecified. Grinnell (1983) however has suggested that they may be due to conformational effects on macromolecules required for growth. The analysis here suggests that the important difference is the control which a three-dimensional structure can provide on the movements of salts and water. Furthermore, this represents a physical requirement which must be provided for most cells in order to maintain a differentiated state *in vitro*.

REFERENCES

ARMSTRONG, W. W. McD. & KNOEBLE, S. B. (1966). *J. Cell Physiol.* **67**, 211.
BENECKE, B-J., BEN-ZE'EV, A. & PENMAN, S. (1978). *Cell* **14**, 931.
CARTER, S. B. (1967). *Nature* **210**, 256.
CASTOR, L. R. (1970). *J. Cell Physiol.* **75**, 57.
CHALMLEY-CAMPBELL, J., CAMPBELL, G. R. & ROSS, R. (1979). *Physiol. Rev.* **59**, 1.
CHAYEN, J. (1978). *Int. Rev. Cytol.* **58**, 333.
CHERNEY, A. B., VASILIEU, J. M. & GELFAND, M. (1975). *Exp. Cell Res.* **90**, 317.
CLEGG, J. S. (1979). In: *Cell Associated Water* (Drost-Hansen, A. & Clegg, J. S. eds.), p. 135. New York: Academic Press.
COMPER, W. D. & LAURENT, T. C. (1978). *Physiol. Rev.* **58**, 255.
CULP, L. A. (1983). In: *Biocompatible Polymers, Metals and Composites* (Schyzer, M. ed.). Westport: Technomic Publishing Co.
DIAMOND, J. M. (1966). *J. Physiol.* **183**, 58.
DIETSKY, J. M. (1978). In: *Microenvironments and Metabolic Compartmentation* (Srere, P. A. & Estabrook, R. W. eds.), p. 401. New York: Academic Press.
DIPASQUALE, A. (1975). *Exp. Cell Res.* **95**, 425.
DISALVIO, S. A. & DE GIER (1983). *J. Chem. Phys. Lipids* **32**, 39.
ELSDALE, T. & BARD, J. (1972). *J. Cell Biol.* **54**, 626.
FARQUAHR, M. G. & PALADE, G. (1966). *J. Cell Biol.* **30**, 359.
FOLKMAN, J. & GREENSPAN, H. J. (1975). *Biochim. Biophys. Acta* **417**, 211.
FOLKMAN, J. & MOSCONA, A. (1978). *Nature* **273**, 345.
GOLDMAN, R. D. & KNIPE, D. M. (1973). *Cold Spring Harbor Symp. Quant. Biol.* **37**, 523.
GOSPODAROWICZ, D. & TAUBER, J. P. (1980). *Endocrinol. Rev.* **1**, 201.
GRANGER, H. J. (1981). In: *Tissue Fluid Pressure and Compositions*, p. 41. Baltimore: Williams and Wilkins.
GRINNELL, F. (1978). *Int. Rev. Cytol.* **53**, 67.
GRINNELL, F. (1983). In: *Biocompatible Polymers, Metals and Composites* (Schyzer, M. ed.). Westport: Technomic Publishing Co.

HARRIS, A. (1973). In: *Locomotion of Tissue Cells, CIBA Foundation Symposium*, p. 10. Amsterdam: Elsevier.
HAWROT, E. (1980). *Dev. Biol.* **74,** 136.
HEATH, J. &. DUNN, G. A. (1978). *J. Cell. Sci.* **29,** 67.
HERZ, F. & SCHOUSBOE, A. (1975). *Int. Rev. Neurobiol.* **18,** 41.
HONNA, U., KASHUKABE, T., OKABE, J. & HOZUMI, M. (1979). *Exp. Cell Res.* **124,** 421.
KAIKE, T. & PFEIFFER, S. E. (1979). *Dev. Neurosc.* **2,** 177.
KLEINZELLER, A. (1972). In: *Metabolic Pathways VI Metabolic Transport* (Hokin, L. E. ed.), pp. 91-131. New York: Academic Press.
KREGENOW, F. M. (1973). *J. Gen. Physiol.* **101,** 59.
KREGENOW, F. M. (1981). *Ann. Rev. Physiol.* **43,** 493.
LEIGHTON, R., BRADER, Z., ESTES, C. W. & JUSTH, G. (1970). *Cancer* **26,** 1023.
MACKNIGHT, A. D. C. & LEAF, A. (1977). *Physiol. Rev.* **57,** 510.
MAROUDAS, N. G. (1973). *Exp. Cell Res.* **81,** 104.
MERCHANT, D. J., HELLMAN, K. B., SCHNEIDER, H. & MUIRHEAD, E. E. (1962). *Bact. Proc.* **62,** 141.
NICOSIA, R. F., TEHAO, R. & LEIGHTON, J. (1982). *In Vitro* **18,** 528.
O'HARA, P. T. & BUCK, R. C. (1979). *Exp. Cell Res.* **123,** 235.
PATTERSON, R. E. & MAINS, P. H. (1973). *J. Cell Biol.* **59,** 329.
POLLARD, T. D. (1976). *J. Supramol. Struct.* **5,** 317.
RAPPAPORT, C. (1960). *Exp. Cell Res.* **20,** 479.
REES, D. A., LLOYD, C. W. & THOM, D. (1977). *Nature* **267,** 124.
REISKE, E. (1968). *Z. Mikro. Anat. Forsch.* **79,** 485.
ROBINSON, J. M. (1975). In: *Pathology of Cell Membranes* (Arstila, N. & Trump. B. P. eds.), p. 173. New York: Academic Press.
ROTI ROTI & ROTHSTEIN, A. (1973). *Exp. Cell Res.* **79,** 295.
SATO, G. & REID, L. (1978). *Int. Rev. Biochem.* **20,** 219.
SAVAGE, C. R. & BONNEY, R. J. (1978). *Exp. Cell Res.* **114,** 307.
STOKER, M., O'NEILL, C. & WAXAMAN, V. (1968). *Int. J. Canc.* **3,** 683.
TODARO, G. & GREEN, H. (1964). *Proc. Soc. Biol. Med.* **116,** 688.
TOSTESON, D. C. (1964). In: *Cellular Functions of Membrane Transport* (Hoffman, J. F. ed.), p. 3. New York: Prentice-Hall Inc.
TRIBLE, H. R. & HAGUCHI, K. (1962). *Bact. Proc.* 141.
TURNER, G. S. (1962). *Nature* **193,** 164.
VAIL, J. M. & GLINOS, A. (1974). *J. Cell Physiol.* **83,** 425.
VASILIEV, J. M. & GELFAND, M. (1977). *Int. Rev. Cytol.* **50,** 159.
WEISS, L. (1969). *The Cell Periphery.* Amsterdam: North-Holland.
WEISS, P. & GARBER, B. (1952). *Proc. Natl. Acad. Sci. U.S.A.* **38,** 264.
WHITE, M. T. & NAND, S. (1977). *J. Cell Physiol.* **93,** 335.
WHITFIELD, J. G., BOYNTON, J. P., MACMANUS, R. H., RIXON, R. H. & KOSSKA, M. S. (1980). In: *Growth Regulation by Ion Fluxes* (Laffert, H. L. ed.), p. 216. Annals of the New York Academy of Sciences.
WHITTAM, R. (1964). In: *Cellular Functions of Membrane Transport* (Hoffman, J. F. ed.). New York: Prentice-Hall.
WILLMER, E. N. (1965). In: *Cells and Tissues in Culture.* Vol. I, pp. 144-173. New York: Academic Press.
WILLMER, E. N. (1962). *Exp. Cell Res.* Suppl. **8,** 3.
ZETTERBERG, A. & AUER, G. (1970). *Exp. Cell Res.* **62,** 282.