STUDIES ON ISOLATED NORMAL AND TUMOR MAMMARY CELLS

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CHAPTER I

INTRODUCTION

The basic unit of organization of all living organisms is the cell. Cells vary in their properties with some having a very simple internal organization while others have an extremely intricate organization.

Living organisms are made up of two distinct cell types. The procaryotic cell such as bacteria utilize the plasma membrane and the structures derived from it to perform a variety of functions without isolating these functions into compartments. The eucaryotic cell found in higher organisms has a highly organized system of cellular parts and organelles which allow units of the cell to have distinct functions.

A plasma membrane about 100 Å thick isolates the internal environment from the surrounding external environment in every cell. The plasma membrane does more than just provide a boundary. It is the site of many complex processes such as active transport, hormone binding, antigenic response, cellular communication, cellular recognition and cellular adhesion.

A multicellular organism exhibits a division of labor between cells. Cells are organized into tissues and tissues into organs. In an embryo cells by random or directed movement organize into tissues and organs in response to internal or external stimuli. Several factors may act together or independently in morphogenesis. Cell migration may be due to differences in cell-cell adhesions (1). Cells pass over cells where

adherence is lacking. A component may orient cells as they leave the neural crest. A directional component or a chemical gradient may give direction to the migration (2). Cells from which the heart forms migrate en masse toward the midline of the heart region following oriented paths formed, perhaps, by the underlying endoderm cells (3).

Other examples of directed cell movement are known. A chemical gradient of cAMP (4) attracts amoeboid cells of Dictyostelium discoideum and causes aggregation (5). Schwann cells guide axons across a wound (6) providing contact guidance (7) for the movement of axons in regenerating cut nerve connections.

Adhesiveness must play a part in all movements otherwise the cell could not push or pull forward on the underlying surface (8). Abercrombie (9) considered the leading edge of cytoplasm, the ruffled membrane, to be the locomotory organ of fibroblast motion. The ruffle forms by an upward folding of the flattened margin of the cell. When the leading edge of a cell collides with some part of another cell it stops, loses its ruffle and contracts. The contraction may drag the leading edge back, sometimes snapping it entirely free showing the firmness of the adhesion. If the adhesion is strong both cells may be drawn closer together without breaking the adhesion. This is called contact inhibition and may be considered the primary response of a cell in contact with another. Normally cell contact regulates movement and cell division (10). Epithelial cells act in sheets in the same fashion as do single cells (11).

Hlinke and Sanders (12) have devised a system to view both the real and reflected images of cells simultaneously. A pair of images separated by a gap shows that the cell is not attached to the substrate. Time-lapse films using this method do not support Abercrombie's concept of

cell locomotion. The films suggest that selective adhesion or non-adhesion followed by detachment and bridge formation may be more important to movement. The detached part of the cell appears to be under tension and to have elastic properties. The details of movement involve the nucleus with retraction, contraction and relaxation of the cytoplasm.

Comparison of normal, SV40 transformed, and revertant Balb/c 3T3 cells in electron micrographs show a felt-like mesh, often parallel rows of alpha filaments in the leading edge of normal and revertant cells but not in transformed cells. Alpha filaments are abundant near cell-to-cell attachments. β-Filaments and microtubules are also less in transformed cells. Surface replicas of normal cells show bundles of alpha filaments extended from the tips of microvilli back into the ectoplasm (13).

Heavy meromyosein produced spiked surfaces or "arrowhead complexes" on the α-filaments such as are found with F-actin. No binding appeared on the β-elements (13).

Interactions between nonembryonic cells have been found to be equally important. Adhesiveness is important in cellular immunology. Interaction of adherent and nonadherent cells has been described in graft-versus-host reactions, delayed hypersensitivity, antigen stimulated blast transformation of lymphocytes, mixed leucocyte culture and mitogenic response to phytohemagglutinin (14).

It has become clear that for every embryonic induction the cell and tissue interactions involved still exist in adult life. Cells grown under tissue culture conditions have shown that antibody formation in vitro requires reconstruction of tissue organization, cooperative interaction of different cell types and an intact structural equivalent of lymphoid follicles. If mechanisms exist for permanent inductive

interactions then it should be possible to utilize the mechanisms for repair or replacement (15). Understanding cellular adhesion is but one part of understanding cellular interactions.

CHAPTER II

LITERATURE REVIEW

The Cell Membrane and Its Surface

Composition of Plasma Membranes

Only the gross structure of the plasma membrane is known at present. There are three major classes of membrane components, proteins, lipids and oligosaccharides. Singer (16) has proposed that globular proteins and glycoproteins alternate with a phospholipid bilayer. The polar regions are associated with the aqueous phase and the nonpolar regions with no carbohydrate or ionic residues are embedded in the hydrophobic interior of the membrane.

Proteins can be peripheral or integral to the membrane. Peripheral proteins require only mild treatment such as an increase in ionic strength or presence of a chelating agent to dissociate them from the membrane. They are relatively soluble in neutral aqueous media and dissociate free of lipids. Membranes require more drastic treatment such as detergents or organic solvents to isolate the integral proteins. The integral proteins remain associated with lipids and if free of lipids remain insoluble in aqueous buffer (16).

Sodium dodecyl sulfate acrylamide gel electrophoresis patterns of erythrocyte membranes of eight mammalian species reveal more than twenty proteins which show extensive similarities among the different species.

When identical gels were stained with a periodic acid-Schiff procedure to reveal the glycoprotein patterns, marked differences were found from species to species (17). Differences in cell function may be correlated with differences in glycoprotein composition.

Basement membranes are characterized by the presence of hydroxylysine, hydroxyproline [3,4], proline and high amounts of glycine. They contain glucose and the uronic acids (18). The connective tissue polymers, the acidic glycosaminoglycans *in vivo* are bound to proteins. These are found to be 80% polysaccharide and 20% protein. The protein-carbohydrate linkage is a serine-xylose linkage. They are composed for the most part of glucose, galactose, and iduronic acid. Glucose and galactose are in the hexosamine or uronic acid form (19). Collagen precipitated from glomerulus lens capsule and Descemet's membrane have only glucose and galactose present (18).

Plasma membranes are similar in one respect to collagen and basement membrane in that glucose is present. The Hyl-Gal-Glc nucleus identified by Butler and Cunningham (20) is now recognized as a common structural feature of vertebrate collagens (21). Glucose also occurs in glycolipids as the glucosyl residue closest to the lipid moiety (22).

Table I shows some representative values of carbohydrates present in membranes of normal and transformed cells. Codington, *et al.* (24) found glucose present in TA_3 mammary carcinoma ascites cells of strain A mice. They also identified ribose by both gas-liquid chromatography and mass spectroscopy. Warren (25) reports that RNA might be an integral component of the membrane itself. Smith and Walborg (26) isolated sialoglycopeptides from the rat ascites hepatoma AS-30D before and after tumor progression. The progression was characterized by a morphological

TABLE I

CONTENT OF CARBOHYDRATES IN NORMAL AND VIRUS TRANSFORMED CELLS (μMOLE/MG PROTEIN X 10^{-4})[1]

Sugars	Chinese Hamster Cells			Chick Embryo Fibroblasts		BHK_{21}/C_{13}[2]		
		Transformed			Transformed		Transformed	
	Control	SV	Polyoma	Control	RSV	Control	C_{13}/B_4[3]	C_{13}/SR_7[4]
L-Fucose	24 ± 9	15 ± 9	10 ± 2	45	26	26	15	16
D-Mannose	196 ± 13	165 ± 28	184 ± 29	213	224	114	126	148
D-Galactose	221 ± 32	217 ± 3	198 ± 63	158	232	82	77	81
N-Acetyl-D-Glucosamine	---	---	---	327	358	121	181	191
N-Acetyl-D-Galactosamine	---	---	---	83	72	20	68	59
Sialic Acid	199 ± 47	121 ± 14	210 ± 42	244	326	67	68	69

[1]Hartman, J. F., Buck, C. A., Defendi, V., Glick, M. C., and Warren, L. J. of Cell Physiol., 80:159-166, 1972.

[2]Baby hamster kidney cells.

[3]Transformed by a Byron strain Rous Sarcoma.

[4]Transformed by a Schmidt-Ruppin strain Rous Sarcoma.

alteration and an increase in virulence. Composition of these sialopeptide fractions were similar except for a marked decrease in the amount of glucose as the tumor became more virulent. Shen and Ginsberg (22) compared the carbohydrates of glycopeptides from Hela cell membranes before and after the glycopeptides were removed from the membrane by trypsin treatment. Two such studies were made, one of Hela cells grown in suspension culture and the other of cells grown in monolayer culture. The amount of glucose present was given for cells grown in suspension culture. 19% of the glucose was removed by trypsin treatment. More total carbohydrate was found in cells in monolayer culture than in suspension culture and less could be removed by trypsin treatment. Table II gives the comparison of carbohydrates present in the membrane of Rat Ascites Hepatoma 7974H before and after lipid extraction of the membranes (27). Glucose is present in both cases.

Two cell lines which grow well in suspension culture, the L5178Y murine lymphoma cell line and the Erlich ascites tumor have a low level of surface sialic acid content, about 2.0×10^8 molecules/cell (28). Circulating normal human lymphocytes contain only 0.84×10^8 sialic acid molecules per cell. Warren, et al. (29) found increased sialic acid in glycoproteins from membranes of hamster, mouse, chick and human cell cultures after they were transformed by either DNA or RNA oncogenic viruses.

Contacts Between Membranes

When cells adhere special contacts can often be seen with the electron microscope (30). Membranes of adjacent cells are parallel with an interspace of 150 to 200 Å outside the special contact areas. The

TABLE II

CARBOHYDRATE COMPOSITION OF PLASMA MEMBRANE OF RAT ASCITES HEPATOMA AH7974[1]

	Carbohydrate (μmoles/mg protein)	
	Intact Membranes	Lipid-free Membranes
Fucose	33.6	16.1
Mannose	57.2	57.2
Galactose	97.8	43.2
Glucose	56.4	11.4
Galactosamine	52.2	19.9
Glucosamine	59.2	53.7
Sialic Acid	28.8	20.7

[1]Shimizu, S. and Funakoshi, I. Biochim. Biophys. Acta, 203:167-169, 1970.

junction between cells forms a terminal bar at the apex of the cell which is on the luminal border of epithelial cells. The tight junction or zonula occludens is at the tip of the cell. It circles the apex forming an unbroken attachment to adjacent cells. Within this space the outer membrane surfaces of the two contacting cells may fuse. It is thought to serve a permeability function since it offers an area of low electrical resistance allowing for increased permeability to ions. From this area the spread of surface excitation is promoted from cell to cell. It forms a linear seal between the lumen and the intercellular space.

Freeze-cleave replicas of the zonula occludens, tight junctions, show long branching and anastomosing chains of particles on one membrane face and chains and grooves on the opposite membrane face (31). Revel (31) stabilized the tight junctions with OsO_4 and tried acetone extractions to remove the material in both areas. 60% acetone was required before the material disappeared. Thin-layer chromatography showed the presence of three major phospholipids and several less important ones. Protein was found in all extracts, but no new major protein was present in the 60% extraction.

Gap junctions are a second continuous girdle found next to tight junctions (31). The membranes are not fused but are in a close cell-to-cell apposition and are separated by an interspace of about 20 Å. The inner surface of the membrane appears thickened and a dense network of fibrils extend from the membranes into the adjacent cytoplasm (30). In oblique sections through gap junctions containing lanthanum, a polygonal lattice of substructures is outlined by the heavy metal. The particles are packed irregularly into what appears to be an hexagonal lattice. The subunits show a center-to-center spacing of 90-100 Å.

There is a third type of cellular contact called the desmosome. The desmosome or macula adhaerons forms discrete contacts rather than a continuous contact. They may form with a particular pattern or orientation or in particular restricted regions (32). There is a 100-250 Å space between the adjacent cell membranes and this is filled to a variable extent with dense material. There is a thickening of the inner cell surface of the plasmalemma and attachment of cytoplasmic fibrils which frequently link desmosomes. Desmosome structure varies between different cell types and in different species. The cytoplasmic fibrils may represent a fibrillar framework which helps maintain cellular shape and rigidity in relation to its neighbors (30).

Electron microscopy shows that the first step in the process of forming cellular contacts is the formation of desmosomes. Initially the cell membranes are seen lying parallel to each other at a distance of about 100-250 Å. Then material is deposited in the cytoplasm adjacent to the region of contact. The deposit becomes very dense and is known as a desmosome. Successive layers of material are secreted between the cells. Once this happens cell contacts become extremely stable (33).

Mouse L cells in Eagle's MEM solution with 10% calf serum form tight junctions within about three minutes, or ten at the most, after making contact. Increased adhesiveness and contact inhibition may result from the formation of tight junctions between cells (34).

Membrane Surfaces

A continuous layer of moderately-dense material is on the outer surface of the plasma membrane of most cell types (35). The layer covering the outer surface of endothelial cells is usually called the basement

membrane (36). Fawcet (37,38) proposed that the layers which underlie epithelium and invest capillaries be called the basal lamina while the layers which surround the cells be called the external lamina.

Most cultured mammalian cells have surface cytoplasmic projections. Cornell (39) has described them from examination by the light microscope and the transmission electron microscope. They have been called micro-extensions or microspikes (40) or filopodia (41). Hodges and Muir (42) using the scanning electron microscope found a relatively smooth-textured nuclear region in interphase cells. A rough textured area surrounded the nucleus, overlying endoplasmic reticulum and the Golgi. The cytoplasmic region was relatively smooth. Cytoplasmic projections varied in size and distribution over the upper free surface and around the lateral edges. The distribution, density and length of the microprocesses depended on the conditions of culture. Bubble-like protrusions are characteristic of anaphase and early teleophase.

The interaction between cells has been considered due to charge density on cell surfaces. Specific groups have been stated as responsible for the charges: sialic acid, charged side chains of membrane proteins, membrane RNA and membrane lipids (43). No convincing evidence for electrostatic interactions has yet been given (44,45).

Blood group determinants were the first carbohydrates found on the cell surface. Later sialic acid was found (46) as well as immunologically active polysaccharides (47) and enzymes (48). Concanavalin A sites (49) wheat germ agglutinin sites (50) and other lectin sites have also been found. Many experiments have been done using proteolytic enzymes such as trypsin to cleave glycoproteins from the membrane surface. Slayter and Codington (51) using glycoproteins released from the membrane, showed by

metal contrast in electron micrographs that the glycoproteins were highly asymmetric rods.

When ^{32}P labeled lymphocytes were incubated with glycosidases the number of ^{32}P labeled cells accumulating in lymphoid tissue were reduced. The effect of the enzyme was specifically inhibited by L-fucose and N-acetyl-D-galactosamine (52). Booyse (53) localized thrombosthenin and Nachman (54) localized fibrinogen on the membrane surface of platelets by antigen-antibody reactions.

Phosphotungstic acid stains carbohydrates for electron microscopy. Basement membranes, goblet cell mucous, coats associated with the brush borders of the intestinal and kidney epithelia, the matrix of cartilage, glycogen in cartilage cells, the contents of the Golgi apparatus, intercellular material, the clefts of terminal bars, desmosomes, synapses, aortic elastin and the luminal surface of endothelial cells have been stained with phosphotungstic acid indicating that these structures contained carbohydrates (55).

Aggregates formed from cells from a four-day chick limb bud were stained with lanthanum to check for the presence of an intercellular substance. An oriented fibrillar material coated the cells and there was a 35 Å center-to-center spacing. The material was nearly 20 A in diameter (56).

There is no doubt that structures on the cell surfaces are important in cellular adhesion. Burger (57) has stated that the lack of agglutination in nontransformed cells may be due to a careful balance between anabolic and proteolytic (catabolic) processes. The transformed cell which agglutinates might have the balance shifted toward the catabolic state. Different cell types may have different mechanisms of recognition

and adhesion.

Cellular Aggregation

Morphogenetic Studies

Cellular aggregation is a normal process of development and differentiation. Tissue removal is followed by a dispersal of cells, aggregation of homologous cells and formation of specific interactions of cells to form tissues and organlike groupings of tissue.

Aggregation studies originated in studies of morphogenesis, with translocation of cells over long distances, invaginations of prechordal material and emigration of cells from the neural crest and early germinal areas. Studies have shown that there is often an increase in cell population density before histogenesis occurs. The cell density may increase by aggregation of cells, contraction of the whole cellular mass or by a local increase in cell division.

The germ cells migrate through blood vessels and are concentrated in the future gonadal regions. They accumulate in the small vessels leading from the dorsal aorta. They accumulate due to lack of passage or because of some subtle factor such as differential adhesiveness (58).

Neural crest cells incubated with tritiated thymidine were found to follow two well defined paths, one leading ventrally into the mesenchyme between the neural tube and the myotome to form spinal and sympathetic ganglia. The other led into the superficial ectoderm where the cells stopped moving and quickly accumulated in their expected destinations. The orientation and direction of the ventral movement followed the orientation of the neural tube (59).

Trinkhaus (60) found an increased adhesiveness during the transition

of the Fundulus blastula to gastrula. Adhesiveness was shown by an increased flattening of blastomeres. This was associated with a newly acquired capacity to move. Ectoderm of an amphibian gastrula adheres readily to both mesoderm and endoderm, but after a few days less readily to endoderm (61).

The importance of the underlying substrate is emphasized by the role it plays in contact guidance. Contact guidance explains certain movements. No indisputable evidence for its existence has been shown. Pigment cells migrate along blood vessels, myotome boundaries and along the remains of degenerating nerve fibers. Dendritic extensions of melanocytes orient along rows of barbule cells in a feather germ. Other examples include the posterial movement of the lateral line organ and the Wolfian duct, the oriented movement of clusters of heart-forming cells on an endodermal substrate, and the ventral movement of neural crest cells along the neural tube (62).

Chemotaxis, responsible for aggregation in collective amoebae, may also be a mechanism. It is well known that leukocytes and lymphocytes are chemotactically attracted to a focus of infection. Thyrotropin has been shown to cause aggregation of thyroid cells (63).

Modern investigations of tissue and organ reconstitution began with the formation of pronephris systems from dissociated prospective pronephric cells (64). When Moscona (65) obtained characteristic tissue from mesonephric or chondrogenic cells of four-day chick embryos reconstitution of tissues and organs appeared to be biologically significant and vertebrate embryos became better material for study than sponges.

When embryonic cells aggregated, Holtfreter and Townes (66) found that ectodermal cells accumulated at the outer surface and endodermal

cells in the interior, or at the surface, depending on the cells present. In all cases mesoderm cells moved to the interior. Moscana (67) found that cells of one organ sorted out from those of another. He also reported for five combinations of tissue cells of different species that no species specific sorting out of cells occurred (67-70). Burdick (71) showed that there was also a species specific sorting out of cells in mixed cell aggregates of limb mesoblast cells of chick and mouse embryos. The mouse cells sorted out from the chick cells after 21 to 33 hours in culture.

Steinberg (72-74) developed an hypothesis for cell sorting in studies of this kind. He assumed that sorting out results entirely from random mobility and quantitative differences in the general adhesiveness of cells. He treated sorting out as a thermodynamic equilibrium. In order to achieve equilibrium there must be maximal adhesion of cell surfaces. If these conditions apply and the cells are motile, they tend to exchange weaker for stronger adhesions. The distribution of the tissues reflect the strengths with which the differing cells adhere to partners of like and unlike cells.

This hypothesis was attractive because it explained adhesion of both like and unlike cells and also explained the origin and maintenance of organ structure. The cohesion of like cells forms the individual tissues, but the adhesion of unlike cells binds different tissues together to form organs.

Originally Weiss (75) proposed that selective adhesion might depend on complementary macromolecules on the opposed surfaces of like cells. Townes and Holtfreter (76) argued that movements of cells relative to one another in a cohesive mass do not confirm that the bonds involved in

adhesion are of an irreversible type.

Steinberg's selective adhesiveness depended on differential adhesiveness. The theory relied on the differences in quantity, kind and pattern of linkages at the cell surface. If the cells are alike the adhesive sites are equivalent and differences in adhesion must be due to differences in the quantity of randomly distributed linkage sites. In reality, cells recognize like and non-like cells. The ability of a cell to interact with other cells in varying degrees ultimately lies in the differences in the bonds linking cells (77).

Jones (77) suggested that linkage sites may move between two positions, one favorable and the other unfavorable to adhesion. The oscillation of the cell between these two positions might have a frequency characteristic of that cell. Differential adhesiveness might be due to differences of the oscillations of the equivalent linkage sites in the cell and between cells. Constractile proteins of the actomyosin type at the cell surface might be directly or indirectly involved in the movement of the equivalent linkage sites. A site would be unavailable in the contracted state and available in the relaxed state.

Studies of Cellular Adhesion

The many qualitative experiments exploring changes in mutual adhesiveness of cells emphasizes the importance of the cell surface. There have been three approaches to the study of cellular adhesion: a study of cellular adhesion to glass or plastic surface (78-80); a study of cellular adhesion to serum or fatty acid monolayers on glass slides or at a liquid, liquid interface (81-83); and a study of intercellular adhesion which is adhesion between cells (84-89).

Cellular Adhesion to Glass or Plastic Surfaces

Adhesion to a solid substrate is considered due to a reduction of the electrostatic potential between the cell and the substrate as the cell touches the substrate. After the first contact is made the cells spread and become more firmly attached due to the growing mutual interfacial area between the cells and the substrate (90). This is then followed by the extension of small cellular processes.

Bangham and Pethica (91) suggested that the potential energy barrier preventing cell contact can be reduced by reducing the radius of curvature of mutually approaching cell processes. Physical adhesion may be considered a function of wetting between surfaces. Wettability is a measure of molecular contact between water and a surface. The contact angle of 0° indicates total wetting and 180° total non-wetting. The order of wettabilities of surfaces is the same whether the drop is water, oil, a cell surface or a microexudate. The adhesive bond involves short range forces and molecular contacts of 5-10 Å. Grinnell and Srere (92) found that better cell attachment occurs on surfaces of high wettability since the cells make closer or more molecular contacts. Cells are observed to adhere to the substrate by processes on the cell surface facing the substrate (12). A surface of high wettability offers a lowered radius of curvature and a reduction of the potential energy barrier which increases the adhesiveness of cells to the substrate.

Weiss, et al. (93) found that trypsin treatment of tumor cells retarded their attachment to coverslips. Kolodny (94) found that initial adhesion and spreading of trypsinized 3T3 cells were not inhibited by lack of DNA synthesis, RNA synthesis or protein synthesis. Detachment occurred after inhibition of DNA and RNA synthesis for 16 hours and

protein synthesis for 6 hours. Inhibition of glycoprotein synthesis had no effect on adhesion. Replating of trypsinized cells in media containing neuraminidase did not alter the time for adhesion and spreading of 3T3 cells.

Weiss and Chang (95) found that attachment of Ehrlich ascites tumor cells to glass and plastic was increased by incubation with cyclohexamide, puromycin or actinomycin D. This could be interpreted as inhibition of synthesis of some molecule that interfered with adhesion of cells. Grinnell and Srere (96) showed that inhibitors of transport, respiration and glycolysis had no significant effect on rat hepatoma cell adhesion to a solid support. They found that c-AMP and theophylline decreased detachability of transformed baby hamster kidney cells, but did not increase the adhesion of either normal or transformed kidney cells. Johnson and Pastan (97) found that dibutyryl c-AMP, theophylline and PGE_1 increased the adhesiveness of L-929 cells and SV40-transformed 3T3 cells. They also found that addition of serum lowers intracellular levels of c-AMP and decreases the adherence of cells to the substratum. Maderspach and Farkas (98) showed that catacholamines increased adenyl cyclase activity, the concentration of c-AMP, and decreased cell motility in L-929 cells. This action was blocked by a β-blocking drug.

Grinnell and Srere (92) showed that animal cells adhered best to high energy surfaces, surfaces of high wettability. Takuchi and Okada (99) showed that when cells adhered to glass in serum free media, no divalent ions were needed. As pH increased, cell adhesiveness increased.

Only reduced temperature and colchichine had any real effect on limiting adhesiveness. Colchichine (5×10^{-7} M) significantly inhibited cellular adhesion and spreading. Both reduced temperature and

colchichine are known to interfere with mitosis. This is probably due to interference with the action of microtubules. Microtubules may be important in cell movements (94).

Grinnell and Srere (96) showed that various types of sulfhydryl reagents blocked adhesion of rat hepatoma cells as well as normal and transformed baby hamster kidney cells to varied substrates with high surface energy. Adhesion would appear to be mediated by surface components containing SH groups. Since inhibition of cell respiration, glycolysis and transport had no effect on adhesion it was concluded that the SH groups must be on some cell surface component involved in the adhesion process.

Adhesion to Monolayers

Studies of adhesion to monolayers resulted from the simple observation that cells adhere better to a hydrophilic glass surface than to a hydrophobic siliconized surface. The presence of an undefined cell surface and an undefined substratum made the study of adhesion difficult. Taylor (100) attempted to define a hydrophilic and hydrophobic surface by measuring the contact angle the substratum made with water. However, he reported that the percentage of cells adhering had no correlation to whether the surfaces were hydrophilic or hydrophobic. Grinnell and Srere (96) when they repeated this work did show that a positive correlation existed. Normal and transformed baby hamster cells and rat hepatoma cells were more adherent to the more hydrophilic surfaces.

Substrates have been coated with serum (101) or chemicals such as fatty acids (102,103) or metal (104). Takeichi and Okada (99) showed that cell attachment of chick embryo fibroblasts was greatest to a

protamine coated plastic surface, than to the plastic surface itself. Attachment decreased in the order of protamine, plastic, bovine serum albumin, γ-globulin, gelatin and fetal calf serum surface.

Cell attachment to plastic was independent of pH. With bovine serum albumin greater adhesion occurred at a lower pH. The attachment of cells to albumin, γ-globulin, gelatin or fetal calf serum coated plastic required either Ca^{2+} or Mg^{2+}. Mg^{2+} was more effective. In comparing cell-to-cell adhesion with Mg^{2+} and Ca^{2+}, Ca^{2+} was more effective.

Rosenberg (105) found that layers of molecules on a substrate were influenced by inhomogeneities, or crystalline type imperfections of the underlying substrate. To study the effect of a monolayer he studied adhesion at a liquid/liquid interface. The lower phase was a slightly positive layer of a perfluoramine $(C_4F_9)_3N$ or a neutral mixture of C_8F_{18}-$C_8F_{16}O$. The upper phase was the nutrient phase. The attachment and spreading of Hela and human conjunctiva cells differed for the two surfaces with the cells adhering and spreading rapidly on the slightly positive perfluoranine layer. The cells remained spherical, attached, spread and proliferated at a lower rate on the neutral surface.

Interest is turning to cellular microexudates which are deposited on the substrate. Rosenberg (106) reported that a material exuded from cells and deposited on glass prevented cellular release by trypsinization. Weiss (107) showed that cells were more adhesive to the surface from which cells had been mechanically removed. Maslow and Weiss (108) have recently used the release of ^{51}Cr from labeled cells as an index of cell exudation. The release of labeled material from cells closely paralleled the number of cells adhering to the substrate.

Intercellular Adhesion

Aggregation may be studied in self aggregating systems of stationary cultures (109), by contact in rotating systems (110) or by gyratory shakers (111). Several studies used pellets formed by centrifuging cellular suspensions (112,113).

Cellular aggregation has been studied in many systems. Roux (114) reaggregated amphibian and avian embryonic cells, Wilson (115) sponge cells, Moscona (65) amphibian and avian embryonic cells, Hadorn (116) embryonic insect cells, Scott (117) embryonic protochordate cells, Guidice (118) embryonic echinoderm cells, Ansevin (119) adult Rana Pipiens liver, Halpern, et al. (120) and Dodson (121) normal human and human tumor cell lines.

Cellular aggregation has been used in a great variety of studies. It has been used to study reaggregation of embryos from the 8-cell stage to late blastocyst and their reimplantation (122), aggregation of dissociated cells of different parts of the brain (123), enhancement of brain aggregation with conditional media (124), histogenesis of fetal mouse isocortex and hypocampus (125), aggregation of the optic lobe of chick embryos (126), bioelectric discharges from reaggregated rat brain (127), and formation of neuromuscular junctions in embryonic cell cultures (128).

Cellular aggregation has been used to study the synapse between dissociated nerve and muscle cells (129), communication between cells (130), increased repair capacity of cells grown in contact, to sublethal radiation damage (131), reaggregation of embryonic heart myoblasts, to study the assembly of myofibrils (132), incorporation of muscle cells into a common multinucleated muscle unit (133), reconstruction of skin

from single cells and integumental differentiation in cell aggregates (134), melanin pigmentation resulting from the interaction of melanocytes and keratinocytes (135), and reaggregation of isolated thyroid cells with thyrotropin (136).

Aggregation of dissociated cells passes through three phases. There is an initial random clumping of mixed cells where cells move in relation to each other and cohesive forces are weak. After 24-36 hours there is a secondary aggregation where cells have moved to homologous regions of functionally matching cells. Cell motility is reduced, structural stabilization sets in and rapid cell multiplication begins. In tertiary aggregation growth and differentiation proceed leading to higher stages of development (137).

If cellular and histodifferentiation proceed beyond the stage of the cells at the moment of dissociation induction has occurred. This reflects the interplay between the cells themselves and between the cells and their environment. Intercellular materials are produced by the cells in their aggregated state. These materials or pseudopodial extensions may be responsible for cell contact. Usually cells from the same tissue in the same functional and developmental state aggregate. Depending on the system cellular aggregation is dependent on the culture media, pH, temperature protein and RNA synthesis (137).

A Model for Intercellular Aggregation

The General Model

Wilson (109) did the first reaggregation experiment with dissociated sponge cells in 1907. Spiegel (138) verified that antigen-antibody-like combinations gave specific cell adhesion as proposed by Tyler (139) and

Weiss (75). He prepared antisera against two species of sponges. The aggregation was species specific. Weiss (140) has recently reviewed aggregation phenomenon but Heinmetz (137) has aptly summarized the common concepts believed to be involved in aggregation.

He visualized "extensor-enzymes" on micro-filaments projecting out from the membrane. Metabolites, degradation products, monomeric or polymeric, mucoidal filament type materials, "extensor-substrates", lie in the peripheral cellular regions. As two cells come into contact the "extensor-enzymes" of one cell act on the "extensor-substrates" of the other cell. He calls this process of cellular sensing by the exoenzyme system the first phase of recognition.

Basic recognition arises from the enzyme systems linked to the sensing enzymes. Enzyme reaction products are transported into the cell. These may be inducers or repressors. They interact in certain patterns leading to information exchange. The metabolic decoding leads to cell recognition or rejection.

The matrix responsible for intercellular adhesion is in a state of dynamic equilibrium. Structural elements are synthesized and degraded continuously providing a constant turnover of material. The metabolic activity is controlled at the genetic level by means of external stimuli (137). There are four systems of aggregation that fit at least partially into this theory of aggregation: aggregation of Dictyostelium discoideum, aggregation of platelets, induction of cellular immunity, and aggregation mediated by reaction of glycosyltransferases on complex carbohydrate substrates.

Experimental Models of Aggregation

Dictyostelium discoideum. There must be some basic aspects of differentiation common to all organisms despite the differing processes of forming multicellular organisms. The common biochemical aspects of life, DNA, gene replication and protein synthesis support this view. One of the simplest systems of differentiation is that of the cellular slime mold, the collective amoebae.

In *Dictyostelium discoideum* (141) aggregation begins when the food supply is gone. Grouped cells, foci, appear within the cell monolayer. These are aggregation centers for the surrounding cells. The founder cells release a chemotactic agent in the maturated state. This guides cells to the founder cell. The center now becomes multilayered due, not only to the chemotactic response, but also to a change in the surface properties of the cells themselves. The change allows cells to move over one another. Contact inhibition is no longer active in this center. After about five hours the center spontaneously dissolves. When leaving the center, the cells, tightly bound to each other, form streams of cells. The streams may flow to other centers or may contract to form rounded cell groups which continue to develop into sporophores.

The biology and the biochemistry of differentiation go hand in hand (142). In *Dictyostelium discoideum* aggregation was mediated by a chemotactic agent named acrasin (143). Acrasin was found to be destroyed by an extracellular enzyme acrasinase (144). Konijn (145) showed that adenosine-3',5' cyclic phosphate attracted amoebae. Bonner (5) collected substantial amounts of c-AMP from *Dictyostelium discoideum* as well as a phosphodiesterase that specifically converted c-3',5'AMP to 5'AMP. Sonneborn, *et al*. (146) showed that a new antigen appeared on the cell

surface at the aggregation stage. Beug and Gerisch, et al. (147) blocked adhesiveness with a univalent antibody prepared from antisera of aggregation cells. The loss of aggregation did not interfere with their ability to move in response to a chemical gradient.

Aggregation of Blood Platelets. Platelets have three fundamental functions (148): adhesion, aggregation, and production of clot retraction. Adhesion of platelets is distinct from aggregation (149). Exposure to subendothelial connective tissue initiates adhesion of platelets to endothelium and aggregation between platelets (150).

There are two phases of platelet aggregation (151). It is believed the initial phase is controlled by α-adrenergic receptors while the second phase of disaggregation and subsequent aggregation is induced by β-adrenergic receptors.

Helem (152) found a factor in red blood cells that caused platelet clumping. Gaarder (153) demonstrated that ADP was probably factor R. Chandler and Hutson (154) showed that thrombin induced aggregation was caused by ADP. ADP, epinephrine and thrombin cause both phases of platelet aggregation.

Collagen also stimulates aggregation but it stimulates the second phase of disaggregation and reaggregation. Release, or second phase aggregation was found to be connected with ATP-hypoxanthine conversion (155). Hydroxylamine known to inhibit ATPase did inhibit platelet aggregation (156).

Adenyl cyclase is found only in the plasma membranes, not in storage organelles nor their membranes. c-AMP, mono-cyclic phosphonucleotides and 5-hydroxytryptamine are stored in subcellular organelles (157). [^{14}c]ADP was shown to bind to specific receptors on the platelet membrane

(158) and aggregation response was proportional to the bound ADP present. Prostaglandin E_1 inhibited both aggregation and binding of [^{14}c]ADP on the membrane. It also reversed ADP aggregation when added after ADP. [^{14}c]ADP binding also decreased.

PGE_2 synthesis is known to be inhibited by aspirin. Second phase or collagen initiated aggregation is inhibited by aspirin (159). Carageenin which stimulates prostaglandin formation also stimulates aggregation. Smith and Willis (160) showed that thrombin caused formation and release of PGE_2 and $PGE_{2\alpha}$ in platelets. This could also be blocked by aspirin. Shio and Ramwell (161) proposed that PGE_1 and PGE_2 may have a regulatory effect on aggregation in platelets.

Boullin, et al. (158) showed that the first effect of ADP on platelets was a rapid shape change. The disc shaped platelet was transformed into a "spiny sphere". Cell contact and shape change were not enough to induce the release action. A divalent cation and a plasma cofactor were also needed. This description of events was confirmed by electron microscopy (162).

The nonactivated platelets have a smooth flat surface with a well defined edge. About 1-2 sec after activation, the edge seems to disappear or relax and becomes amorphous or fluffy. At 3-4 sec the platelets interact and the amorphous material interacts to form well-defined interplatelet bonds. At 4-6 sec the bonds appear to contract and fusion of platelets begins. Booyse (162) has done immunohistochemical staining of distinct interplatelet bonds to show that thrombosthenin, a contractile protein is present. Nachman (163) has isolated thrombosthenin from platelet membranes. Inhibition of ATPase activity inhibits the platelet retraction step (157).

Booyse (162) suggests that thrombosthenin is extruded from the cytoplasm by action of ADP on the membrane. Rapid mixing of the proteins of two cells takes place. In a medium of low ionic strength, high Ca^{2+} and pH 6.8-7.4 rapid polymerization and gel formation of the contractile proteins occur. Contraction follows.

Aggregation in Cell Mediated Immunity. It is now understood that delayed hypersensitivity, tissue and organ allograft rejection, destruction of antigenic tumor cells and graft-versus-host reactions are cell-mediated (164). Two types of cell-mediated target cell lysis mechanisms are known. Thymus derived lymphocytes with no detectable amount of immunoglobulin may interact directly with target cells causing lysis. Lymphoid cells may interact with target cells in the presence of antibody to give specific target cell lysis. It is most likely that the antibody assisted cell causing target cell lysis is the macrophage.

Stem cells usually found in bone marrow are the source of circulating lymphocytes called B-lymphocytes (165). This cell is somehow activated to make a limited number of antibodies, probably just one. The thymus lymphoid cells are dependent upon marrow cells for development (166) and are even thought to be descendents of marrow stem cells.

The interaction of two or more cell types appears to be required to induce the primary immune response (167). Clusters of lymphoid cells are seen, both in vivo and in vitro, during the immune response. Most, probably all, antibody cells in vitro come from cell clusters. The interaction of cells in a cluster appears to be specific and also to be mediated by antigen and/or antibody on the surface of the interacting cells. Continued division of antibody cells during the early primary response requires that these clusters be present.

Claman and Mosier proposed (14) that an adherent cell, which may or may not be a macrophage cell, holds antigen on its surface. It functions as a meeting point for thymus derived cells and the bone marrow derived lymphoid cells. There appear to be more thymus-derived cells than bone marrow cells around a macrophage. After initial interaction of thymus cells with antigen on the surface of the antigen-holding cell, thymus cells remain attached or close by while bone marrow cells come and go from the cluster.

Two different kinds of cells appear to be required for the antibody response to some antigens (14). The thymus-derived cells react best with large antigenic carriers, but synthesize little or no exportable antibody. Marrow-derived cells react with haptens (after previous exposure by means of thymus derived cells). They synthesize large amounts of anti-hapten antibody for export. Both thymus derived cells and marrow-derived cells undergo mitosis when exposed to an antigen.

The complete model proposes that antigen carrier hapten is held on the surface of the macrophage. The thymus cell specific for the carrier meets the carrier on the surface of the adherent cell, divides and synthesizes more receptors for carrier. The activated thymus cell may or may not detach itself from the macrophage. The activated thymus cell reacts with more carrier. The antigen acts as a bridge between the thymus cell and the marrow derived lymphocytes. The marrow cells are specific for hapten, are activated, divide, and synthesize more receptors; they also manufacture and secrete anti-hapten antibody. Since marrow cells come and go, it is proposed that this allows for high levels of marrow cell activation and secretion of antibody (14).

It is not known if cell contact, processing of antigens by

macrophages, antigen, antigen-RNA or messenger RNA from a macrophage activates the lymphocytes to make antibody.

The Glycosyltransferase-Complex Carbohydrate Substrate Mechanism of Aggregation. A variety of experiments have suggested the involvement of complex carbohydrates in the mechanism of aggregation. MacLennan (168) aggregated sponge cells with an antisera against the cells. A glycoprotein extracted from the cells neutralized the antisera. Experiments of the Moscona group have repeated and verified these results. They have also pioneered studies on aggregation of embryonic cells (169,170).

Moscona (70) showed that periodate inhibited the reaggregation of embryonic chick cells to some extent. Garber (171) reported that glucosamine inhibited aggregation of chick embryonic neural retina cells.

Ginsburg (52) incubated ^{32}P labeled thoracic duct lymphocytes with mixed glycosidoses and found that lymphocyte accumulation was decreased in the spleen and in the lymph nodes while the activity was increased in the liver. Both L-fucose and N-acetyl-D-galactosamine increased the number of lymphocytes in the spleen and lymph nodes.

Orr and Roseman (172) showed that there was an active factor in horse serum, similar to a macroglobulin which stimulated aggregation of chick neural retina cells. Pessac and Defendi (173) isolated aggregation factors from monolayer cultures of ten cell lines, cells isolated from tissue, transformed cells or cells from established cell lines. They also isolated aggregation factors from four sera: fetal calf, horse, calf and chicken serum. Treatment of the aggregation factors with periodate for 24 hours at 4° C destroyed the aggregation activity. Bovine hyaluronidase treatment also destroyed aggregation activity.

Further involvement of complex carbohydrates in intercellular

adhesion was supported by the work of Oppenheimer (174). He showed that L-glutamine was required for the synthesis of carbohydrates necessary for adhesion of mouse ascites teratoma cells. He subsequently showed that L-glutamine incorporation was required for adhesion of three mouse ascites tumor strains and for chick neural retina cells (175).

With this type of experimental evidence Roseman (176) proposed that the mechanism of cellular adhesion was the formation of a substrate-enzyme complex. He proposed that the elements of the complex were complementary molecules on opposing cell membranes, complex carbohydrate substrates on one cell membrane and glycosyltransferase enzymes on the other cell membrane.

In support of this theory Roth (177) provided evidence to suggest that β-D-galactopyranosyl groups were involved in specific adhesion of neural retina cells. He also showed that chick embryonic neural retina cells catalyzed the transfer of galactose-^{14}C from UDP-galactose to both endogenous and exogenous acceptors (88). Roth (89) also provided evidence for the presence of galactosyltransferases and galactosyl acceptors on Balb/c 3T3 cells and Balb/c 3T 12 cells. Daday (178) found that cellular aggregation of chick embryo retina cells required a period of 24 hours. His results indicated that two phases of the aggregation process took place. The first phase, lasting four hours, was independent of protein synthesis while the second phase required a biosynthetic process such as protein synthesis. Fujisawa (179) found no distinct structural differentiation in chick retina cells after 36 hours. He found six to seven days were required for development of overall histological features of the normal retina.

Assays for Cellular Adhesion

Since the mechanism of cellular adhesion is unknown, direct measurement of adhesion is not possible. Some operational criterion must be chosen which is measurable (180). The criterion should be some meaningful indicator of adhesion or some consequences of the adhesion process. The criterion is defined by some operation which must be open to reliable and unequivocal measurement.

Moscona's group used the diameter of the aggregate as a measure of adhesiveness (124). The greater the size the more adhesive were the particles. Cell types forming very small aggregates were classed as non-adhesive. Curtis (181) believed that the important distinction was between single cells and adhesive cells. By using a hemocytometer to follow the disappearance of single cells, the number of single cells left at timed intervals was a good estimate of adhesion. Steinberg and Granger (182) filtered aggregates on a millipore filter at timed intervals and counted the number of each cluster size present. Roth and Weston (180) collected labeled cells on formed aggregates. The labeled cells were counted by a radioautographic method. Roth (177) modified the previous method and counted the labeled cells by liquid scintillation. Kemp (183) used a turbidometric assay. The suspension was stirred at a constant rate. Initially as small aggregates formed the turbidity as reflected by absorption at 600 mμ seemed to rise. Later as larger aggregates formed absorption decreased with time and was used as a measure of aggregation. Orr and Rosman (87) counted disappearance of single cells similarly to Curtis, but used a Coulter counter instead of a hemocytometer. Table III compares different assays for aggregation.

Roseman's group measures just the initial adhesion process which is

TABLE III

COMPARISON OF ASSAYS FOR AGGREGATION

Cell Type	Dissociation Technique	Cell Conc. Per ml	Volume Used	Rotation Speed	Assay Method	Timing of Assays	Total Time	Test Reagent	Reference
Chick Embryo Muscle Fibroblast	1% trypsin 10-15 min.	10×10^6	2 ml	Magnetic Stirrer	Turbidometric 600 mμ	10 min	5 hrs	p-benzoquinone	184
Chick Embryo Muscle Fibroblast	(a) 0.25% trypsin (b) 0.0002 M EDTA Each 10 min.	7×10^6	2 ml	Magnetic Stirrer 450 rev/min	Turbidometric 600 mμ	30 min	4 hrs	puromycin	190
Chick Embryo Muscle Fibroblast	0.25% crude 0.1% trypsin crystalline 10 min.	7×10^6	2 ml	Magnetic Stirrer 450 rev/min	Turbidometric 600 mμ	30 min	4 hrs	neuraminidase	183
Chick Embryo Neural Retina	0.25% trypsin 30 min.	1×10^5	3 ml	70 rpm Gyratory	Coulter Counter	5 min	45 min	Cell density pH, temperature, Ca^{2+}, Mg^{2+}	172
Chick Embryo Neural Retina and Liver	1% trypsin 30 min.	1.5×10^5	3 ml	70 rpm Gyratory	Number and size of aggregate (diameter)	4 hrs 20 hrs	20 hrs	Glucosamine	171
Sponge Cells	Pressed through fine silk cloth	1.5×10^6	3 ml	80 rpm Gyratory	Number and size of aggregate (diameter)	30 min.	4 hrs	Binding Factor	169
Embryonic Brain Chick and Mouse	1% trypsin 30 min.	1.5×10^6	3 ml	70 rpm Gyratory	Number and size of aggregate (diameter)	Every 24 hrs	20 days	(a) none (b) conditicned media	123
Chick Embryo Neural Retina	0.1% trypsin 0.1% collagenase 10 min.	2.2×10^6	4 ml	75 rpm Gyratory	No. of cells collected per aggregate	10 min.	70 min	ozide cyanide dinotrophenol fluoride ion puromycin cycloheximide proteases glycosidases	177
Chick Embryo Neural Retina	0.1% trypsin 0.1% collagenase 10 min.	2.2×10^6	4 ml	75 rpm Gyratory	No. of cells collected per aggregate	1st hour every 15 min then every hr	2-3 hrs	galactosyl acceptors	88
Balb/c 3T3 Balb/c 3T12	----------	2.2×10^6	4 ml	75 rpm Gyratory	----------	----------	-------	UDP-galactose	178
Chick Embryo Neural Retina	0.25% trypsin 10 min.	1.5×10^6	3 ml	72 rpm Gyratory	(a) % cells aggregated (b) mean diameter	(a) 4 hrs (b) 24 hrs	every hr	(a) intercellular retinal protein (b) cyloheximide (c) dinitrophenol (d) ATP, ADP	179

the earliest detectable process of adhesion (185). Orr (87) found that DNase, ATP, ADP, 2',3' AMP and metabolic inhibitors had no effect on this early process of adhesion. Lowering the temperature did slow down adhesion. Roth (177) found that azide, cyanide, dinitrophenol and low temperature did affect aggregation of neural retinal cells. Fluoride ion, puromycin, cycloheximide, and proteases had little or no effect on aggregation. Glycosidases increased aggregation, particularly β-galactosidase which increased aggregation approximately five-fold. Daday (179) found two phases in neural retina cell aggregation. The first four hours was independent of protein synthesis whereas the second phase which was after the first four hour period depended on protein synthesis. Cycloheximide and dinitrophenol inhibited this phase. Daday (179) trypsinized the cells for increasing time periods short of causing cellular death and found that increasing tyrpsinization inhibited the first phase of aggregation.

Disaggregation of Tissues

Normal tissues and cells are desirable for biochemical studies. Cell lines established from a single cell can vary in morphology, rate of growth, metabolism and in nutritional requirements when cultivated *in vitro* for long periods of time. Cell strains can also undergo spontaneous malignant transformation. Freshly isolated cells have the disadvantage of not being pure populations, but this can sometimes be overcome by differential flotation or centrifugation. Freshly isolated cells may lose some of their extracellular coat, but in many cases, it has been shown that this can be resynthesized (186).

Intercellular materials differ in different tissues and may consist

of mucopolysaccharides, fibrous protein and inorganic salts as in bone (187). Different tissues require different disaggregation techniques. Embryonic tissue, adult and neoplastic tissue with little matrix can often be disaggregated by physical disruption, chelating agents or light enzymatic treatment. Most intercellular materials have been found to be glycoproteins (187). Usually a mixture of proteolytic enzymes and the enzyme hyaluronidase are used. The enzymes that are used either (1) cleave the linkage between the polysaccharide and the protein, (2) hydrolyze peptide bonds, or (3) depolymerize the carbohydrate. The method chosen is one which usually preserves the cell membrane and retains the known cellular functions.

Mechanical methods, mincing, homogenizing, and tissue presses give less than 10% of the tissue as single cells in the final suspension. Jacobs and Bhargava (188) physically disrupted adult rat liver. The liver was first perfused and by gradually increasing the pressure during perfusion, the liver became fully distended. It was excised, cut into small pieces and homogenized. The yield of viable cells was reported to be close to 80%. It is reported that embryonic tissue (189) and tissue from newborn and postnatal mice up to nine days can be dissociated after mincing. The tissue is repeatedly flushed through smaller bore hypodermic needles in culture media.

Enzymes are the most commonly used cell dispersing agents. EDTA has been used for dissociating avain tissues (190). DNA, carbohydrate and protein are often contaminants released by EDTA treatment. Trypsin was introduced by Moscona to dissociate embryonic tissue (191). Trypsin is an endopeptidase and may do irreparable harm to the cell. A recommendation has been given that cells having trypsin treatment not be used in

preservation of cells by freezing in glycerol at liquid nitrogen temperatures (192). Papain has sometimes been used to disaggregate cells as well as pronase.

Collagenase and elastin attack fibrous structures such as keratins, collagen and elastins. Both are true proteolytic enzymes, splitting peptide bonds. Hyaluronidase attacks mucopolysaccharides. The testis preparation of hyaluronidase also contains both a glucosaminidase and glucoronidase (187).

The role of chealting agents for dissociating cells is rather ambiguous. Meyer and Rappaport (193) suggested that mucopolysaccharides acted as cationic exchange resins capable of binding Ca^{2+} and Mg^{2+}. For this reason Ca^{2+} and Mg^{2+} have been strongly favored as cell-cell binding agents. Various chelating agents such as citrate, oxalate, pyrophosphate, ATP, EDTA and tetraphenylboron have been used in disaggregating embryonic and adult mouse tissue supposedly by binding divalent ions.

In any disaggregation procedure the method which best preserves cellular function is the one to use.

CHAPTER III

PREPARATION AND PROPERTIES OF ISOLATED NORMAL AND TUMOR MAMMARY CELLS

Introduction

Cell culture has become a very important tool to many disciplines because it reduces the complexity of study required in organ or tissue culture. The conditions of culture such as temperature, pH, media, and effect of additives may be more tightly controlled at the cellular level and the response to a single change of one condition may be more easily recognized. Enzymic dispersion of tissue into single cell suspensions bypasses the necessity of maintaining cells for long periods of time and allows for the study to be done on cells with a metabolic integrity closer to that present in the tissue.

Many of the recent innovative studies of hormone action, differentiation, the mammalian nervous system, cellular immunology, cellular communication and cellular aggregation have been attempted only under these simpler conditions allowed by use of enzymic dispersion and cell culture (194).

For the study of cellular adhesion and the mechanisms by which cells recognize and adhere to each other viable cells with intact membranes are necessary. A method of mild enzymic dispersal of cells was needed which would allow the isolation of intact, viable cells. A culture medium was needed which would preserve the viability of the cells under the

conditions needed to study cellular adhesion and aggregation. Oxygen uptake by the isolated single cells was chosen to assess these conditions. Initial oxygen uptake before cellular repair could take place would be a simple criterion for selecting the conditions of enzymic dispersal and for selecting the conditions of routine maintenance of the cells.

Endogenous respiration is possible only when an intact cellular system of enzymes, cofactors and substrates and a functional membrane preventing molecular leakage is present (195).

Early procedures for dispersing cells generally used mechanical means or trypsin. Such cellular preparations had low endogenous respiration rates. Soluble enzymes from the cytosol leaked out into the medium easily from such cells (196). Leakage occurred at the time of cellular dispersion and was due to injury of the membrane during the isolation procedure (197).

Howard, et al. (198) developed an isolation procedure for intact liver cells utilizing 0.15% collagenase with 0.15% hyaluronidase and showed that the isolated cells retained high endogenous respiration rates. The isolated cells failed to absorb vital stains indicating the presence of an inctact membrane (199). With liver cells isolated by trypsin and mechanical methods, Tsai and Ebner (200) could not induce tyrosine aminotransferase using cortisol. The cells also showed enzyme leakage. However, using a revised Howard procedure, with 0.05% collagenase and 0.10% hyaluronidase, Huang and Ebner (201) isolated rat liver cells which allowed dexamethasone phosphate induction of tyrosine aminotransferase in normal liver cells. Takeda (197) had found that enzymic leakage remained high despite the tightening effect of dexamethasone on membranes isolated under procedures which caused membrane leakage.

A second technique was used to test the viability of the isolated cells. Isolated R3230 AC mammary tumor cells were injected subcutaneously into rats at various timed intervals after isolation. Growth of the tumor would show that the cells were viable and that they were very similar to the original cells of the tumor.

Experimental Procedures

Materials

The following reagents were obtained from Sigma: xanthine oxidase, hyaluronidase, bovine testis type I, collagenase, cl. histolyticum, type I containing both protease and peptidase activity; trypsin, bovine pancreatic, type XI, DCC treated, bovine serum albumin, fraction V (BSA), Hepes, penicillin-G, potassium salt, streptomycin sulfate and neomycin sulfate. Pronase, B grade, was from Calbiochem, EDTA from Fisher and CDTA from Fluka AG Bucks SG. Uric acid was from Eastman Organic Chemicals. Fetal calf serum (FCS), Medium 199, 10X concentrated, F-10, F-12 and McCoy's dry media, consisting of three separate fractions, amino acids, vitamins and essential salts were obtained from Grand Island Biological. Nutrient agar was from General Biochemicals. Xanthine was obtained from the Nutritional Biochemical Company. Virgin female Fischer CDF rats were obtained from Charles Rivers. Lactating females were from Charles Rivers or bred in the animal facilities of the biochemistriy department. Holtzman rats were obtained from the Holtzman Company. The R3230 AC tumor was obtained from Dr. Russell Hilf through the courtesy of Squibb Institute of Medical Research and from the Mason Tumor Bank.

Methods

Preparation of Tissue

A female Fischer rat, 100-120 grams, carrying an R3230 AC mammary tumor for 3-4 weeks or a normal rat lactating about 15 days, provided with standard laboratory diet and drinking water ad libitum was killed by decapitation and drained of blood. The R3230 AC tumor or mammary glands were aseptically removed in the Tenney laminar flow hood and weighed. The mammary tumor weight was also recorded. The tissue was placed into Hanks minimal media containing antibiotics. Any soft, yellow, necrotic material was removed. The firm white peripheral tissue was cut by surgical scalpel into one millimeter cubes. Mammary tissue was minced with surgical scissors. One millimeter cubes (mm^3) of firm tissue from the outer one-third of the tumor were transplanted by a sterile trocar technique to the region of both front mammary glands, two transplants per female Fischer rat weighing 80-100 grams. Transplantation was done on a weekly basis to maintain the tumor. The tumor is specific to the Fischer rat and grows at the same rate in both the male and female rat (202).

Preparation of R3230 AC Mammary Tumor Cells

After the R3230 AC tumor was aseptically removed, the tissue was minced in the Tenney laminar flow hood. R3230 tumor tissue was minced by a bank of razor blades (2 mm between blades) held in a small gel cutter. The tissue was placed in a stainless steel sieve, 100 mesh screen, and washed with Hanks minimal media to remove red blood cells. About 3 g of minced tissue was placed in 10 ml collagenase (0.05% w/v)-hyaluronidase (0.1% w/v) solution in Medium 199, pH 7.5 at 25° C (at 37° C, the pH will

be 7.3 (203)), containing BSA (0.1% w/v) and antibiotics: streptomycin (100 µg/ml), neomycin (100 µg/ml) and penicillin (100 units/ml). The tissue was incubated in the enzymic solution for 45 minutes at 37° C in a rotary water bath shaker (Fermentation Design, Inc.), set at 120 rpm. The tissue was removed by passing the suspension over a 100 mesh stainless steel sieve and the cells were centrifuged at 1/3 of the maximum speed of the International Clinical centrifuge (500 xg) for one minute. The remaining tissue was again incubated and the procedure repeated until enough cells were obtained to provide an initial count of 2-3.0 x 10^6 cells/ml. Approximately 0.5 ml of packed cells/gram of tissue were isolated and this approximates 2.5 x 10^7 cells/gram of tissue. A general scheme for the isolation procedures is presented in Figure 1.

Preparation of Normal Mammary Cells

The normal mammary glands were aseptically removed and adhering tissue was removed. About 20 gm of tissue was minced into millimeter cubes by means of surgical scissors, placed in a stainless steel sieve, 100 mesh screen, and washed with Hanks minimal media to remove red blood cells. About 3 g of minced tissue was placed in 10 ml collagenase (0.5% w/v)-hyaluronidase (0.1% w/v) solution in Medium 199, pH 7.5 at 25° C, containing BSA (0.1% w/v) and antibiotics: streptomycin (100 µg/ml), neomycin (100 µg/ml) and penicillin (100 units/ml). The tissue was incubated in the enzymic solution for 45 minutes at 37° C and 120 rpm in a Fermentation Design rotary water bath shaker. The tissue was removed by passing the suspension over a 100 mesh stainless steel sieve. The remaining tissue (3 g/10 ml of enzyme) was placed in pronase (0.1% w/v) solution in Medium 199, pH 7.5, containing BSA

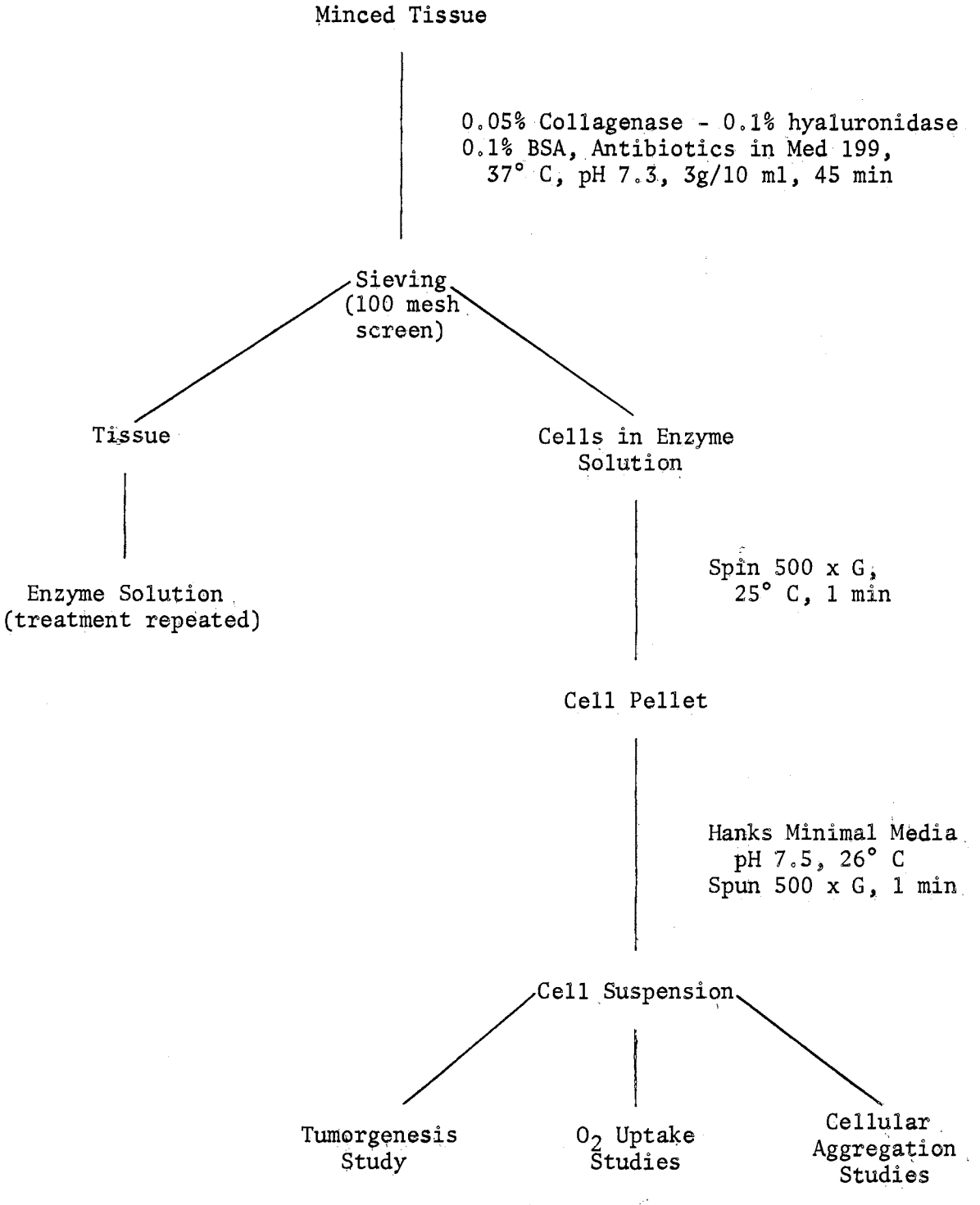

Figure 1. General Scheme for Isolation of R3230 AC Tumor Cells

(0.1% w/v) and antibiotics: streptomycin (100 µg/ml), neomycin (100 µg/ml) and penicillin (100 units/ml). The tissue was incubated for 30 minutes at 37° C and at 120 rpm in the rotating water bath shaker. The cells were centrifuged from the collagenase-hyaluronidase enzymic solution at 1/3 of the maximum speed of the International Clinical centrifuge (500 xg) for one minute. The procedure was repeated until enough cells were present to provide an initial count of 1.0×10^6 cells/ml. About 0.3 ml of packed cells/gram of tissue were isolated which approximates 2.0×10^8 cells/gram of tissue.

Alternate Methods of Isolation of R3230 AC Mammary Tumor Cells

Three other methods of enzymic dispersal were also used. Hyaluronidase (0.1% w/v) and pronase (0.3% w/v) solutions each in the usual Medium 199 with 0.1% BSA was used to incubate tissue (3 g/10 ml) alternately for fifteen minutes each. In the second method the usual collagenase (0.05% w/v)-hyaluronidase (0.1% w/v) solution in Medium 199 and 0.1% BSA was alternated with pronase (0.7% w/v) in Medium 199 + 0.1% BSA every fifteen minutes. In the third method the usual collagenase (0.05% w/v)-hyaluronidase (0.1% w/v) in Medium 199 and 0.1% BSA was alternated with pronase (0.1% w/v) in Medium 199 and 0.1% BSA every fifteen minutes. The last two methods are a revised Wiepjes-Prop procedure (204). The tissue was prepared and the cells were isolated and centrifuged according to the procedures outlined above.

Preparation of the Cells for Suspension Culture

Both the normal mammary cells and the R3230 AC tumor cells were washed and centrifuged three times in 20 mls of Hepes minimal media containing BSA (0.1% w/v) before being resuspended in the culturing media. Ten to fifteen mls of suspended cells (2-3 x 10^6 cells/ml of R3230 AC tumor cells, or 1.0 x 10^6 cells/ml normal mammary cells) were placed in a sterile 50 ml Erlenmeyer flask and rotated at 80 rpm, 37° C, in a Fermentation Design rotary water bath shaker. The cells were counted on a Model B Coulter Counter at the settings 1/amplification = 4 and 1/aperature current = 1/2. The upper setting was 100 and the lower setting was 10.

Preservation of Tumor Tissue in Liquid Nitrogen

Sterile media containing 10% glycerol (v/v) was used for preservation of the tumor tissue. One ml of medium with added glycerol was placed in each of eight sterile marked vials provided with screw caps. Three to four pieces of tumor (1 to 2 mm squares) were placed in each tube. The vials were gased with CO_2, capped and the caps were tightly screwed in place and sealed with tape. Each tube was wrapped in gauze, taped and labeled. The tubes were held one hour at 40° and then put in the deep freeze for two hours, and finally placed in a Biostat maintained with liquid nitrogen.

When tissue was needed for transplant a vial was placed in a 37° C water bath to thaw the tissue. It was transplanted by the usual aseptic trocar method and took approximately two months to develop a tumor.

Oxygen Uptake Studies

Initially the Gilson Differential Respirometer was used to measure oxygen uptake. Each flask contained two ml of cell suspension (2 x 10^6 cells/ml) in the appropriate incubation medium. The center well contained Whatman No. 42 filter paper moistened with five drops of 10 M NaOH. Equilibrium with air was maintained for ten minutes, after which the system was closed and oxygen uptake was recorded for 3 hours at 37° C at 60 reciprocal strokes per minute. Two determinations of oxygen uptake were made for each treatment. Oxygen uptake was expressed as $\mu l/hr/1 \times 10^6$ cells. Measurements of oxygen uptake were made also with the oxygen electrode, Yellow Springs Instrument Company (205), and compared to those obtained with the Gilson respirometer.

All glassware and instruments used in removing the tumor tissue, in isolating cells, culturing and carrying out oxygen uptake were sterilized by autoclaving for thirty minutes in the American Sterilizer autoclave, 18 psi, 107° C.

Preparation of Media

Hanks minimal media and Medium 199 were prepared with 2 mg/liter glucose, penicillin G, potassium salt (0.15 mg/ml), streptomycin sulfate (0.1 mg/ml) and neomycin sulfate (0.1 mg/ml). Medium 199 was buffered with 10 mM Hepes, pH 7.5 at 25° C. Antibiotics were added at the same concentration to media F-10 and F-12. If proteins were added, BSA (0.1% w/v) or FCS (10%) was added separately or together. Hanks was prepared with or without BSA. Hanks is buffered (pH 7.4) with $NaHCO_3$ (0.4 g/l). When Hepes was used in place of $NaHCO_3$ as the buffer (2.4 g/l), Hanks was called Hepes. Solutions of Hepes buffer, pH 7.5 at 25°C were

prepared with 2 mg/l glucose, antibiotic as above, with or without BSA (0.1% w/v). After preparation the solutions were sterilized by filtration with a 0.22 μ Millipore filter. Sterile FCS was added to sterile media when needed. Routine plating of media from cell culture on nutrient agar was done to determine if bacteria were were present. Values from oxygen uptake were used only from experiments where bacteria were shown to be absent.

Standardization of the Oxygen Electrode (206)

Two solutions, monosodium xanthine (10 mg/100 ml) and uric acid (10 mg/100 ml) were dissolved in 0.033 M KH_2PO_4, pH 7.4. Xanthine oxidase from Sigma was used to make a solution such that the A_{290} was 0.100. Using the A_{270} of the xanthine solution and the A_{290} of the Uric acid solution along with the extinction coefficient of each solution, the concentration of both the xanthine and the uric acid solutions was determined. A standard curve was prepared from solutions of uric acid ranging from 0.005 mg/ml to 0.025 mg/ml. Duplicate samples of xanthine solution (0.673 mM) varying from 0.2 ml to 0.75 ml were reacted with 20 μl xanthine oxidase both in the oxygen monitor and in test tubes. Phosphate buffer was added to make a final volume of 2.68 ml. The deflection per inch of chart paper was recorded along with the A_{290}. Full deflection, 5 inches (100%), required 705 nmoles of oxygen/2.8 ml which is 250 μM. The literature value is 265 μM (205). 250 μM of dissolved oxygen is equivalent to 5.6 μl O_2/ml. Ringers solution contains approximately 5 μl O_2/ml at 37° C and 760 mm Hg (207).

Results

Tumors were regularly used at three to four weeks after transplant. When two transplants per rat were made the tumor size averaged 3.6 cm in length and 2.9 cm in cross-sectional diameter for ten tumors. The average weight was 12.5 grams. When four transplants per rat were made, two ventral and two dorsal to the front legs the average length was 2.6 cm and average cross-sectional diameter was 1.9 cm. The average weight for twenty such tumors was 5.6 grams.

Transplants made in a Fischer male rat also grew and the size and weight appeared comparable to that in the female (two experiments). Tumor transplants made in two Holtzman rats did not grow.

A study of initial oxygen uptake with the Gilson Differential Respirometer established an average initial uptake of 8 $\pm$ 2 µl/hr/10^6 cells for R3230 AC mammary tumor cells. In similar experiments using the Gilson Differential Respirometer the normal mammary cells were found to have an average oxygen uptake of 3 µl/hr/10^6 cells. Using the Lowry procedure R3230 AC mammary tumor cells were found to contain 3 mg protein/10^6 cells wet weight. The oxygen uptake for R3230 AC tumor cells was determined as 2.6 µl/hr/mg of protein wet weight. Assuming the cells contain 80% water, the oxygen uptake becomes 3.25 µl/hr/mg of protein dry weight. Abraham and Smith (208) determined that oxygen uptake for normal mammary cells of C_3H/Cryl mice was 9.1 $\pm$ 1.4 µmoles O_2/mg/min. This approximates 12 µl/hr/mg of protein wet weight or 9.6 µl/hr/mg of protein dry weight. Sandstrom (209) reported that O_2 uptake for liver cells was 12 $\pm$ 1.0 µl/hr/mg of protein dry weight. Mouse embryonic muscle cells were shown to have an uptake of 3.8 $\pm$ 0.27 µl/hr/10^6 cells (210). Human embryonic muscle was reported as 3.4 $\pm$ 0.25 µl/hr/10^6 cells. The values

for oxygen uptake of R3230 AC mammary tumor cells and normal mammary cells are well within the range found for other cells.

Values of oxygen uptake obtained with the oxygen electrode correlate well with values obtained with the differential respirometer (Figure 2). The values range from 6.0 µl O_2/hr/10^6 cells in Hepes minimal media to 9.8 µl O_2/hr/10^6 cells in Medium 199. Each value is an average of three experiments. The value of oxygen uptake for the normal mammary cell obtained using the oxygen electrode was 3.3 µl/hr/10^6 cells (Table 7).

In an intact cell the amount of oxygen uptake is dependent on the enzymes and substrates of the Krebs cycle in the cytosol (195). In a cell with a damaged membrane endogenous respiration is low since substrates and enzymes can diffuse into the medium (196). Many cells isolated in an 0.05% collagenase-0.1% hyaluronidase solution had a high endogenous respiration which indicated that the procedure seemed best for isolating the R3230 AC mammary tumor cells. The same oxygen uptake was obtained whether the cells were incubated in the enzymic solution for 35 or 70 minutes. There was no difference in oxygen uptake from cells isolated from tumors maintained for increasing periods of time, 2, 2 1/2, 3, or 3 1/2 weeks. Addition of glucose or antibiotics had no effect on oxygen uptake nor did insulin. Fetal calf serum appeared to stimulate O_2 uptake (Table IV).

Disaggregation of cells seemed to take place more rapidly at pH 5.0-6.0. Cells were isolated at varying pH and retained in Hepes + 0.1% BSA at pH 7.3 (Figure 3). Cells isolated at a pH below 6.5 showed no recovery of oxygen uptake. The negative oxygen uptake may be due to an equilibration period required for the cells to resume their normal metabolic activity at 37° C.

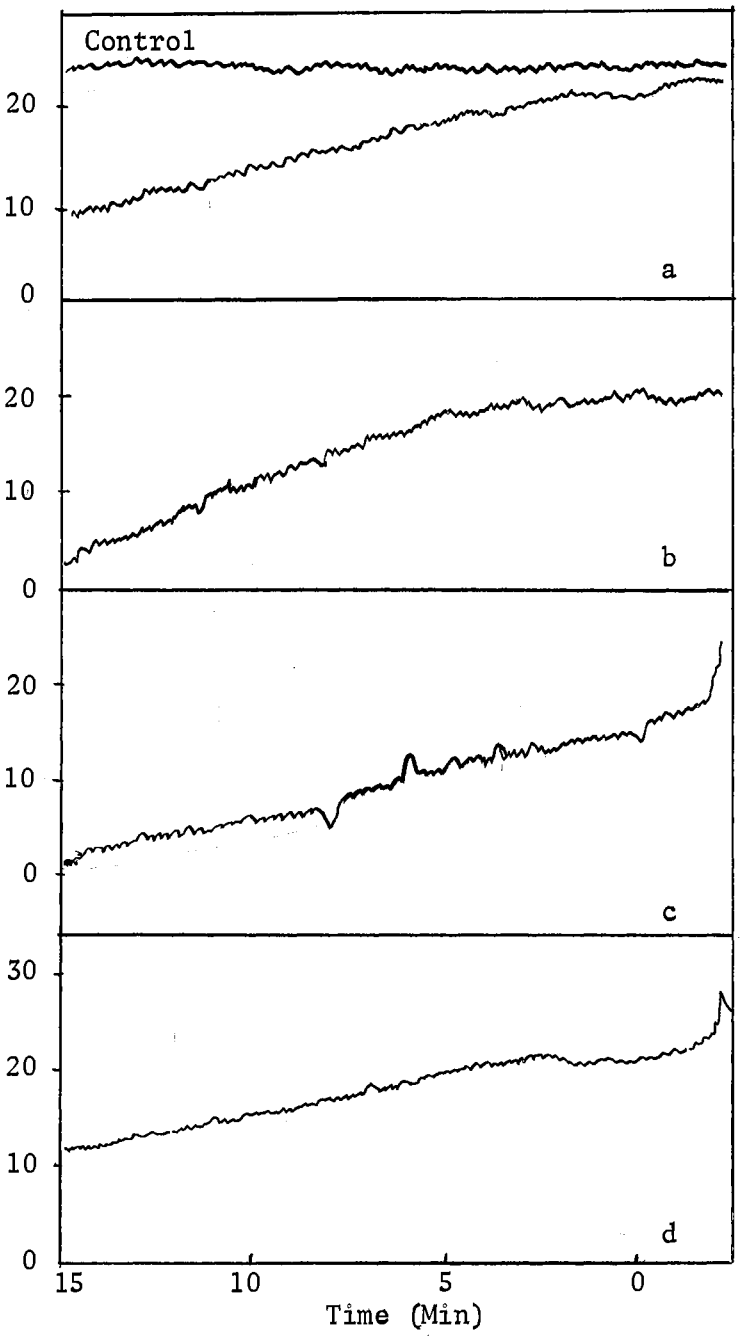

Figure 2. Initial Oxygen Uptake of R3230 AC Mammary Tumor Cells Measured With the Oxygen Electrode

Oxygen Monitor Yellow Springs Instrument Company, 37° C. Rate was one inch/minute. (a) Hepes media, 36%/hr/1x10^6 cells ≡ 5.4 µl/hr/1x10^6 cells; (b) Medium 199, 65.8%/hr/1x10^6 cells ≡ 9.8 µl/hr/10^6 cells; (c) Hepes, 40%/hr/1x10^6 cells ≡ 6.0 µl/hr/10^6 cells; (d) Medium 199, 40%/hr/10^6 cells ≡ 6.0 µl/hr/10^6 cells.

TABLE IV

INITIAL OXYGEN UPTAKE OF R3230 AC TUMOR CELLS AND NORMAL MAMMARY CELLS OF THE FISCHER RAT

	O_2 Uptake (µℓ/hr/10^6 cells)			
	n[1]	R3230 Tumor[2]	n	Mammary Cells[2]
Hanks + Ca^{2+}	3	7.3 ± 0.4	-	-
Hanks + Ca^{2+} + Glucose	3	7.7 ± 0.6	-	-
Hepes + Glucose	3	7.6 ± 2.7	3	2.2 ± 1.9
Hepes + Glucose + Insulin	1	4.0	-	-
Hepes + Glucose + FCS (10%)	1	10.7	2	4.0 ± 1.6
Hepes + Glucose + FCS + Insulin	1	10.8	-	-
Medium 199 + Antibiotics	3	8.9 ± 4.6	6	3.1 ± 0.8

[1]n = no. of experiments.

[2]Cells were isolated by an enzymic mixture of collagenase (0.05% w/v)-hyaluronidase (0.1% w/v) in Hanks media which contained Ca^{2+} (0.14 g/l), Mg^{2+} (0.1 g/l) and glucose (2g/l). Hepes medium is Hanks medium buffered with Hepes (2.38 g/ℓ), pH 7.5 at 25° C. Hanks media is buffered with NaHCO (0.35 g/l). Values were obtained by Angela Tedesco, except for the last experiment with medium 199.

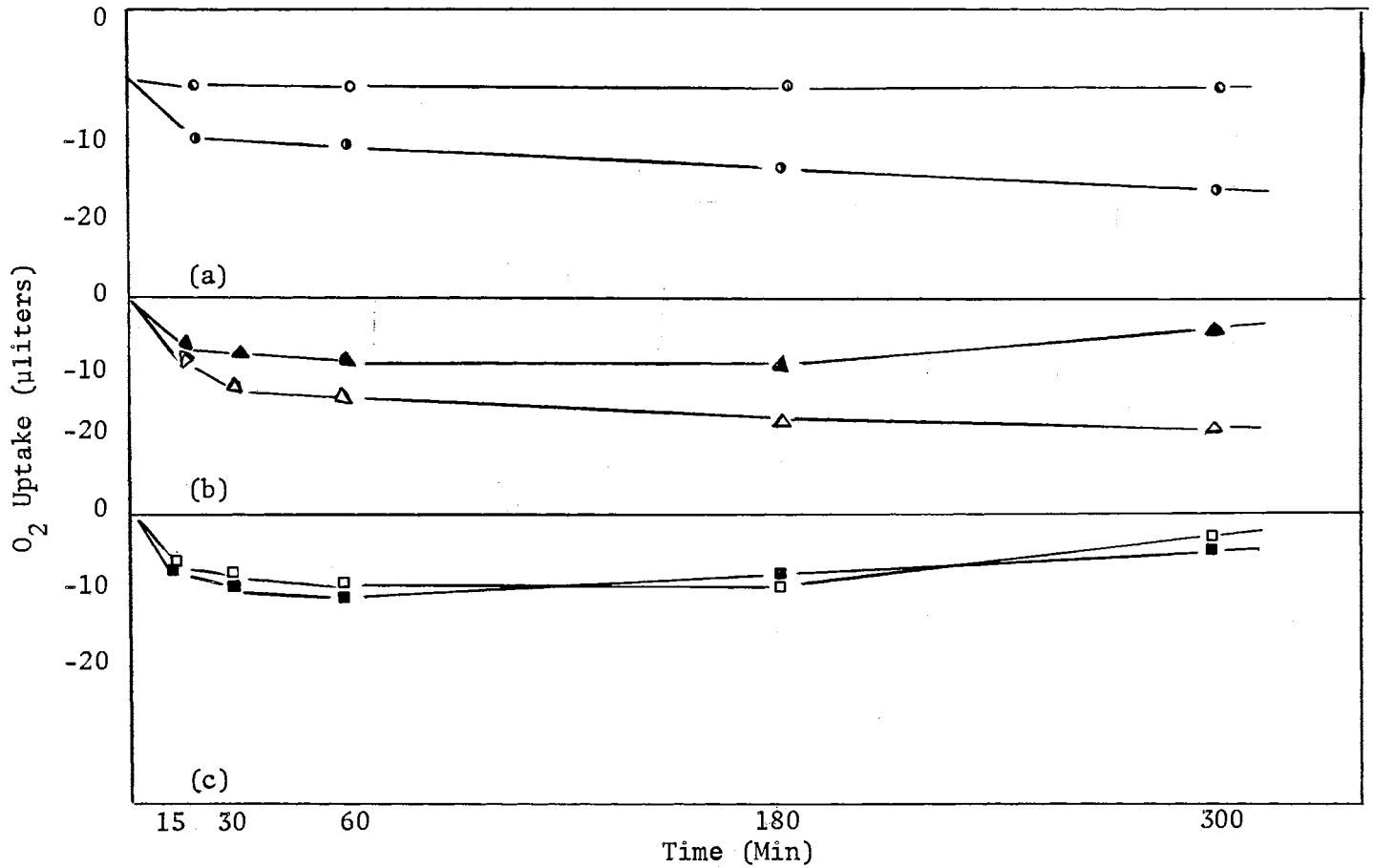

Figure 3. Initial Oxygen Uptake of R3230 AC Tumor Cells Disaggregated and Cultured at Differing pH Values

Gilson Differential Respirometer. Oxygen uptake was done in Hepes media containing 2x glucose and 0.02% BSA, pH 7.2, 37° C. The cell concentration was between 2-3 x 10^6 cells/ml. (a) — O —, control, pH 7.3; — ◐ —, pH 5.6; (b) — Δ —, pH 6.0; — ▲ —, pH 6.5; (c) — □ —, pH 7.0; — ■ —, pH 7.4.

There was a gradual decline in oxygen uptake when oxygen uptake was followed continuously for eight hours (Table V). In addition, there is a gradual decrease in pH. As oxygen was depleted in cultures of mouse LS cells, there were decreased levels of some of the enzymes involved in terminal repiration and increased levels of enzymes involved in glycolysis and the hexose monophosphate pathway (211). Both aerobic and anaerobic glycolysis are inhibited by a drop in pH. The concentrations of both lactate and pyruvate decrease as the pH decreased. Fructose diphosphate levels and triose phosphate levels also decreased (212). The tumor and mammary cells after enzyme incubation had been washed with a Hepes buffered Hanks solution, pH 6.8, to prevent aggregation. Even after this short exposure to acidic pH, there was inhibition of O_2 uptake; cells isolated at pH above 6.5 showed a recovery of only a low level of endogenous oxygen uptake after two hours of incubation (Figure 3).

Exposure of Ehrlich ascites cells to acidic pH below pH 7.0 produced large uptake vacuoles with no increase of pinocytotic vessels. Ehrlich ascites tumor cells when exposed to acidic pH below 6.1 failed to undergo cellular division when reinjected intraperitoneally into living mice (213). The pH in the media of R3230 AC mammary tumor cells was approximately 6.5 to 6.8 after five hours of culture. Despite such a low pH drop the cells continued to aggregate and viability remained since tumors grew when one ml of packed cells was reinjected subcutaneously in the region of the front mammary gland.

In a preliminary tumorogenesis study one ml of packed R3230 AC tumor cells was injected subcutaneously at 6, 12 and 24 hours after isolation. Two basic culture media were used: Medium 199 with and without 10% FCS serum. Each suspension was divided into two fractions. In one fraction

TABLE V

INITIAL OXYGEN UPTAKE OF R3230 AC TUMOR CELLS DURING A PERIOD OF 8 HOURS

Media	Total Oxygen Uptake[1] at the Hour Read (µl)					
	Hours of Incubation					
	1	2	3	4	5	6
Hepes-No Glucose	15	28	38	47	57	67
Hepes + Glucose	12	24	35	44	54	66
Hepes + Glucose + FCS	17	32	46	58	73	92

[1]Values taken by Angela Tedesco. Cells were cultivated according to the procedure for Table IV. Only one value was obtained for each media. Cell concentration was 2.0×10^6 cells/ml.

the media was not changed whereas it was changed once in the other fraction. One ml of packed cells from each of these four treatments was injected into female rats. After six hours of suspension, cells from the unchanged media without serum developed a tumor. No other tumors developed from cells suspended in media without fetal calf serum. Only cells maintained in media containing fetal calf serum developed tumors when reinjected and of these cells only those in unchanged media developed tumors. Only one rat was used for each of these conditions though two injections/rat were made. The results were similar for both injections.

Using rats where injected cells produced no tumors, a series of cell injections (1 ml of packed cells/injection) were given each of 4 rats one week apart for three weeks. Cell suspensions with no fetal calf serum present were used. After this series of cell injections, a regular tumor transplant using tumor tissue was made. No tumors grew. A second series of injections was made to each of four rats using 1 ml of tumor membrane suspension per injection, again one week apart for three weeks. After this series of membrane injections, a regular tumor transplant of tumor tissue was made and no tumors grew. The rats had become immune to the foreign tumor tissue and rejected the tumor.

Table VI lists the time after cell isolation when cell injection was made, the number of animals used and the number of animals developing tumors. After 96 and 120 hours only one third of the cell injections caused tumors. Cell viability remains high up to seventy-two hours of suspension.

Control of pH is a constant problem in actively metabolizing cell populations. Normal cells undergoing division under cell culture conditions resemble malignant cells <u>in vivo</u> (214). Cell density rather than

TABLE VI

TUMOROGENESIS STUDY. A STUDY OF PRESERVATION OF CELL FUNCTION VERSUS TIME IN SUSPENSION[1]

Time After Cell Isolation (hrs)	Number of Animals	Number of Animals Developing Tumors
6	4	3
12	4	3
24	5	2
48	4	3
72	6	5
96	9	3
120	9	3

[1]Injection of one ml of packed R3230 AC mammary tumor cells, injected subcutaneously at 0, 2, 4, and 6 hrs (2 rats/time period) produced tumors at each time period. In this set of experiments the media was Medium 199 with 10% FCS.

growth rate was shown to determine the ratio of lactate to CO_2 when normal cells grow at the same rate but at differing populations. This also occurs in cultures of transformed cells where cell density does not limit the growth rate (214). This indicates that one control of acid production and rapid pH change in suspension culture is to control the density of the cell population. The optimum culturing density of the R3230 AC tumor cell lies between 2-4 x 10^6 cells/ml (Table VII).

Wiepjes and Prop (204) developed a method of isolating epithelial cells of mouse mammary gland where a solution of 0.125% collagenase-0.1% hyaluronidase containing 4% BSA was used for 45 minutes of incubation followed by one hour of incubation in 1.25% pronase in Medium 199 at 36° C, 120 rpm in a water bath shaker. This method was modified such that all enzymes were in Medium 199 each containing 0.1% BSA. Three modifications were developed to test enzyme isolation, as indicated in the section Alternate Methods of R3230 AC Mammary Tumor Cell Isolation. Pronase concentration and the incubation time were varied to find the most favorable procedure of isolating viable cells. Oxygen uptake was used to check cell viability both in Hepes minimal media and in Medium 199 after R3230 AC tumor cell dispersion (Figures 4 and 5). The values of initial oxygen uptake for each procedure is given in Table VIII.

The only cell preparation method which showed a constant value of oxygen uptake for each media used was the collagenase (0.1% w/v)-hyaluronidase (0.1% w/v) method. With this method of cell isolation, cells suspended in Medium 199 consistently had a slightly higher initial oxygen uptake (Table IV and Table IX) than those in Hepes. The high cell concentration, 7.1 x 10^6 cells/ml, exerts an inhibitory effect on oxygen uptake resulting in lower values of initial oxygen uptake. It would

TABLE VII

EFFECT OF CELL CONCENTRATION ON INITIAL OXYGEN UPTAKE OF THE R3230 AC MAMMARY TUMOR CELLS OF THE FISCHER RAT

Number of Cells/ml	Average O_2 Uptake	
	n[1]	µl/hr/10^6 cells
2.5 x 10^6	2	10.3
4.0 x 10^6	2	12.0
7.0 x 10^6	2	4.5
8.0 x 10^6	2	6.0
13.0 x 10^6	2	3.0
15.0 x 10^6	2	2.0

[1]n = number of determinations. Gilson Differential Respirometer. All oxygen uptake was done on cells suspended in Medium 199 at 39° C. The oxygen electrode determinations were: 6.0 µl/hr/10^6 cells for 1 x 10^6 cells and 1.0 µl/hr/10^6 cells for a cell concentration of 13.0 x 10^6 cells for R3230 AC tumor cells in both Hepes and Medium 199. Normal mammary cells gave 3.3 µℓ/hr/10^6 cells at a concentration of 2.0 x 10^6 cells/ml but only 1.0 µl/hr/1 x 10^6 cells when the cell concentration was 7.0 x 10^6 cells.

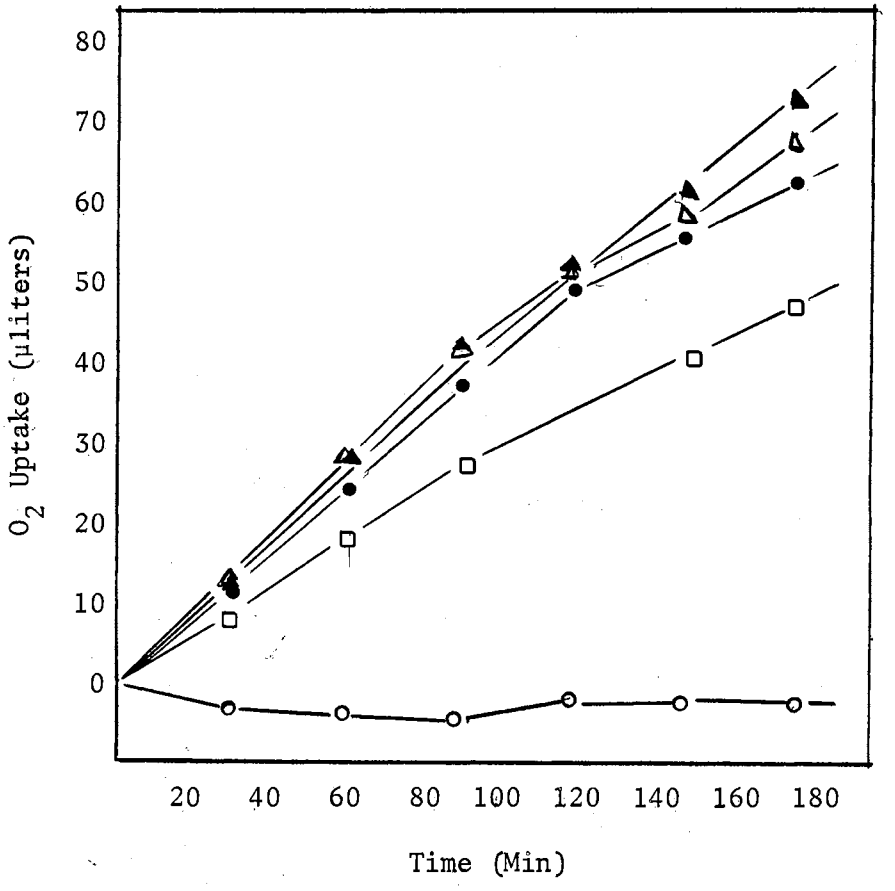

Figure 4. Initial Oxygen Uptake of R3230 AC Tumor Cells in Minimal Media After Disaggregation by Four Differing Procedures

Suspension of cells was in Hepes. Gilson Differential Respirometer, 37° C, 60 rpm. — O —, control; — ● —, hyaluronidase (0.1%)-pronase (0.3%); — Δ —, collagenase (0.05%)-hyaluronidase (0.1%)-pronase (0.7%); — ▲ —, collagenase (0.05%)-hyaluronidase (0.1%)-pronase (0.2%); — ■ —, collagenase (0.10%)-hyaluronidase (0.1%). Cell concentration given in Table VIII.

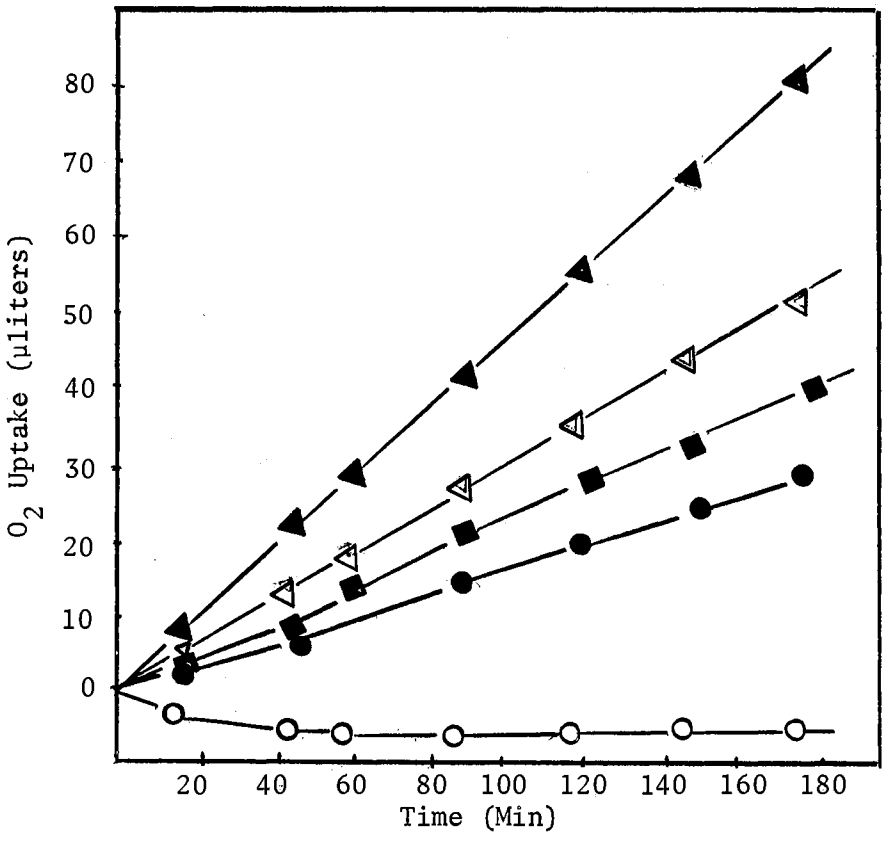

Figure 5. Initial Oxygen Uptake of R3230 AC Tumor Cells in Medium 199 After Disaggregation With Four Differing Procedures

All enzyme solutions were dissolved in medium 199 + BSA. Gilson Differential Respirometer, 37° C, 60 rpm. — O —, control; — ● —, hyaluronidase (0.1%)-pronase (0.3%); — Δ —, collagenase (0.05%)-hyaluronidase (0.1%)-pronase (0.7%); — ▲ —, collagenase (0.05%)-hyaluronidase (0.1%)-pronase (0.2%); — ■ —, collagenase (0.10%)-hyaluronidase (0.1%). Cell concentrations given in Table VIII.

TABLE VIII

INITIAL OXYGEN UPTAKE OF R3230 AC TUMOR CELLS ISOLATED WITH DIFFERING PROCEDURES OF CELL DISPERSION[1]

Method of Enzyme Isolation	Initial Oxygen Uptake			
	Hepes		Medium 199	
	cells/ml	µl O_2/hr/ 10^6 cells	cells/ml	µl O_2/hr/ 10^6 cells
Control	-	-	-	-
Hyaluronidase (0.1%)-Pronase (0.3%)	3.1×10^6	7.4	1.8×10^6	10.3
Collagenase (0.05%)-Hyaluronidase (0.1%); Pronase (0.66%)	3.0×10^6	7.8	2.6×10^6	12.6
Collagenase (0.05%)-Hyaluronidase (0.1%); Pronase (0.17%)	3.1×10^6	8.2	2.0×10^6	14.7
Collagenase (0.1%)-Hyaluronidase (0.1%)	2.3×10^6	7.3	7.1×10^6	7.7

[1]Tissue was removed from the enzymic solution and alternately incubated in the second enzymic solution every 15 minutes. Values are from Figures 3 and 4. Media given are the media used for initial oxygen uptake in the Gilson Differential Respirometer, 37° C.

TABLE IX

INITIAL OXYGEN UPTAKE OF NORMAL MAMMARY CELLS AND R3230 AC MAMMARY TUMOR CELLS IN DIFFERING MEDIA AND WITH SINGLE SUBSTRATES ADDED TO MINIMAL MEDIA

Media + Substrate	Oxygen Uptake ($\mu l/hr/10^6$ cells)			
	n	R3230 AC Tumor Cells	n	Mammary Cells
Hepes + Glucose	3	7.6 ± 2.7	3	2.2 ± 1.9
Medium 199	3	9.4 ± 2.7	3	3.6 ± 0.3
F-10	3	8.1 ± 1.4	2	0.8 ± 0.01
F-12	3	7.0 ± 1.5	2	3.0 ± 2.0
Hepes + FCS	3	7.2 ± 0.6	2	4.0 ± 1.6
Medium 199 + FCS	3	8.0 ± 0.9	1	5.8
Hepes + BSA	3	11.8 ± 0.4		
Medium 199 + BSA	3	8.7 ± 1.3		
Hepes + Glutamine	3	4.9 ± 3.0		
Hepes + Alanine	3	5.4 ± 1.6		
Hepes + Glutamate	3	6.5 ± 2.2		
Hepes + McCoy's Amino Acids	3	6.1 ± 1.0		
Hepes + McCoy's Vitamins	3	4.4 ± 0.2		
Complete McCoy's	3	5.2 ± 0.4		

appear from these results that any of these enzymic methods would provide cells with good oxygen uptake. The Weipjes-Prop method was used to isolate normal mammary cells from the Fischer rat. Initial oxygen uptake for normal mammary cells is given in Tables IV, VII, and IX.

Since addition of single substrates such as glucose, glutamine, glutamate or alanine to Hepes had little effect on oxygen uptake indicates that these cells had intact membranes. Addition of glutamine would be expected to have no effect on oxygen uptake even if the membrane were leaky since it is potentially a Kreb cycle intermediate. Ehrlich ascites tumor cells and isolated mitochondria from the ascites cell have a high oxidative metabolism in the presence of glutamine. The degree of malignant alteration is highly correlated with the activity of glutaminase (215). If isolated cells have intact membranes the addition of glutamine or glutamate should have no effect on oxygen uptake which is the case when intracellular availability of substrate is the limiting factor in respiration. If the cell membrane is impaired and substrates escape, added substrate increases the endogenous respiration. The lower the endogenous respiration the higher the percentage activation of added substrate (195).

Initial oxygen uptake of the isolated cells appears to be nearly the same for all four media used and is within the experimental error. The R3230 AC mammary tumor cells have the highest endogenous oxygen uptake with the more complete media or Medium 199. Table IV shows that addition of fetal calf serum stimulated oxygen uptake. From visual observation through the microscope addition of bovine serum albumin or fetal calf serum increased the viability of cells suspended for 72-120 hours. Less debris was present and aggregates appeared more like tissue. Oxygen

uptake was measured initially after 19 hours and after 48 hours and the results are shown in Figure 6. Depression of oxygen uptake is believed to be due to accumulation of acid during the suspension period. Values for oxygen uptake are given in Table X.

Cells in medium 199 appeared to have the best oxygen uptake especially when supplemented with 0.1% bovine serum albumin or 10% fetal calf serum. Hepes minimal media appeared attractive for studies of short duration, particularly in simple studies to determine the effect of a single substrate.

Oxygen uptake studies are a simple method for defining conditions for cell isolation, for determining cell viability and for determining the intial or preliminary conditions for short-term or long-term suspension culture. The most useful purpose for measuring oxygen uptake is to assess the integrity of the cell membrane and the potential biosynthetic ability of the cell.

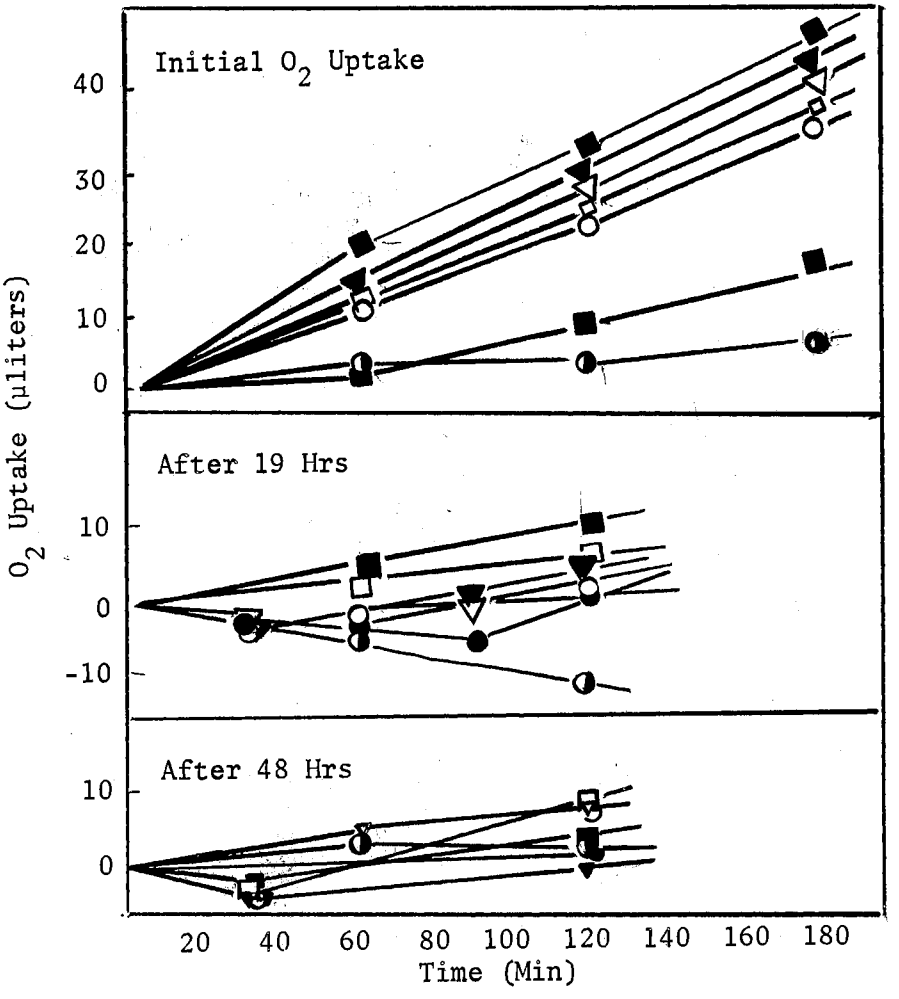

Figure 6. Oxygen Uptake of R3230 AC Tumor Cells. Initial Uptake and Uptake After 19 Hours and 48 Hours of Culture

Comparison of the effect of BSA and FCS on O_2 uptake. — ◐ —, control; — ● —, Hepes + FCS; — ○ —, Hepes + BSA; — △ —, medium 199 + FCS; — ▲ —, medium 199 + BSA; — ■ —, F-12 + FCS; — □ —, F-12 + BSA. Cell concentration is given in Table X.

TABLE X

OXYGEN UPTAKE OF R3230 AC MAMMARY TUMOR CELLS: INITIAL UPTAKE AND UPTAKE AFTER 19 HOURS AND 48 HOURS OF CULTURE

Media	O_2 Uptake (µl/hr/10^6 cells)		
	Initial	19 Hours	48 Hours
Control	-	-	-
Hepes + FCS	2.4	1.3	0.5
Hepes + BSA	6.0	1.6	-
Medium 199 + FCS	8.7	1.0	0.5
Medium 199 + BSA	8.0	2.2	0.3
F-12 + FCS	7.3	2.0	1.0
F-12 + BSA	6.0	2.4	0.5

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF CELL MEMBRANES OF THE NORMAL MAMMARY CELLS AND R3230 AC MAMMARY TUMOR CELLS OF THE FISCHER CDF RAT

Introduction

The plasma membrane has long been considered the mediating surface involved in cellular aggregation (216-218). Studies with the red cell membrane and other plasma membranes have shown them to be composed of a large number of polypeptides and glycoproteins (219-222). It is not known yet which of these constituents is involved either in cellular recognition or in cellular adhesion.

Several theories of cellular adhesion have been proposed. One theory involves protein synthesis (189-223), another proposes complementary molecules which act as a lock and key such as an enzyme-substrate complex (89) or an antigen-antibody reaction (224-226) and one proposes the formation of intermolecular bonds such as are found in collagen and elastin (227-229).

A preliminary study of the isolation, amino acid and carbohydrate composition of plasma membranes might give some insight into means for testing one or the other theory of aggregation. For example a lack of hydroxyproline or hydroxylysine would rule out the formation of cross-linking such as is found in collagen and elastin. In order to facilitate

such a study the R3230 AC mammary tumor cell membrane and the normal mammary cell membrane from the Fischer CDF rat were isolated. A comparative study of proteins, glycoproteins, amino acids and carbohydrates of these membranes was made. These membranes had not been previously isolated or characterized. These studies also allowed comparison of membranes prepared from the normal mammary cell and the mammary tumor cell. These studies represent a preliminary survey of methods for the isolation and characterization of these membranes, and are not to be considered definitive but rather serve as the basis for a more extensive investigation at a later date.

Three different procedures were compared in isolating the plasma cell membranes from the two cell types: fluorescein mercuric acetate (230,231), zinc chloride (230,231) and Tris-HCl (232) procedures. The use of 5'-nucleotidase (233) was attempted to identify the plasma membrane.

In general the results showed that all three membrane preparations had comparable carbohydrate and amino acid compositions. None of the membranes required a stabilizer to preserve the integrity of the membrane.

Experimental Procedures

Materials

Fluorescein mercuric acetate was obtained from the Nutritional Biochemical Corporation. Glucose-6-phosphate, 5'-AMP, sodium dodecyl sulfate, Tween 20 and glycine were from Sigma. UDP-galactose-^{14}C and 2-mercaptoethanol were from Calbiochem. Tween 80 was from Mann Research laboratories, urea from J. T. Baker, 1-amino-2-napthol-4-sulfonic acid and activated charcoal were from Matheson Coleman and Bell. Gel electrophoresis materials were from Canalco or Bio-Rad while all other chemicals

were reagent grade from Fisher. The galactosyltransferase (234) and α-lactalbumin (235) were isolated as previously described. Lactate dehydrogenase, β-galactosidase and bovine serum albumin were purified standards supplied by Dr. Carraway.

Membrane Preparations

The procedures for removal of tissue and preparation of cells are given in Chapter III. After the isolation of single cells, the cells were repeatedly washed about ten times in 0.9% NaCl and centrifuged in the International Clinical Centrifuge at one-third of its full speed (500 x g) to remove traces of enzymes, red blood cells and hemolysis contaminants. Mammary cells were washed only after first being suspended in physiological saline containing one molar sucrose in a 1:2 mixture to remove adipose cells and fat droplets (236). After standing five minutes the cells were centrifuged (500 x g). Adipose cells and fat droplets were discarded in the supernatant solution. The cells were transferred to a 35 ml glass Dounce homogenizer.

Fluorescein Mercuric Acetate Method

To ten ml of packed cells, approximately 5 x 10^8 cells, thirty ml of fluorescein mercuric acetate (FMA) (0.0022 M, pH 8.1) were added while gently stirring (230,231). After five minutes at room temperature the cell suspension was cooled to 4° C. All steps of the membrane homogenization were done in an ice bucket at 4° C. Membrane suspensions were maintained at 4° C during all steps of isolation.

A Leitz, student model, phase contrast microscope was used to follow membrane ghost formation at intervals during the homogenization step.

Ten to twenty strokes of the tight pestle (B form) produced membrane ghosts with the least amount of destruction. Ten ml of 60% sucrose were added with stirring and this gave a membrane solution which approximated a 15-30% sucrose solution. Ten ml of membrane suspension were layered over twenty ml of 45% sucrose in each of six blunt tipped polypropylene tubes (40 ml volume) and centrifuged at 600 x g for thirty minutes at 4° C in the RC-2B Sorvall centrifuge. Five ml of 60% sucrose formed a cushioning layer. Whole cells and nuclei entered the 60% sucrose layer whereas membranes collected at the 30-45% interface.

The membranes were harvested with a syringe fitted with an 18 gauge needle bent to a 90° angle fifteen millimeters from the beveled end. The upper layer, the 30% sucrose layer, of each tube was combined and saved. The remaining bottom 45% sucrose layer of each tube was combined, diluted with an equal volume of deionized water and relayered over 55% sucrose, again with 5 ml of the cushioning 60% sucrose solution. The solutions were centrifuged at 900 x g for thirty minutes at 4° C. After the membrane bands were harvested the top layer of each tube was added to the first supernatant solution. The bottom layers were discarded. All recovered material was centrifuged after 50% dilution with deionized water for thirty minutes at 7-8,000 x g in the Sorvall RC-2B centrifuge at 4° C. The precipitate was saved as the membrane pellet.

The precipitate or pellet was resuspended in approximately six ml of 35% sucrose and layered either on a continuous gradient of 45-65% sucrose or on a discontinuous gradient of 40-65% sucrose. The gradient consisted of three ml each of 65 and 60% sucrose and five ml each of 55, 50, 45, and 40% sucrose. The tubes were centrifuged in the Spinco L-2 ultracentrifuge, SW 25.1 rotor, at 90,000 x g for one hour at 4° C. The membranes

formed a band at an approximate density of 1.405 (42% sucrose) or at the interface between the 40 and 45% sucrose layers. The membranes were collected using the syringe, then diluted with deionized water and spun down at 8,000 x g in the RC-2B for twenty minutes at 4° C. The membranes were washed by centrifugation three times with water to remove sucrose and finally lyophilized.

Zinc Chloride Method

Ten ml of cells (5 x 10^8 cells) were reacted with 90 ml of 0.001 M $ZnCl_2$ and allowed to stand for ten minutes at room temperature (230,231). Five ml of 1% Tween 20 were added slowly while stirring. After two minutes, an additional five ml of 0.01 M $ZnCl_2$ were added. The solution stood two minutes before being cooled to 4° C on ice prior to homogenization. Thirty ml of membrane suspension were homogenized at one time in a glass Dounce homogenizer. One hundred strokes of the loose pestle (A pestle) were used. Only fragments of membranes were observed.

An equal volume of 60% sucrose was added to the membrane suspension. Twenty-five ml of the membrane suspension were layered over 50 ml of 45% sucrose in 250 ml bottles. A cushioning layer of 10 ml of 60% sucrose was placed in each bottle before preparing the discontinuous gradient. The suspension was centrifuged at 1,000 x g for one minute. The membranes collected at the interface above the 45% sucrose layer. Whole cells and nuclei entered the 60% layer. The interface material along with membranes precipitated on the side of the centrifuge bottle and at the interface were collected and relayered over 20 ml of 45% sucrose and centrifuged at 7-8,000 x g for twenty minutes at 4° C. The top layer and precipitate were again pipetted off. Both supernatant suspension and precipitates

were pooled. The supernatant suspension was diluted 50% with deionized water and centrifuged at 12,000 x g at 4° C for thirty minutes. The precipitates were pooled.

The pellet was suspended in six ml of water and layered either on a continuous gradient of 30-65% sucrose or on a discontinuous gradient of 35-60% sucrose. Centrifugation was carried out in the Spinco L-2 ultracentrifuge, SW 25.1 rotor at 90,000 x g. A main band appeared at the 50-55% interface, density 1.230, and a minor band between the 40 and 45% interface, density 1.176. The membranes were collected, centrifuged at 8,000 x g for twenty minutes at 4° C in the RC-2B, washed three times with water and lyophilized.

Tris-HCl Differential Sedimentation Method

Ten to fifteen ml of packed cells were suspended in twenty times their volume of 10 mM Tris-HCl, pH 8.0 for five minutes (232). At this time tumor cells were homogenized with about ten strokes of the Dounce tight-fitting (B pestle) homogenizer. Mammary cells were given about five strokes. A solution of 30 mM $MgCl_2$, 100 mM NaCl was added at about 0.1 of the volume of the cells in order to stabilize the nuclei. The suspension was centrifuged at 1,000 x g for one minute in the RC-2B Sorvall at 4° C. The supernatant suspensions were pooled. The pellet was resuspended in four times its volume of Tris-HCl buffer, 3 mM $MgCl_2$, 10 mM NaCl, pH 8.0 and centrifuged again at 1,000 x g for one minute. The supernatant suspensions were pooled and the pellets containing nuclei and whole cells were discarded.

Ten ml of the supernatant solution were layered onto discontinuous sucrose gradients composed of ten ml of 25% sucrose layered over ten ml

of 45% sucrose. All sucrose solutions contained 10 mM Tris-HCl buffer, pH 8.0. The gradients were centrifuged at 7,000 x g for 20 minutes at 4° C in the RC-2B Sorvall. The 25% sucrose layers were pooled as well as the 45% sucrose layers. The precipitate was collected, resuspended in 10 mM Tris and relayered over a discontinuous gradient composed of ten ml of 25% sucrose layered over 10 ml of 45% sucrose and centrifuged at 7,000 x g for 20 minutes at 4° C. All 25% sucrose layers were pooled. The membranes at the 25%-45% interface were collected and saved. The 45% sucrose layers were pooled, diluted 1:1 with 10 mM Tris-HCl, buffer pH 8.0 and centrifuged at 7,000 x g for thirty minutes at 4° C. The membranes and 25% sucrose layers were pooled with their respective fractions. The 25% sucrose supernatant suspensions were centrifuged at 27,000 x g for fifteen minutes. All membrane fractions were combined and the supernatant solutions were discarded. Depending on the pellet size, the pellet was suspended in three to nine milliliters of Tris buffer.

The membranes were layered on a discontinuous gradient composed of eight milliliters each of 60%, 50%, 45%, 40%, 35%, and 25% sucrose. From 1-3 ml of membrane suspension were layered on each tube of the SW 25.2 rotor and centrifuged at 13,000 rpm for one hour in the Spinco L-2 centrifuge. The membranes collected between the 25-30% interface, the 30-45% interface and the largest amount was between the 45-60% sucrose interface. The membranes were harvested, centrifuged at 7-8,000 x g, washed three times with deionized water and lyophilized.

Membrane Prepared From Tissue Homogenates of Rat Mammary and Mammary Tumor Tissues

Tumor Tissues. Tumor tissue was minced into 1-2 mm cubes. About 10 ml of tissue was made up to 5 x its own volume with homogenizing solution. The homogenizing solution was either 10 mM Tris-HCl, pH 8.0 or 0.001 M zinc chloride solution. To 35 ml of homogenizing solution was added 5 ml of 100 mM NaCl, 30 mM $MgCl_2$ solution to stabilize nuclei. This 40 ml of homogenizing solution was added to 10 ml of tumor tissue before homogenizing. The tissue was homogenized about three minutes at 100 rpm with an Eberbach model teflon homogenizer, 2.53 cm in diameter with 4 mm of clearance at 100-200 rpm/minute. The suspension was passed through a 100 mesh stainless steel screen. The filtered solution was diluted to 100 ml with additional Tris or $ZnCl_2$ solution. Four 25 ml fractions were centrifuged at 1,000 x g for one minute. The supernatant solution was saved whereas the precipitate, mostly nuclei and whole cells was resuspended. The precipitate was diluted 1:4 with 40% sucrose made up with homogenizing solution. The suspension was again centrifuged at 1,000 x g for one minute. The supernatant solutions were pooled and the precipitate was discarded. The supernatant solutions were combined and centrifuged at 7-8,000 x g for twenty minutes. The membrane pellets were treated from this point according to the normal procedure for the SW 25.2 rotor.

Normal Mammary Tissue. Normal mammary tissue was minced into 1-2 mm cubes with scissors. Ten ml of mammary tissue was made up to 10 x its volume with homogenizing solution, either 10 mM Tris-HCl, pH 8 or 0.001 M zinc chloride solution. To 70 ml of homogenizing solution was added

10 ml of 100 mM NaCl, 30 mM $MgCl_2$ solution to stabilize nuclei. 80 ml of homogenizing solution were added to 10 ml of mammary tissue. The suspension was homogenized with the Sorval Omni-mixer at full speed for thirty seconds. After filtering through glass wool, fifty ml of solution were layered on a discontinuous gradient of 10 ml of 60% sucrose and 50 ml of 45% sucrose. The solutions were centrifuged at 400 x g for thirty minutes. The supernatant solution was saved. The precipitate was resuspended and layered over the same discontinuous gradient and centrifuged at 1,000 x g for ten minutes. The supernatant solutions were pooled, diluted 1:1 with deionized water and centrifuged at 12,000 x g. The pellet was saved and the supernatant layer was discarded. The resuspended pellet was layered on a discontinuous gradient according to the usual procedure for the SW 25.2 rotor.

Enzymatic Assays

5'Nucleotidase (233)

5'Nucleotidase is considered to be characteristic of the plasma membrane. The substrate for the 5'nucleotidase assay was a solution of 5.5 mM Tris, pH 8.5 containing 11 mM AMP and 5.5 mM $MgCl_2$. Membrane suspension, 0.2 ml, was added to 0.18 ml of substrate. The reaction was stopped after 15 minutes with 0.2 ml of trichloracetic acid. The solution was centrifuged and the supernatant solution was assayed for phosphate. The amount of supernatant containing phosphate was varied from 50 to 100 µl in the assay for phosphate. Water was added to make the volume complete at 0.86 ml. 0.1 ml of molybdate and 0.04 ml of reducing agent (237) were added completing the total volume of 1.0 ml. The absorbance was read after 20 minutes at 660 nm. The assay was carried out at 25° C

with no preincubation. A standard curve for phosphate was made at the same time as the assay was run.

Glucose-6-Phosphatase (233)

Glucose-6-phosphatase is considered to be characteristic of endoplasmic reticulum. The assay procedure is the same as the assay for 5'nucleotidase except for the substrate. Eleven mM glucose-6-phosphate and eleven mM mercaptoethanol in 55 mM Tris buffer at pH 6.6 was used as the substrate.

Galactosyltransferase (238)

Galactosyltransferase is considered to be a marker enzyme for the Golgi apparatus. The incorporation assay was used which measures the incorporation of galactose-^{14}C from UDP-galactose to glucose or to N-acetylglucosamine as the acceptor. The assay with glucose was a four minute assay at 37° C. The assay with N-acetylglucosamine was a 15 minute assay at 37° C.

Amino Acid Analysis

One milligram of membrane protein was hydrolyzed in 6 N HCl in evacuated sealed tubes at 110° C for 28 hours. Twenty-five microliters of norleucine (0.25 µmoles) were added to one ml as an internal standard (239). The analysis was performed on a Beckman Model 120C acid analyzer (240).

Carbohydrate Determination

Neutral sugars and hexasamines were released by hydrolysis of protein in two normal H_2SO_4 for 4 hours at 100° C in sealed tubes. The hydrolysate was through a column (0.9 x 8 cm) of Dowex 50-X4(H^+) (200-400 mesh) coupled to a column (1.35 x 4 cm) of Dowex 1-X8 (formate) (200-400 mesh) (241). The effluent and wash were lyophilized. The neutral sugars were determined by the Technicon automatic sugar chromatography systems (242). Hexosamines were determined on the short column of the amino acid analyzer after elution from Dowex 50 with 2 N HCl. Sialic acid was determined by the thiobarbituric assay of Warren (243) after protein was hydrolyzed with 0.1 N HCl at 80° C for 1 hr. N-acetylneuraminic acid was used as the standard. Protein was determined by the Lowry procedure (244) using bovine serum albumin as the standard.

Preliminary carbohydrate analysis of R3230 AC mammary tumor membranes showed the presence of ribose. This was eliminated by a twenty hour incubation of 5 mg of protein in 5 mls of sodium acetate buffer, pH 5, containing 0.5 mg of ribonuclease.

Lipid-free membranes were analyzed for amino acids and carbohydrate and were prepared in the following manner. Lyophilized membranes were suspended in deionized water. If dry tissue is not rehydrated there may be an incomplete extraction of highly polar lipids. The membrane suspension was homogenized with a 2:1 chloroform:methanol solution. The solution was allowed to stand about ten minutes with frequent agitation. The solution was filtered through a sintered glass funnel. The solution was discarded after the membranes were washed with an 8:4:3 chloroform: methanol:water solution. Methanol was used to wash off chloroform and five washes were needed. Methanol was removed with five washes of

deionized water (245). The membranes were lyophilized and stored frozen.

Gel Electrophoresis Studies

Membrane proteins were separated using gel electrophoresis. Standard procedures for preparing and running gel electrophoresis (246-248) were modified according to the procedures developed by Kobylka and Carraway (249,250). The ratio of acrylamide to N,N'-methylenebisacrylamide (BIS) was constant at 1:0.027. The potassium presulfate was 0.075% and (TEMED), N,N,N',N'-tetramethylenediamine was 0.066% regardless of the gel percentage. The buffer was 0.1% sodium dodecyl sulfate-0.10 M phosphate buffer, pH 7.2. Lyophilized membrane samples were dissolved, 10 mg/ml, in 3.0% (w/v) 0.1% sodium dodecyl sulfate-0.10 M phosphate buffer, pH 7.8, containing 0.14 M mercaptoethanol and 20% (w/v) glycerol. The solution was incubated in a boiling water bath for five minutes. Approximately 100-150 µg of membrane sample was applied to each gel. The period of electrophoresis was about four hours. Protein bands were fixed and stained in 0.05% Coomasie blue-10% methanol-7% acetic acid and destained with 7% acetic acid in a Hoefer type destainer. The gels were scanned at 550 nm with a Gilford 2000 spectrophotometer fitted with a Model 2410 linear transport module.

Results

Enzymic Assays in Rat Mammary and Mammary Tumor Plasma Membranes

Differential centrifugation was used for purification of the plasma membranes. Whole cells and nuclei were sedimented from the homogenized cellular suspension at 600-1,000 x g. Membranes were isolated from the

supernatant suspensions at 7-8,000 x g. Lysosomes, mitochondria, microsomes, endoplasmic reticulum and Golgi apparatus should sediment well above 8,000 x g and were discarded in the supernatant suspension. Both the zinc stabilized membranes and Tris prepared membranes were approximately 70% protein. The Fluorescein mercuric acetate prepared membranes were approximately 50% protein.

5'-Nucleotidase was used to assay for the plasma membrane. Glucose-6-phosphatase, an enzymic marker for endoplasmic reticulum and the galactosyl transferase, an enzymic marker for the Golgi apparatus were also measured. The results are given in Table XI.

Specific activity in μmoles/min/mg protein for 5'-nucleotidase ranges from 1.5×10^{-3} in rabbit leukocyte membranes to 0.67 in rat liver membranes and 12.5 in membranes from rabbit macrophages found in the lung (251). The values in both the normal and tumor membranes for the 5'-nucleotidase is quite low for the plasma membrane. Rodbell's (252) procedure which uses 3.3 mg/ml collagenase has been found to change the enzymatic activity of 5'-nucleotidase found in plasma membrane (251).

Solyom and Tram (251) believe that 5'-nucleotidase is not a true marker enzyme specific for the plasma membrane. The activity in whole tissue is the result of a group of isoenzymes and one or two of these seem to be the true plasma membrane enzymes. They found that there was no correlation between the yield of membrane protein and enzyme recovery of 5'-nucleotidase (251). The lysosomal membrane 5'-nucleotidase at pH 7.5 has the same specific activity as the liver plasma membrane 5'-nucleotidase (253). Cytoplasmic 5'-nucleotidase has a pH optimum between 6.2 and 6.9 and a K_m about half for the enzyme from the plasma membrane (254). Mammary tumor cell activity from Table XI is about ten times

TABLE XI

ENZYMIC ACTIVITIES OF MAMMARY AND TUMOR MEMBRANES

Specific Activity[+] µmoles/min/mg	Mammary Cell	Tumor Cell
5'Nucleotidase	$2.6 \times 10^{-3} \pm 0.3$	$3.6 \times 10^{-2} \pm 0.3$
Glucose-6-phosphatase	N. D.	N. D.
Galactosyl transferase[++]	$2.3 \times 10^{-5} \pm 0.3$	$3.2 \times 10^{-6} \pm 1.0$

[+]Each value is an average of three determinations. N.D. = not detectable.

[++]N-acetylgalactosamine was the substrate. Assay time = 15 min.

higher than the 5'-nucleotidase activity of the normal plasma cell membrane.

Glucose-6-phosphatase is not considered to be a good microsomal marker since it is not exclusively a microsomal enzyme. In pig platelet preparations 65% of the glucose-6-phosphatase activity was in the soluble phase. The remaining 35% was evenly distributed. 17% was in the granular and 17% was in the membrane fraction (255). Glucose-6-phosphatase activity could not be demonstrated in the plasma membranes of normal and tumor membranes which would suggest little contamination by endoplasmic reticulum. NADH-cytochrome C has been suggested to be a better marker enzyme for the endoplasmic reticulum (251).

Endogenous incorporation of UDP-galactose-^{14}C with R3230 AC mammary tumor cell membranes for four minutes at 37° C using glucose as an acceptor was 6% and 10% in two experiments, each using 25 µl of membrane suspension. The incorporation into lactose was in each case about 3.5% above endogenous levels. Galactosyltransferase activity for the tumor cell membrane in a fifteen minute incorporation assay with N-acetylglucosamine as the acceptor was 3.2×10^{-6} µmoles/min/mg of protein while the activity for the normal membrane was 2.3×10^{-5} µmoles/min/mg of protein. These values are extremely low and do not represent any significant activity. Unfortunately, the enzymic activities were not measured in the crude homogenate so that an enrichment factor could be calculated.

Gel Electrophoresis

The lyophilized membranes were difficult to solubilize. Lyophilized R3230 AC tumor plasma cell membranes were not soluble when incubated at

room temperature overnight in 3% sodium dodecyl sulfate. Solubility improved when the Tris prepared membranes were incubated with 3% sodium dodecyl sulfate at 37° C for two minutes and became soluble when incubated in 3% sodium dodecyl sulfate at 100° C for five minutes. Protein separation in disc gels was still poor.

No improvement in solubility was seen by varying the sodium dodecyl sulfate concentration from 4 to 10%. When membrane protein was increased to 10 mg/ml and electrophoresis in sodium dodecyl sulfate-acrylamide gel was done well developed protein bands were found after staining with 0.05% Coomasie blue in 7% acetic acid containing 10% methanol as a fixative. There was considerable background smearing from top to bottom in the gels. Adding urea to a concentration of 4% (w/v) to the membrane solution in 3% sodium dodecyl sulfate did not enhance solubilization and band formation. Background smearing became more enhanced.

In the general procedure, 10 mg/ml of lyophilized membrane protein were solubilized in 3% SDS at 100° C for five minutes and kept overnight at 4° C. The preparation was warmed to room temperature before use. Tris prepared erythrocyte membranes (RBC) were soluble under all conditions and were clearly more soluble than the zinc or Tris prepared tumor cell membranes. Fluorescein mercuric acetate stabilized membranes gave no protein bands when electrophoresed.

Tris prepared mammary cell membranes seemed to be more soluble than zinc stabilized mammary cell membranes. Bands of higher molecular weight proteins were distinguished for Tris preparations whereas the same bands were faint for zinc prepared membranes (Figure 7). Clearly there are seven or eight main protein bands in both preparations. Lower molecular weight proteins give clearer bands with the zinc preparations.

1 2 3 4 5 6 7 8 9

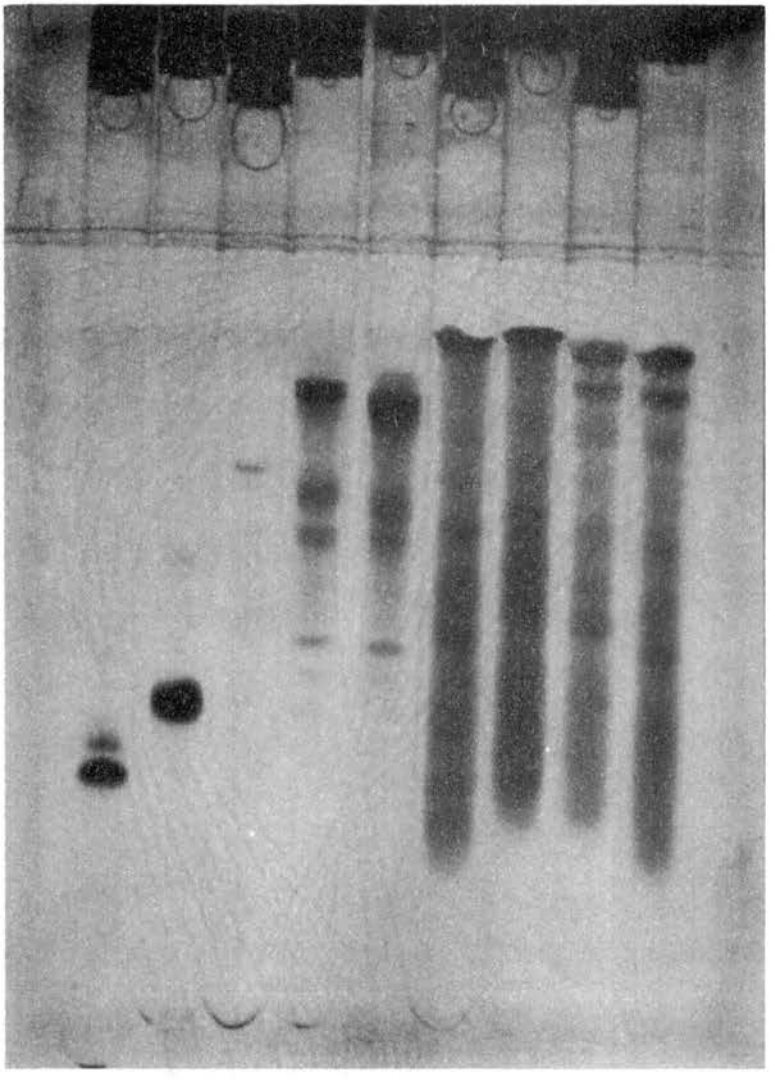

Figure 7. Gel Electrophoresis Patterns of Rat Mammary Cell Membranes in SDS-Acrylamide Gels

Standards: 1, BSA; 2, LDH; 3, β-galactosidase; 4,5, RBC standards; 6,7, Zn mammary; 8,9, Tris mammary.

This same observation was also true of zinc prepared R3230 AC tumor cell membranes. The higher molecular weight proteins do not seem to enter the gel while the lower molecular weight protein bands are quite distinct (Figure 8). Four proteins in both the mammary cell membranes and tumor cell membranes appear to correspond to four main bands of the red cell membrane. There are approximately twenty bands in both the mammary cell membrane and the tumor cell membrane.

The -galactosidase dimer has a molecular weight of 260,000 daltons, bovine serum 68,000 daltons and catalase 60,000 daltons (256). The molecular weight of red blood cell proteins vary from 20,000 to 190,000 daltons when compared to a series of protein standards. From Figure 9 the molecular weights would seem to vary from 20,000 to over 250,000 daltons.

Gel scans (Figure 10) show high background staining with Comassie blue for both normal mammary cell and tumor cell membrane proteins. Trypsinized membrane preparations have been shown to give a high background stain in Dr. Kermit Carraway's laboratory at Oklahoma State University. There is a possibility that some proteolytic degradation of membranes has taken place.

Amino Acid and Carbohydrate Analysis of Membranes

From a study of the graphs from the amino acid analyzer of all three membrane preparations, Fluorescein mercuric acetate, zinc and Tris, two small peaks before aspartate showed that small amounts of cysteic acid and hydroxyproline were present. There was a small shoulder on histidine which indicates that a small amount of hydroxylysine was probably present.

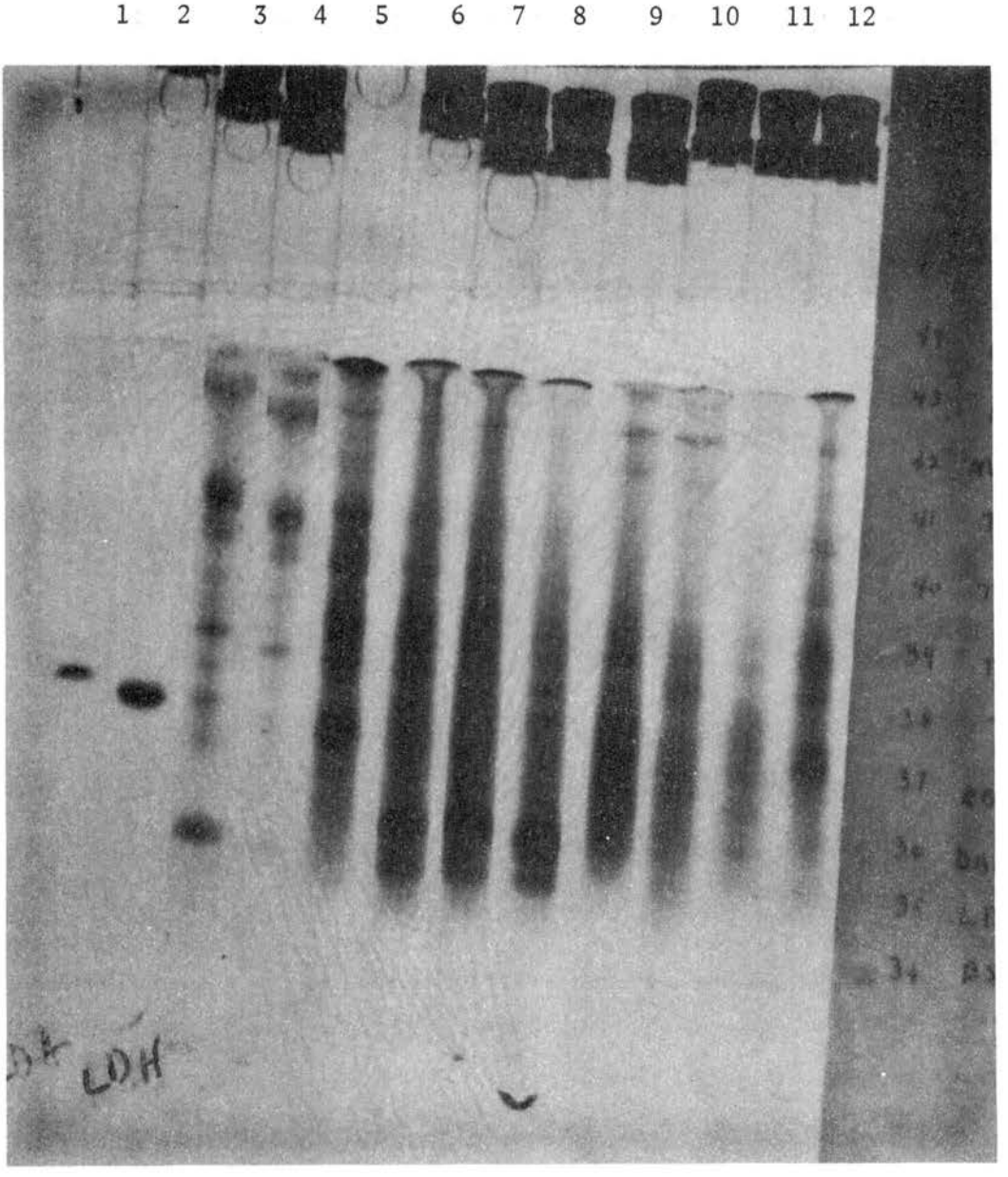

Figure 8. Gel Electrophoresis Patterns of R3230 AC Mammary Tumor Cell and Mammary Cell Membranes in SDS-Acrylamide Gels

1,2, LDH standards; 3,4, RBC standards; 5,12, Tris membranes-tumor; 6,7,8, Zn membranes-tumor; 9,10,11, mammary.

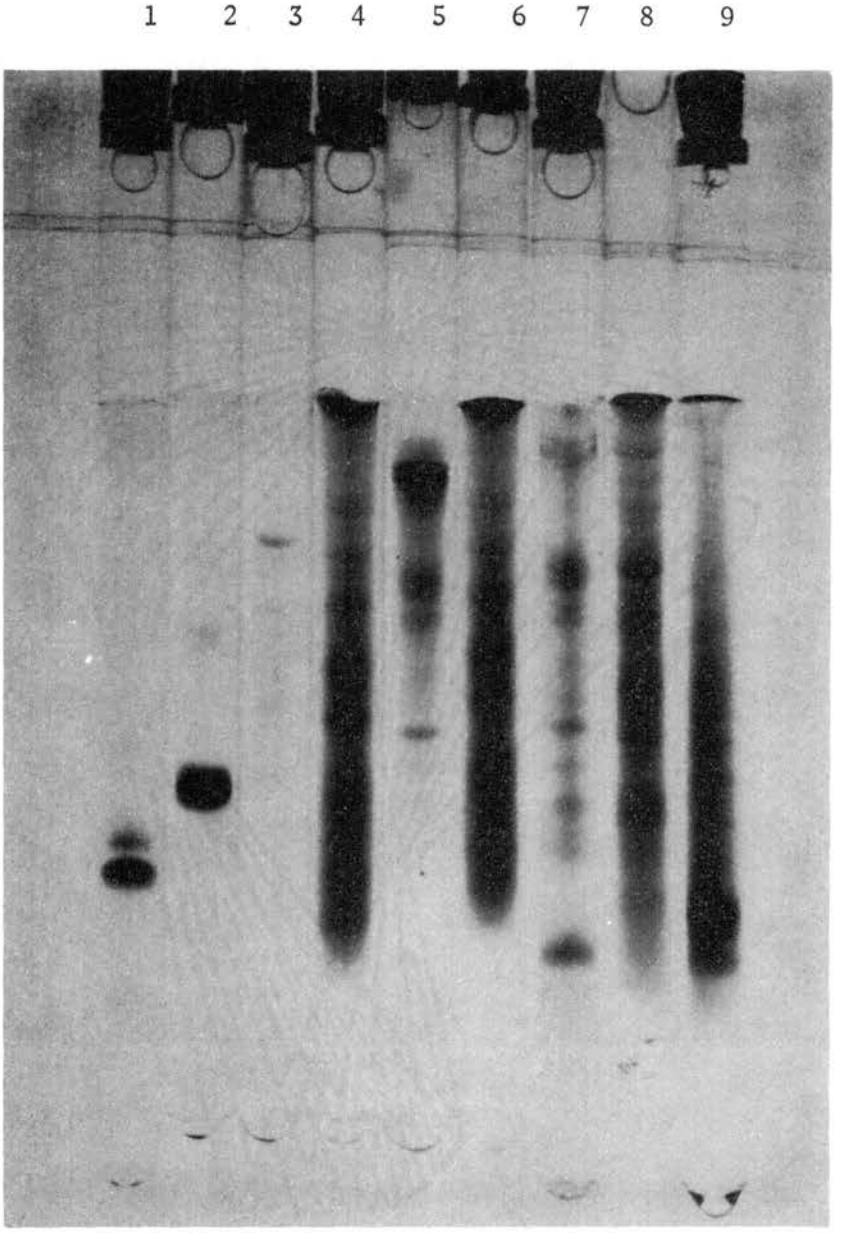

Figure 9. Molecular Weight Approximations From Gel Electrophoresis Patterns and Mammary Cell Membranes

Standards: 1,2,3, BSA, LDH, and β-galactosidase; 5,7, RBC standards; 6, Tris membranes-mammary; 8, Tris membranes-tumor; 4, Zn membranes-mammary; 9, Zn membranes-tumor.

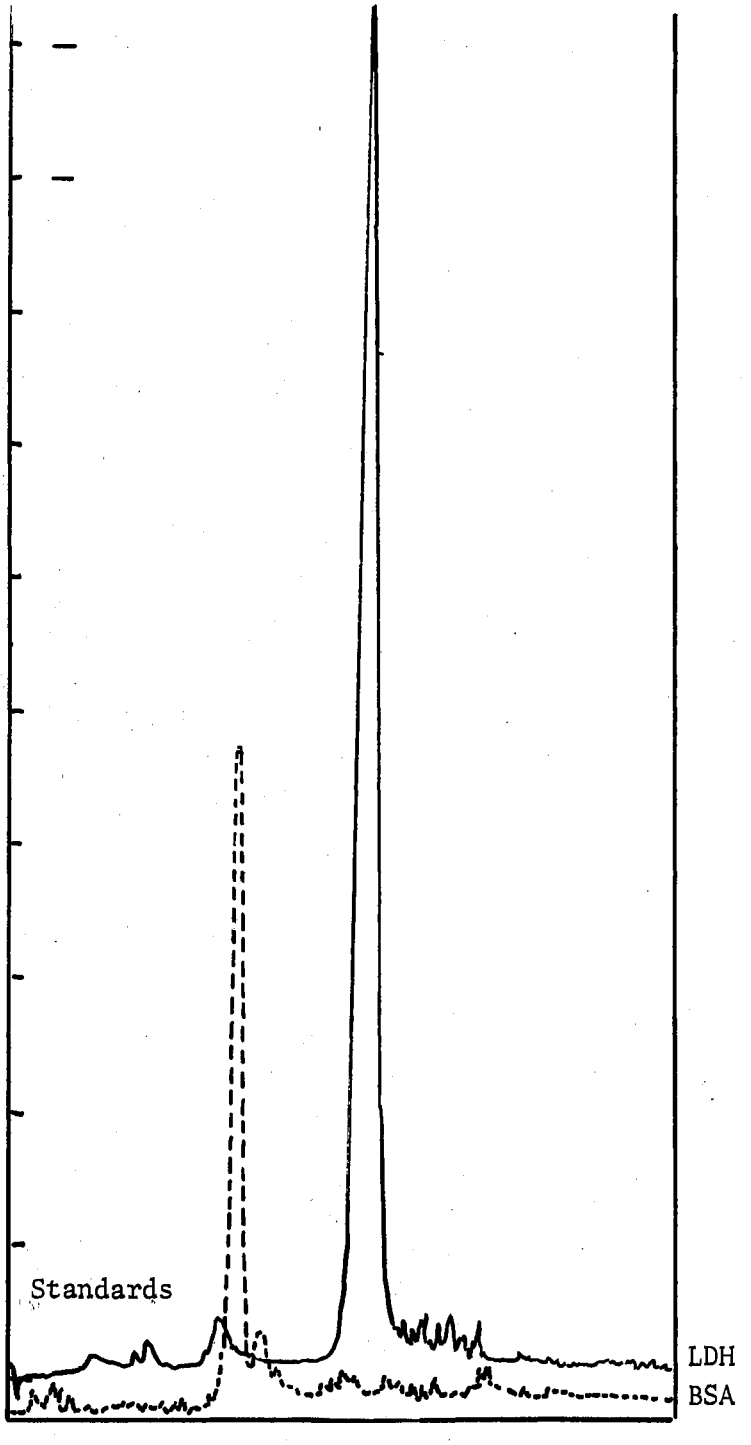

Figure 10. Gel Scans of Representative Gels of Tris and Zinc Preparations of Normal and Tumor Cell Membranes

____, Zn; ----, Tris, at 560 nm, 0.1.

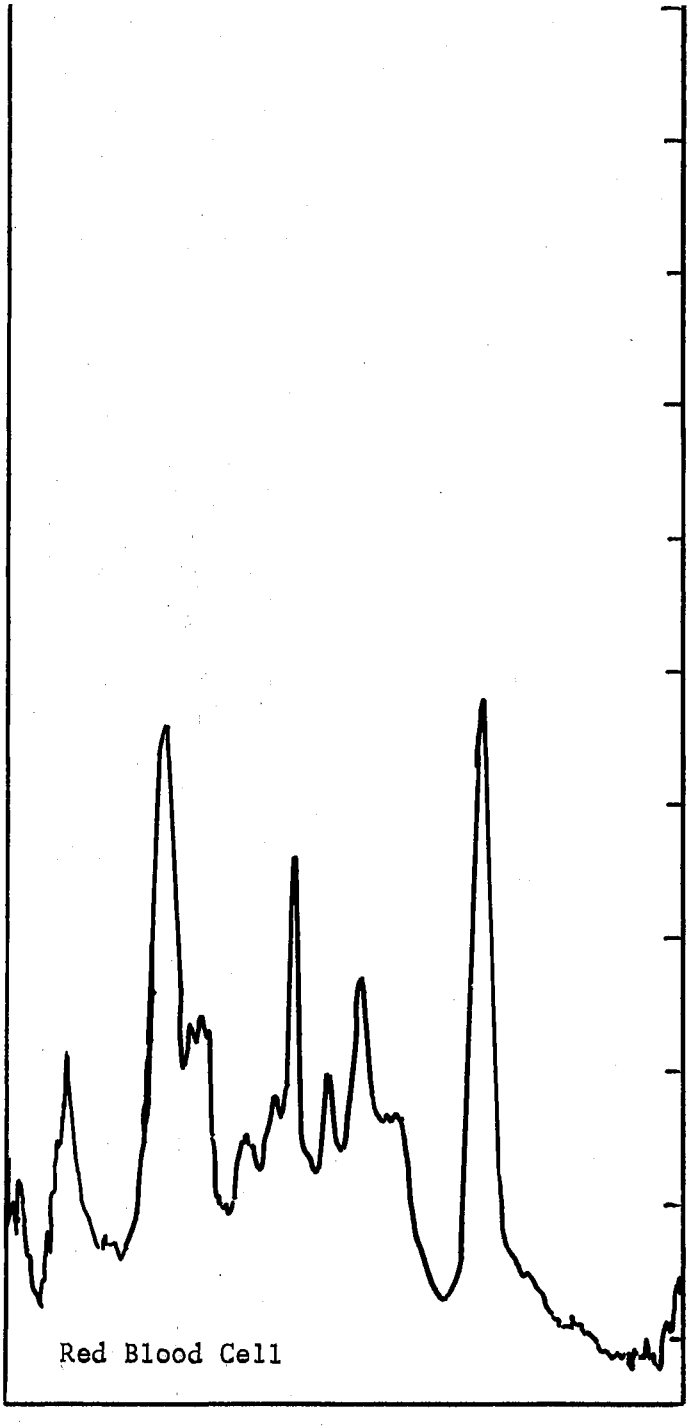

Figure 10 (Continued)

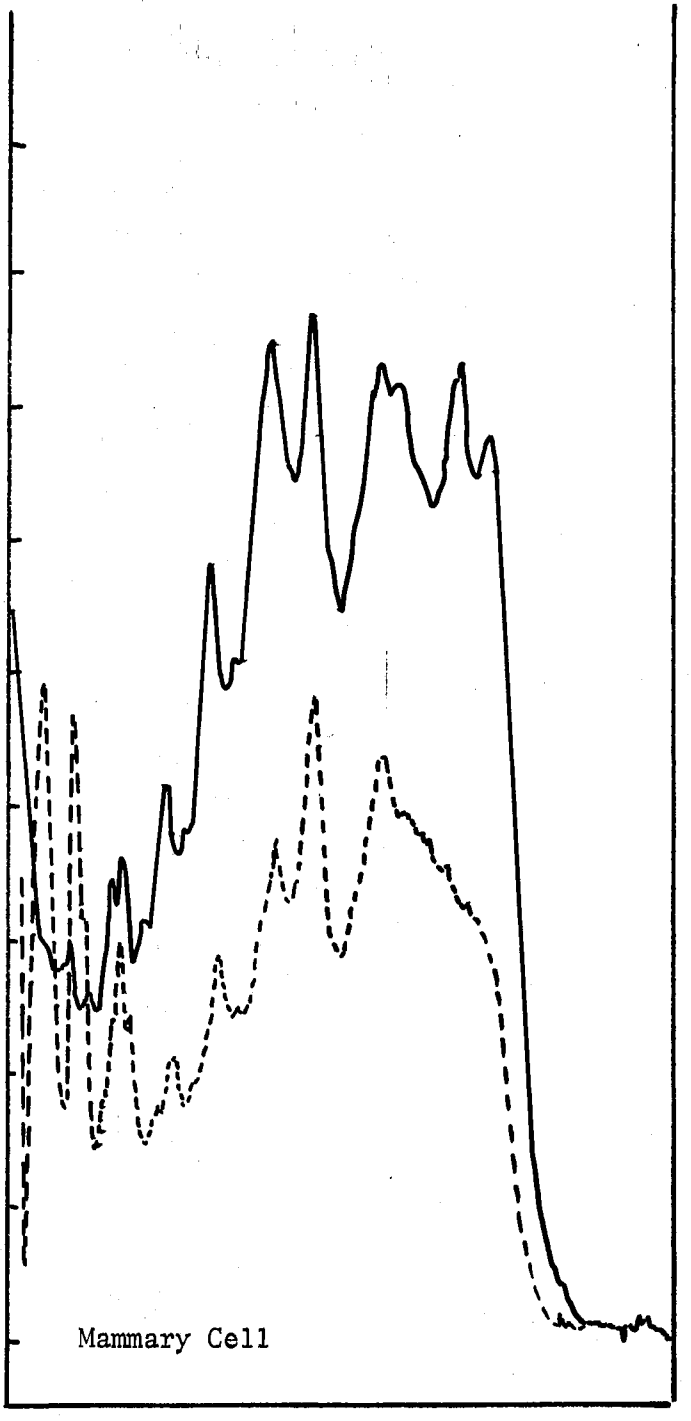

Figure 10 (Continued)

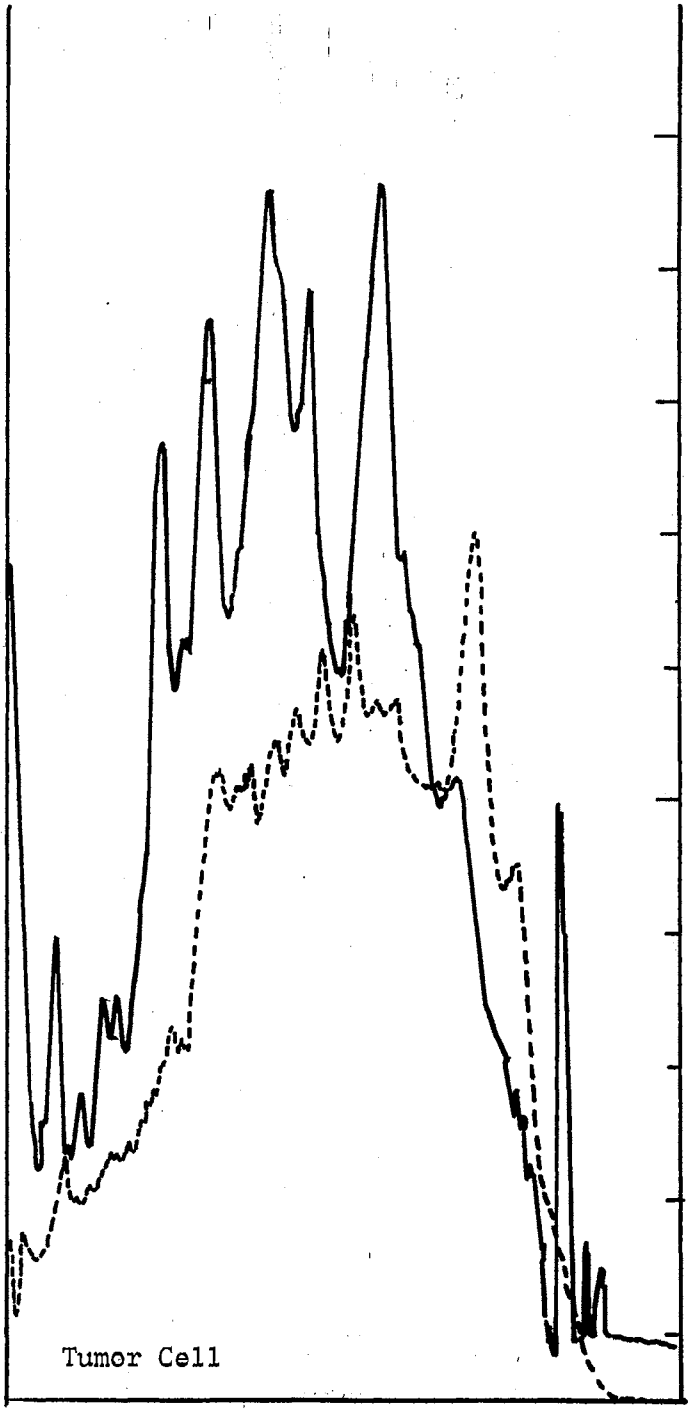

Figure 10 (Continued)

A shoulder on the arginine peak indicates the presence of tryptophan. None of these ninhydrin positive compounds were quantitatively measured. A rather large unidentified peak appeared between the lysine and the ammonia peak which is probably ethanolamine.

Table XII gives the amino acid composition and carbohydrate composition of Fluorescein mercuric acetate prepared tumor cell membranes derived from cells isolated by a collagenase-hyaluoronidase incubation as well as from cells derived by a Wiepjes-Prop procedure. The cells in the Wiepjes-Prop procedure undergo an additional pronase incubation. Pronase treated cells had lower values for seven nonpolar amino acids which were threonine, proline, glycine, alanine, valine, methionine, and isoleucine. Seven polar amino acids in these cells had higher values. They were lysine, histidine, arginine, aspartic acid, glutamic acid, leucine and tyrosine. Phenylalanine and serine had the same value in both preparations. The amounts of galactose, glucose, fucose and galactosamine were less in pronase treated cells. The low amount of galactosamine suggests the loss of a glycoprotein fragment.

Table XIII, sample #9 seems to verify the conclusion that the collagenase-hyaluronidase procedure might be a better cell isolation procedure because the zinc stabilized membranes gave somewhat similar results as shown above with four exceptions, lysine, leucine, tyrosine and aspartic acid. Glutamic acid values showed less of a loss. Zinc chloride may stabilize these amino acids in the membrane.

Table XIV shows the comparison of amino acid composition and carbohydrate composition of three membrane preparations from cells isolated according to the normal enzymatic procedure. The best correlation for neutral sugars is between the zinc and Tris prepared membranes. These

TABLE XII

COMPARISON OF AMINO ACID AND CARBOHYDRATE COMPOSITION OF FMA TUMOR CELL MEMBRANES FROM TWO CELL ISOLATION TECHNIQUES

Component	Residues/1000 Amino Acid Residues	
	Cell Isolation Technique	
	# 2 Wiepjes-Prop Method	# 13 Collagenase-Hyaluronidase
Amino Acids[a]		
Lysine	71.9	59.7
Histidine	24.6	16.5
Arginine	73.2	55.0
Aspartic Acid	135.8	94.6
Threonine	51.0	57.0
Serine	61.5	60.4
Glutamic Acid	136.6	111.6
Proline	41.2	52.8
Glycine	50.9	85.4
Alanine	53.4	89.6
Valine	56.1	67.0
Methionine	6.6	19.8
Isoleucine	45.5	51.6
Leucine	103.0	89.9
Tyrosine	41.7	27.9
Phenylalanine	46.4	46.4
Half-Cystine	---	15.0
Monosaccharides		
Neutral Sugars[b]	20.7	26.3
Mannose	8.3	5.5
Galactose	2.7	4.5
Glucose	7.5	12.4
Fucose	1.8	3.4
Xylose	0.7	0.4
Hexosamines[c]	4.0	4.3
Glucosamine	3.9	3.0
Galactosamine	0.1	1.2
Sialic Acid[d]	2.2	1.1
Total		

[a]Protein samples were hydrolyzed in 6 N HCl in vacuo at 110° for 28 hrs.

[b]Determined on Technicon carbohydrate analyzer after release from protein with 2 N H_2SO_4 at 100° for 4 hours.

[c]Determined on Amino Acid analyzer using short column procedure.

[d]Determined by thiobarbituric acid method after hydrolysis with 0.1 N HCl for 1 hour at 80°.

TABLE XIII

COMPARISON OF AMINO ACID COMPOSITION OF DIFFERENT MEMBRANE PREPARATIONS (RESIDUES/1000 A. A. RESIDUES)[1]

Component	$ZnCl_2$ Stabilized Membranes					FMA Stabilized Membranes		
	Prop Isolation		Collagenase-Hyaluronidase			Prop Isolation	Collagenase-Hyaluronidase	
	# 4	# 6	# 7	# 8	# 8	# 2	# 13	# 9
Lysine	65.7	70.4	74.3	79.5	55.1	71.9	59.7	60.8
Histidine	21.5	31.3	26.0	20.8	19.2	24.6	16.5	23.9
Arginine	60.4	58.9	35.3	53.0	49.6	73.2	55.0	50.3
Aspartic Acid	104.2	117.0	109.0	118.9	115.1	135.8	94.6	105.6
Threonine	54.9	62.2	59.0	50.1	67.6	51.0	57.0	57.6
Serine	61.7	68.7	62.7	59.4	68.4	61.5	60.4	64.8
Glutamic Acid	113.5	121.6	109.8	111.7	105.9	136.6	111.6	128.8
Proline	38.6	42.8	38.0	42.1	44.4	41.2	52.8	48.8
Glycine	93.8	91.5	93.7	95.7	84.5	50.9	85.4	87.9
Alanine	77.6	82.7	83.1	84.0	76.9	53.4	89.6	88.8
Valine	64.6	72.9	79.0	65.8	61.5	56.1	67.0	77.6
Methionine	11.7	6.9	15.7	3.9	10.3	6.6	19.8	8.8
Isoleucine	52.8	44.1	56.6	51.3	47.8	45.5	51.6	39.1
Leucine	97.4	76.7	106.8	102.8	103.1	103.0	89.9	94.4
Tyrosine	26.6	13.1	23.9	15.2	31.5	41.7	27.9	23.1
Phenylalanine	40.7	38.6	45.9	36.9	44.2	46.4	46.4	39.1
Half-Cystine	13.4		14.6	8.1	14.0		15.0	

[1]Protein samples were hydrolyzed in 6 N HCl in vacuo at 110° for 28 hrs.

TABLE XIV

AMINO ACID AND CARBOHYDRATE ANALYSIS OF COLLAGENASE-HYALURONIDASE ISOLATED R3230 AC TUMOR CELL MEMBRANES

Component	Residues/1000 A. A. Residues		
	Method of Preparation		
	FMA (# 13)	Zinc (# 12)	Tris (# 14)
Amino Acids[a]			
Lysine	59.7	73.6	67.9
Histidine	16.5	20.9	20.7
Arginine	55.0	56.2	68.7
Aspartic Acid	94.6	86.2	91.3
Threonine	57.0	53.1	53.8
Serine	60.4	54.5	51.5
Glutamic Acid	111.6	169.1	173.1
Proline	52.8	39.2	51.7
Glycine	85.4	85.8	69.7
Alanine	89.6	87.1	69.6
Valine	67.0	63.1	55.1
Methionine	19.8	15.4	20.1
Isoleucine	51.6	47.7	47.7
Leucine	89.9	69.3	73.9
Tyrosine	27.9	28.7	30.2
Phenylalanine	46.4	36.8	39.7
Half-Cystine	15.0	13.1	15.1
Monosaccharides			
Neutral Sugars[b]			
Mannose	5.5	3.1	2.1
Galactose	4.5	2.5	2.4
Glucose	12.4	6.8	6.3
Fucose	3.4	1.2	1.0
Xylose	0.4	0.2	0.4
Hexosamines[c]			
Glucosamine	3.0	3.0	1.8
Galactosamine	1.2	1.3	2.7
Sialic Acid[d]	1.1	1.5	0.7
Total			

[a,b,c,d]Footnotes as in Table XII.

two preparations appear to offer the best possibility for comparison of amino acids and carbohydrates in the membrane since Fluorescein mercuric acetate is a sulfhydryl reagent and could preferentially remove glycoprotein.

Table XIX verifies this judgement particularly when lipid-extracted membranes are compared. For example carbohydrate values correlate better in the lipid-extracted membranes. A large amount of the glucose in Zn and Tris preparations is extracted by the lipid solvents indicating the loss of a large amount of glycolipid especially in the tumor membrane. Mannose is not removed in Zn preparations, but is removed in Tris preparations.

Tables XV, XVI, XVII, and XVIII show the original data as it is systematically converted to residues/1000 amino acid residues in Table XIX. These tables were retained and included to make comparisons with values reported in the literature.

One unusual feature of both normal mammary and tumor cell membrane is the presence of xylose. The protein carbohydrate linkage of protein to acidic glycosaminoglycans is a serine-xylose linkage (18). Glycosaminoglycans are connective tissue polymers. There is a large amount of glucose and mannose present in R3230 AC tumor cell membrane. Glucose is extracted when glycolipid is extracted. Connective tissue also has a high amount of glucose. Even after lipid extraction mannose levels are high. Ribose was found in some membrane preparations as shown in Table XVI. Since NaCl did not initially remove ribose from an FMA stabilized membrane preparation from R3230 AC tumor cells, the RNA was not cytoplasmic RNA. The ribose presumably comes from attached ribosomes and was removed by incubation with ribonuclease (Table XVI). Warren has

TABLE XV

AMINO ACID COMPOSITION OF FISCHER MAMMARY CELL MEMBRANES AND R3230 AC MAMMARY TUMOR CELL MEMBRANES (μMOLES/100 MG PROTEIN)[1]

Amino Acid	Normal				Tumor			
			Lipid Extracted				Lipid Extracted	
	Zn	Tris	Zn	Tris	Zn	Tris	Zn	Tris
Lysine	12.6	18.9	20.0	24.8	14.7	3.5	20.9	20.2
Histidine	4.7	5.0	6.0	7.8	3.7	1.0	5.9	6.7
Arginine	9.5	14.3	14.1	16.4	10.7	2.9	15.7	17.0
Aspartic Acid	18.1	23.4	24.0	27.8	20.5	5.7	25.3	31.0
Threonine	9.6	13.1	14.3	16.7	10.8	3.3	15.5	16.1
Serine	11.5	15.7	18.5	20.1	12.9	3.9	18.7	19.2
Glutamic Acid	21.5	32.0	29.2	39.7	19.2	6.8	29.9	33.5
Proline	10.3	14.0	11.5	16.3	8.9	3.3	12.1	15.5
Glycine	14.2	16.4	18.7	20.8	14.7	4.5	20.9	21.3
Alanine	15.5	19.7	20.3	24.8	14.9	4.5	20.7	22.0
Valine	11.6	17.7	19.0	20.4	12.4	3.8	18.0	19.1
Methionine	3.5	5.4	5.2	6.9	3.6	0.8	5.1	6.3
Isoleucine	8.1	11.9	11.1	15.0	7.5	2.3	10.8	13.2
Leucine	18.5	23.3	25.3	28.1	16.5	5.1	24.9	27.8
Tyrosine	4.4	7.3	7.1	8.8	5.1	1.6	8.0	8.9
Phenylalanine	7.8	10.4	12.6	12.9	9.1	3.0	12.5	12.9
Half-Cystine	2.8	5.2	2.8	5.5	1.8	0.7	3.4	5.7
Total	185.6	254.6	260.4	313.7	187.9	57.4	269.0	297.5

[1]Protein samples were hydrolyzed in 6 N HCl in vacuo at 110° for 28 hrs.

TABLE XVI

CARBOHYDRATE COMPOSITION OF FISCHER MAMMARY MEMBRANES AND R3230 AC MAMMARY TUMOR MEMBRANES (μMOLES/100 MG PROTEIN)

Monosaccharide	Normal				Tumor			
			Lipid Extracted				Lipid Extracted	
	Zn	Tris	Zn	Tris	Zn	Tris	Zn	Tris
Hexoses[b]	4.9	13.4	4.0	6.8	14.5	38.5	8.7	4.7
Mannose	1.7	6.8	2.3	3.6	4.6	0.9	6.4	2.1
Galactose	1.0	3.6	1.0	1.4	1.2	0.6	1.5	1.6
Glucose	2.1	2.9	0.6	1.8	8.7	36.9	0.8	0.9
Fucose	0.3	0.5	0.3	0.7	0.6	7.0	0.003	0.4
Pentoses[b]	3.8	3.1	5.7	5.3	---	0.5	---	3.0
Ribose	3.8	3.0	5.5	4.3	---	0.5	---	2.7
Xylose	0.1	0.1	0.1	0.9	0.1	0.1	0.3	0.2
Hexosamines[c]	1.8	2.4	2.8	1.9	1.8	0.9	4.1	3.0
Glucosamine	1.1	1.8	1.8	1.2	1.2	0.5	2.6	2.0
Galactosamine	0.7	0.6	0.9	0.6	0.5	0.3	1.5	0.9
Sialic Acid[d]	0.2	0.3	0.6	0.5	0.2	0.0	0.3	0.6
Total	11.3	20.0	13.5	15.3	17.2	47.1	13.4	11.9

[b,c,d]Footnotes as in Table XII.

TABLE XVII

AMINO ACID COMPOSITION OF FISCHER MAMMARY CELL MEMBRANES AND R3230 AC MAMMARY TUMOR CELL MEMBRANES (MG/100 MG)[1]

Amino Acid	Normal				Tumor			
			Lipid Extracted				Lipid Extracted	
	Zn	Tris	Zn	Tris	Zn	Tris	Zn	Tris
Lysine	1.6	2.4	2.5	3.1	1.8	0.4	2.6	2.5
Histidine	0.6	0.6	0.8	1.0	0.5	0.1	0.8	0.9
Arginine	1.4	2.2	2.2	2.5	1.6	0.4	2.4	2.6
Aspartic Acid	2.0	2.6	2.7	3.2	2.3	0.6	2.9	3.5
Threonine	0.9	1.3	1.4	1.6	1.0	0.3	1.5	1.6
Serine	1.0	1.3	1.6	1.7	1.1	0.3	1.6	1.6
Glutamic Acid	2.7	4.1	3.7	5.1	2.4	0.8	3.8	4.3
Proline	1.0	1.3	1.1	1.5	0.8	0.3	1.1	1.5
Glycine	0.8	0.9	1.0	1.1	0.8	0.2	1.1	1.2
Alanine	1.1	1.4	1.4	1.7	1.0	0.3	1.4	1.5
Valine	1.1	1.7	0.9	2.0	1.2	0.3	1.7	1.8
Methionine	0.4	0.7	0.6	0.9	0.4	0.1	0.6	0.8
Isoleucine	0.9	1.3	1.2	1.7	0.8	0.2	1.2	1.4
Leucine	2.1	2.6	2.8	3.1	1.8	0.5	2.8	3.1
Tyrosine	0.5	1.1	1.1	1.4	0.8	0.2	1.3	1.4
Phenylalanine	1.1	1.5	1.8	1.9	1.3	0.4	1.8	1.8
Half-Cystine	0.1	0.5	0.2	0.5	0.1	0.1	0.3	0.5
Total	20.0	28.3	27.8	34.9	20.7	6.3	29.3	33.0

[1]Protein samples were hydrolyzed in 6 N HCl in vacuo at 110° for 28 hrs.

TABLE XVIII

CARBOHYDRATE COMPOSITION OF FISCHER MAMMARY MEMBRANES AND R3230 AC MAMMARY TUMOR MEMBRANES (MG/100 MG SAMPLE)

Monosaccharide	Normal				Tumor			
	% Wt. of Protein				% Wt. of Protein			
			Lipid Extracted				Lipid Extracted	
	Zn	Tris	Zn	Tris	Zn	Tris	Zn	Tris
Hexoses[b]	7.9	21.8	6.5	11.1	2.4	62.4	1.4	7.6
Mannose	2.8	11.1	3.8	5.8	0.8	1.5	1.0	3.4
Galactose	1.6	5.9	1.6	2.3	0.2	1.0	0.3	2.6
Glucose	3.4	4.7	1.0	2.9	1.4	59.8	0.1	1.5
Fucose[b]	0.5	0.8	0.5	1.0	0.02	1.0	---	0.6
Pentoses[b]	5.3	4.3	7.9	7.3	---	0.8	---	4.1
Ribose	5.2	4.1	7.7	6.0	---	0.7	---	3.8
Xylose	0.1	0.2	0.1	1.2	0.1	0.1	0.5	0.3
Hexosamines[c]	3.0	3.9	4.5	3.1	2.9	1.4	6.6	4.9
Glucosamine	1.8	2.9	3.0	2.0	2.0	0.9	4.1	3.3
Galactosamine	1.2	1.0	1.5	1.0	0.9	0.5	2.5	1.5
Sialic Acid[d]	0.7	1.0	1.7	1.4	0.7	0.1	0.9	1.9
Total	17.6	32.0	21.2	24.1	43.1	65.8	9.4	19.3

TABLE XIX

AMINO ACID AND CARBOHYDRATE ANALYSIS OF FISCHER MAMMARY CELL MEMBRANES
R3230 AC MAMMARY TUMOR CELL MEMBRANES

Component	Residues/1000 A. A. Residues							
	Normal				Tumor			
			Lipid Extracted				Lipid Extracted	
	Zn	Tris	Zn	Tris	Zn	Tris	Zn	Tris
Amino Acids[a]								
Lysine	68.1	74.2	76.8	79.0	78.6	61.4	77.9	68.0
Histidine	25.8	19.7	23.0	24.9	20.1	17.9	22.2	22.7
Arginine	51.6	56.3	54.2	52.4	57.1	51.8	58.5	57.3
Aspartic Acid	97.6	92.0	92.4	88.9	109.5	100.8	94.3	104.3
Threonine	51.9	51.5	55.0	53.3	57.6	57.4	57.6	54.1
Serine	62.4	62.0	71.3	64.3	69.0	68.7	69.5	64.7
Glutamic Acid	116.2	126.0	112.3	126.7	102.2	118.4	111.3	112.6
Proline	56.0	55.3	44.2	51.9	47.5	57.5	45.2	52.3
Glycine	76.6	64.5	72.1	66.5	78.6	79.4	77.9	71.6
Alanine	83.5	77.6	78.0	79.0	79.5	79.7	76.9	74.1
Valine	62.5	69.7	73.1	65.0	66.4	67.1	67.1	64.3
Methionine	19.0	21.5	20.1	22.0	19.2	15.4	19.0	21.4
Isoleucine	44.0	46.7	42.9	48.1	40.3	41.0	40.3	44.4
Leucine	99.9	91.4	97.1	89.6	88.2	89.0	92.6	93.4
Tyrosine	24.1	28.7	27.5	28.2	27.3	28.3	29.9	30.1
Phenylalanine	42.1	41.2	48.4	41.1	48.5	53.0	46.6	43.3
Half-Cystine	15.0	20.4	10.9	17.6	9.8	12.4	12.7	19.3
Monosaccharides[b]								
Mannose	9.5	26.9	8.9	11.5	24.5	16.8	23.8	7.1
Galactose	5.5	14.3	3.8	4.5	6.4	11.2	5.6	5.5
Glucose	11.3	11.5	2.5	5.7	3.7	642.4	3.0	3.2
Fucose	2.1	2.3	1.3	2.3	0.3	123.2	---	1.5
Ribose	20.5	11.8	21.4	13.9	---	9.2	---	9.2
Xylose	0.2	0.6	0.5	2.9	0.5	0.8	1.3	0.8
Glucosamine[c]	6.1	7.0	7.1	4.0	6.7	10.2	9.6	7.0
Galactosamine[c]	4.0	2.5	3.7	2.1	2.9	5.4	5.7	3.2
Sialic Acid[d]	1.3	1.4	2.3	1.5	1.3	0.9	1.2	2.2

[a,b,c,d]Footnotes as in Table XII.

considered that RNA may be an integral part of the membrane (25).

Virally transformed cells appear to have a high glucosamine content in glycoproteins of nuclei, mitochondria, surface membrane, endoplasmic reticulum, soluble glycoproteins and in nucleotide sugars (257). The glucosamine content of R3230 AC mammary tumor membrane is not much higher than that found in the normal cell membrane. The reported value for rat ascites Hepatoma (Table II) was 59.2 mM/mg (.06 μmoles/m) of protein. The value for the R3230 AC tumor cell membrane is .02 μmoles/mg protein (Table XI). Total hexoses (258) for rat red blood cell membranes is reported as 64 μg/mg of protein, for rat liver cell membranes 45 μg/mg of protein and for kidney brush borders 37 μg/mg of protein. The value for normal mammary cell membrane varies from 70 μg/mg of protein from zinc preparation to 160 μg/mg of protein for Tris preparations. Total hexoses of zinc preparations of the tumor cell membrane had 20 μg/mg of protein and Tris membranes varied. The lipid extracted membranes had 76 μg/mg of protein while the membrane not lipid extracted was 600 μg/mg of protein. The high value was due solely to the presence in one case of extremely high amounts of glucose which is probably an artifact resulting from sucrose contamination which was not completely removed by washing.

Sialic acid values (258) for rat red blood cell membranes was reported as 61 μmoles/mg of protein. For rat liver cell membranes it was 27 μmoles/mg of protein and for rat kidney brush borders it was 34 μmoles/mg of protein. Sialic acid for the rat mammary cell membrane was 43.5 μmoles/mg of protein and for the mammary tumor cell membrane it was 64.4 μmoles/mg of protein.

Values for carbohydrates from normal and transformed cells are given in Table I. A comparison of carbohydrate values before and after lipid

extraction of rat ascites Hepatoma cells is given in Table II.

Summary

Membranes of both normal and tumor cells were isolated by three methods. The zinc chloride and Tris methods gave membranes whose protein content, amino acid and carbohydrate composition correlated well whereas the Fluroescein mercuric acetate membranes of the R3230 AC mammary tumor had 20% less protein than the other two corresponding membrane preparations.

Both normal and tumor membranes have similar acrylamide gel patterns. Each have about twenty protein bands.

There was little difference between the amino acid and carbohydrate composition of the membranes of normal mammary cells and the R3230 AC tumor cell membranes. High glucose and mannose content was found in the tumor cell membranes. Glucose was removed by lipid extraction. This might mean that there is higher glycolipid content in the tumor cell membrane. It could mean also that a methanol soluble glycoprotein, perhaps a connective tissue origin, was extracted. The presence of xylose indicates connective tissue glycans and connective tissue is high in glucose.

5'-Nucleotidase activity of the R3230 AC mammary tumor membrane was ten times higher than in the normal mammary cell membrane. Galactosyltransferase activity was about seven times less than for the normal mammary cell membrane though these levels were extremely low.

As a degradative enzyme one of the functions of the 5'-nucleotidase might be in the control of nucleic acid metabolism (259). Increased activity would diminish the pool of available mononucleotides and

restrict synthesis of nucleic acids. In a detailed study of organs and tumors of rats with low 5'-nucleotidase activity the RNA content and thymidine incorporation were both high. This might explain the high ribose content observed with both membrane preparations.

CHAPTER V

ASSAY FOR AGGREGATION OF NORMAL MAMMARY CELLS AND R3230 AC MAMMARY TUMOR CELLS OF THE FISCHER CDF RAT

Introduction

Studies on morphogenesis emphasized cellular movements and adhesiveness between cells (260). Most of the early studies were qualitative experiments. One quantitative measurement made at this time was the diameter of the aggregate formed through cell contact in a gently rotating media (84).

A more quantitative assay for aggregation was needed for sorting out the molecular events taking place in the adhesive process. Several types of assays were developed. Kemp (183) used a turbidometric assay where the decrease in single cells was measured at 600 nm. Curtis and Greaves (181) used a hemocytometer to count single cells and aggregates at regular intervals during aggregation. The disappearance of single cells was the measure of aggregation. Roth and Weston (180) collected labeled cells on preformed aggregates. The number of adhering cells were determined from a radioautograph. Roth (177) simplified the assay by counting the labeled cells in a liquid scintillation counter. Orr (87) used a similar method to that of Curtis (181) in that he followed the disappearance of single cells with a Coulter counter.

The mediating agent of aggregation of platelets (153) was identified

as ADP. Prostaglandins (161) also are involved in the control of platelet aggregation. Thyrotropin (63) mediates the aggregation of thyroid cells. In general, there seems to be no involvement of energy linked processes or protein synthesis in the early phase of aggregation of most cells (87).

It is thought that molecular groups on one membrane surface may complement molecular groups on the opposite membrane surface (139). Ginsburg (52) found that lymphocytes incubated with glycosidases lost their specificity for lymphatic tissue. Roth (177) found that treating cells with glycosidases, particularly β-galactosidase caused chick embryo neural retina cells to be five times more adhesive. He showed evidence for the presence of cell-surface glycosyltransferases on neural retina cells (88) and on mouse embryo cell lines, Balb/c 3T3 and Balb/c 3T12 (89). Roseman (176) suggested that the complementary molecules on cell membranes are complex carbohydrates on one cell surface and glycosyltransferases on the other cell surface.

This study was involved with intercellular adhesion of enzyme-dissociated cells of the normal mammary gland and of the R3230 AC mammary tumor of the Fischer rat. The rate of adhesion for each cell type was measured by determining the decrease in single cells. Large molecular weight proteins and carbohydrates were tested for their effect on intercellular adhesion.

Experimental Procedures

Materials

Hyaluronidase, bovine testis type I, collagenase, cl. histolyticum, type one containing both protease and peptidase activity, bovine serum

albumin, fraction V (BSA), Hepes, Ficoll penicillin-G, potassium salt, streptomycin sulfate, and neomycin sulfate were obtained from Sigma. Pronase, B grade and colchichine were from Calbiochem, Dextran T-100 and Dextran T-500 from Pharmacia Fine Chemicals and polyvinylpyrrolidine from Matheson, Coleman and Bell. Fetal calf serum (FCS), medium 199, 10 x concentrated, F-10, and F-12 were obtained from Grand Island Biological. Virgin Fischer CDF rats were obtained from the Charles Rivers Laboratories. The R3230 AC tumor was obtained from Dr. Russell Hilf through the courtesy of Squibb Institute of Medical Research and the Mason Tumor Bank.

Methods

Preparation of Tissue and Cells

Preparation of tissue and cells of the normal mammary gland and of the R3230 AC mammary tumor of the Fischer rat were done according to the procedure given in Chapter III.

Preparation of Media

Hanks minimal media (Hanks) and medium 199 (Med 199) were prepared with 2 mg/liter glucose, penicillin G, potassium salt (0.15 mg/ml), streptomycin sulfate (0.1 mg/ml) and neomycin sulfate (0.1 mg/ml). Medium 199 was buffered with 10 mM Hepes. Hanks minimal media buffered with 10 mM Hepes is called Hepes. When it is buffered with $NaHCO_3$ it is called Hanks. The pH was 7.4 unless otherwise mentioned. Solutions were sterilized by filtering through a 0.22 μ millipore filter after the substrates were added. All cell preparations and transfers were done in a Tenney laminar flow hood. Suspension cultures were placed either in

25 ml Erlenmeyer flasks or in 20 ml test tubes and rotated at 80 rpm at 37° C in the Microbiological Associates Waterbath Shaker.

Preliminary Experiments

Measurement of Single Cells. A distribution curve for Mouse L cells was determined manually with the Model B, Coulter Counter. The upper threshold mode switch was set in the locked position. The upper threshold was set at 50 and the lower threshold was changed by values of 5. A cell count was made at each lower threshold value and recorded for systematic combinations of amplification and current settings. From these values a size distribution curve was determined (Figure 11). Mouse L cells have a size which is quite homogeneous and gives the expected distribution curve. The best aperture current setting was 1/2 and the best amplitude was 4. If the lower threshold is set at approximately 20, all the background count is eliminated. If the window settings are 20 and 100, 100% of the L cells will be counted.

Similarly aperture current and amplification settings were systematically changed and counts made for the R3230 AC mammary tumor cells. Figure 12 shows the symmetrical distribution at an aperture setting of 1/4 and an amplitude of 2. As the amplitude is increased the distribution curve shifts to the left. It was difficult to remove all of the contaminating red blood cells from the tumor cells. To determine if their signal could be eliminated at the amplification and current settings chosen for the R3230 AC tumor cell count, red blood cells were mixed 1:10 with the tumor cells. Figure 13 is the distribution curve for the mixture. At an amplification of 1/2, red blood cells are detected. The curve is shifted to the left at an amplification of 2 and only R3230 AC

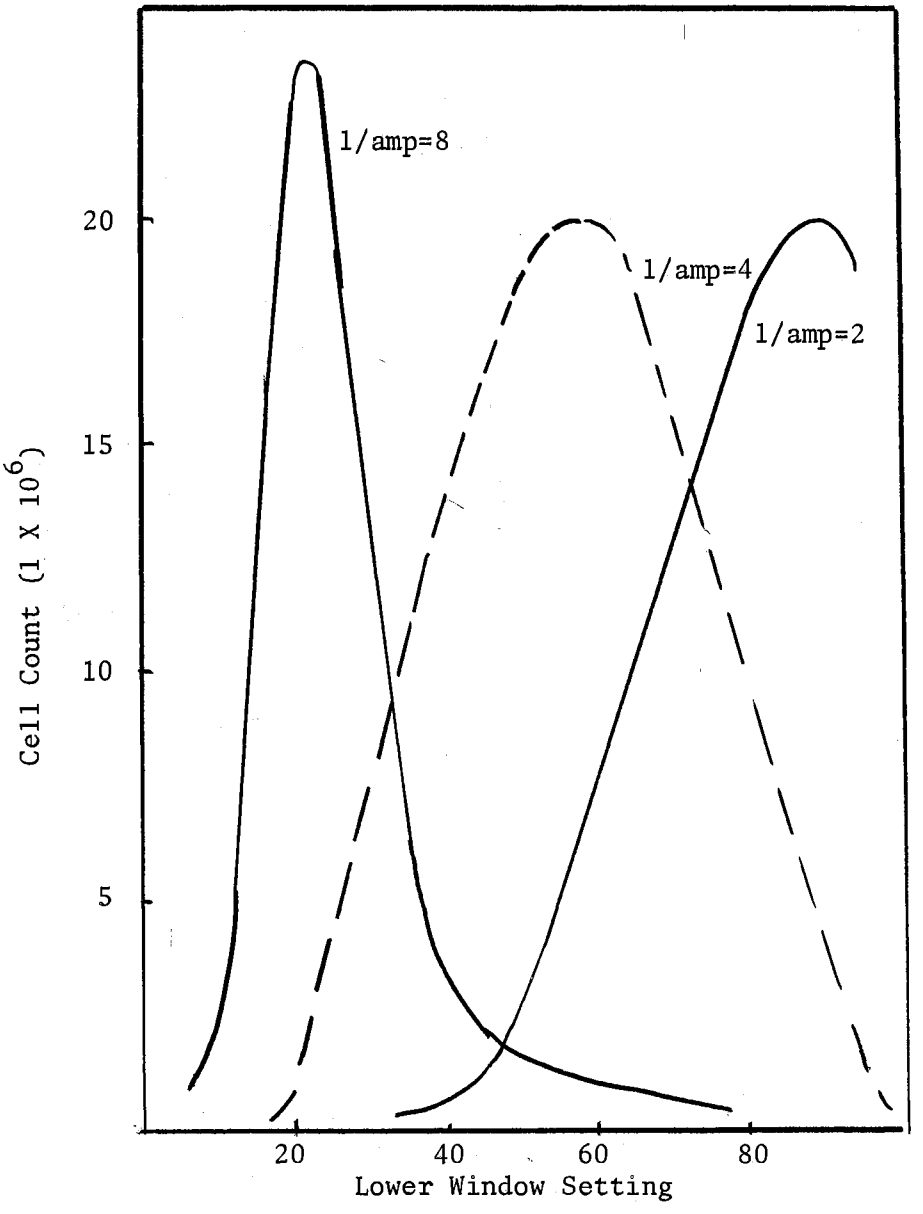

Figure 11. Size Distribution of Mouse L Cells

It is used as a standard for calibration of the Model B, Coulter Counter. Locked position. Upper window 50, lower window settings change by 5. 1/aperature current = 1/2. 1/amplification changes as shown.

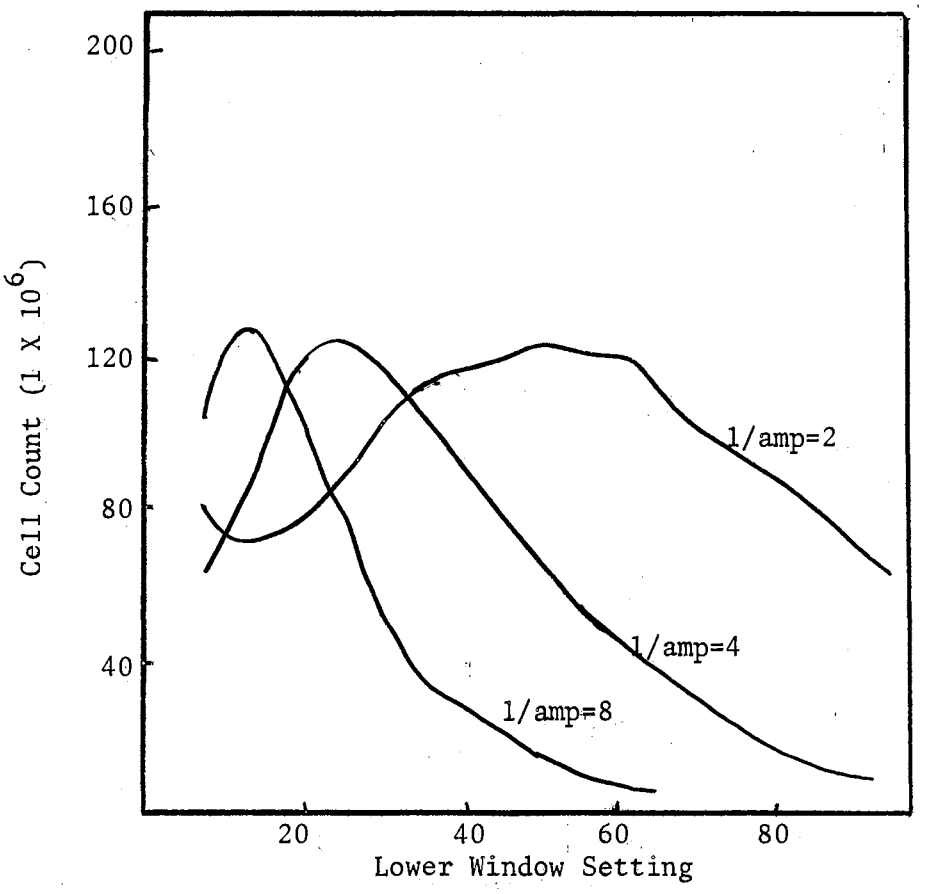

Figure 12. Size Distribution of Isolated R3230 AC Mammary Tumor Cells

Model B, Coulter Counter. Locked upper window 50. Lower window setting changes by 5. 1/aperature current = 1/4. 1/amplification = 2, 4, 8.

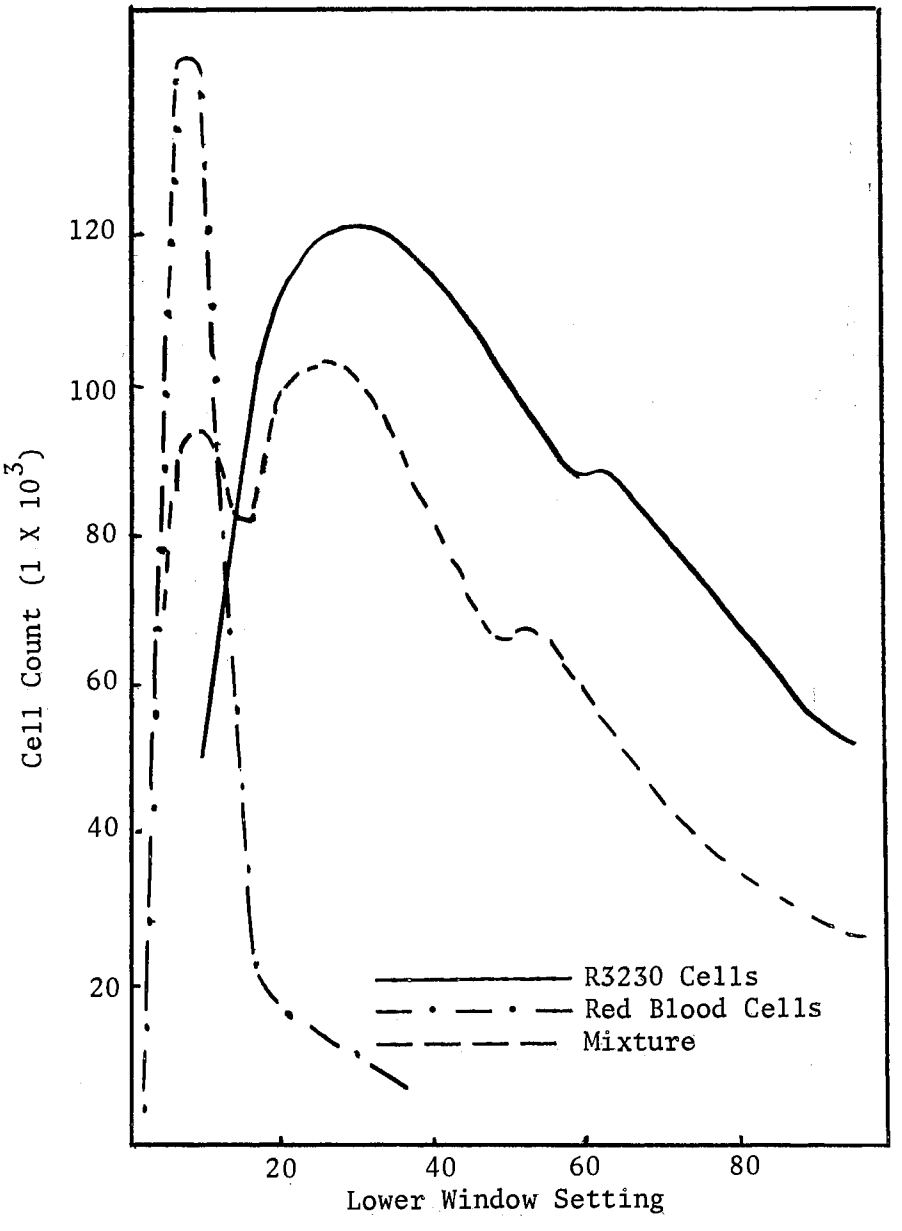

Figure 13. Size Distribution of a Mixture of Rat Red Blood Cells and R3230 AC Tumor Cells (1:10)

Model B Coulter Counter. Locked upper window 50. Lower window settings change by 5. 1/aperature current = 1/4. 1/amplification = 2.

tumor cells are counted between the settings of 20 and 100. Figure 14 shows that normal mammary cells can be counted at the same amplification and current settings as the R3230 AC tumor cells. The shape of both curves, for mammary cells and tumor cells are not symmetrical which shows that the size of cells present is not homogeneous. Cells were sized on a hemocytometer slide and they varied from a diameter of about 10 μ to 25 μ. About 80% of the cells will be counted between the upper and lower threshold values of 20-90.

The hemocytometer count was compared with that of the Coulter Counter. It was from 3-10 times lower than that of the Coulter Counter. The time for a hemocytometer count was approximately 10-15 minutes. No aggregates seemed to find their way into the counting well and direct count of aggregates did not seem to be possible.

Procedure for the Assay of Aggregation. Basis for the Assay. Orr and Roseman (87) developed a convenient assay for quantitatively measuring cellular aggregation using the Coulter Counter. The most reproducible and accurate method was measuring the disappearance of single cells as a function of time. Both the normal mammary cells and the R3230 AC mammary tumor cells were counted at settings of 1/amplification = 2 and 1/aperature current = 1/4 with a window of 20-90. The decrease in the number of single cells over time was used as a measure of aggregation.

_____. Sampling Procedure. To achieve a homogeneous suspension, the culturing flask was quickly flicked with the wrist three times. A sample, 0.2 ml, of the cell suspension was suspended in 19.8 ml of physiological saline, 0.9% NaCl. Three samplings were made for each reading. The Counting solution was carefully aspirated three times with

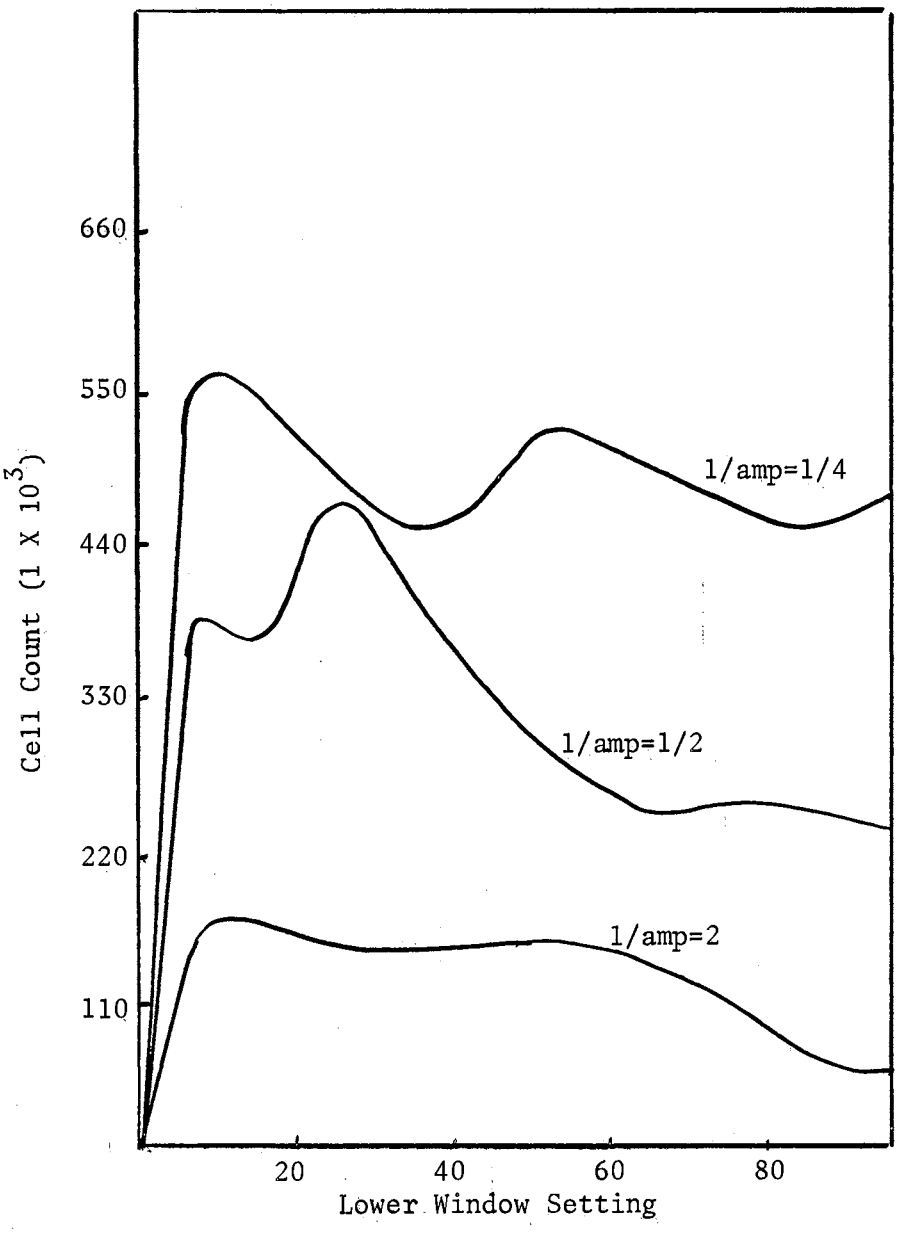

Figure 14. Size Distribution of Isolated Mammary Cells of the Fischer Rat

Model B Coulter Counter. Locked upper window 100. Lower window settings change by 10. 1/aperature current = 1/4. 1/amplification = 2, 1/2, 1/4.

a pasteur pipette to provide a homogeneous suspension for counting. One reading was made of each of the three samples and recorded. The results were averaged and the standard deviation was determined.

_____. Time of Counting. Initially counts were taken every hour for a period of eight hours. After another eight hour period three counts were made at 4 hour intervals and a final count was made at 46 hours.

At 2-4 hours there was often a 5-10% increase of single cells followed by a second increase at 24-46 hours. A representative counting curve is given in Figure 15 which shows that the rate of loss of single cells was a slow process. The initial increase of single cells could be due to disaggregation of an initial lossely formed aggregate. Daday (179) reported an initial disaggregation of some neural retinal cell aggregates after two hours of aggregation. There could also be a small increase in the cell population or it could result from error in the sampling and counting procedures.

Experiments were performed to determine if the experimental design had any influence on the experimental result. A flask was prepared with sufficient cells to allow for continuous removal of samples as aggregation proceeded. This was called the stock culture. Sufficient individual flasks or test tubes were also prepared to allow for removal of samples after eight hour intervals although counts were still made at two hour periods. In this way aggregation was not interrupted by frequent agitation and pipetting. This procedure allowed a comparison of aggregation rate in disturbed cell suspensions and undisturbed cell suspensions.

High molecular weight proteins and carbohydrates were tested to determine if they affected the aggregation process. The effect of pH,

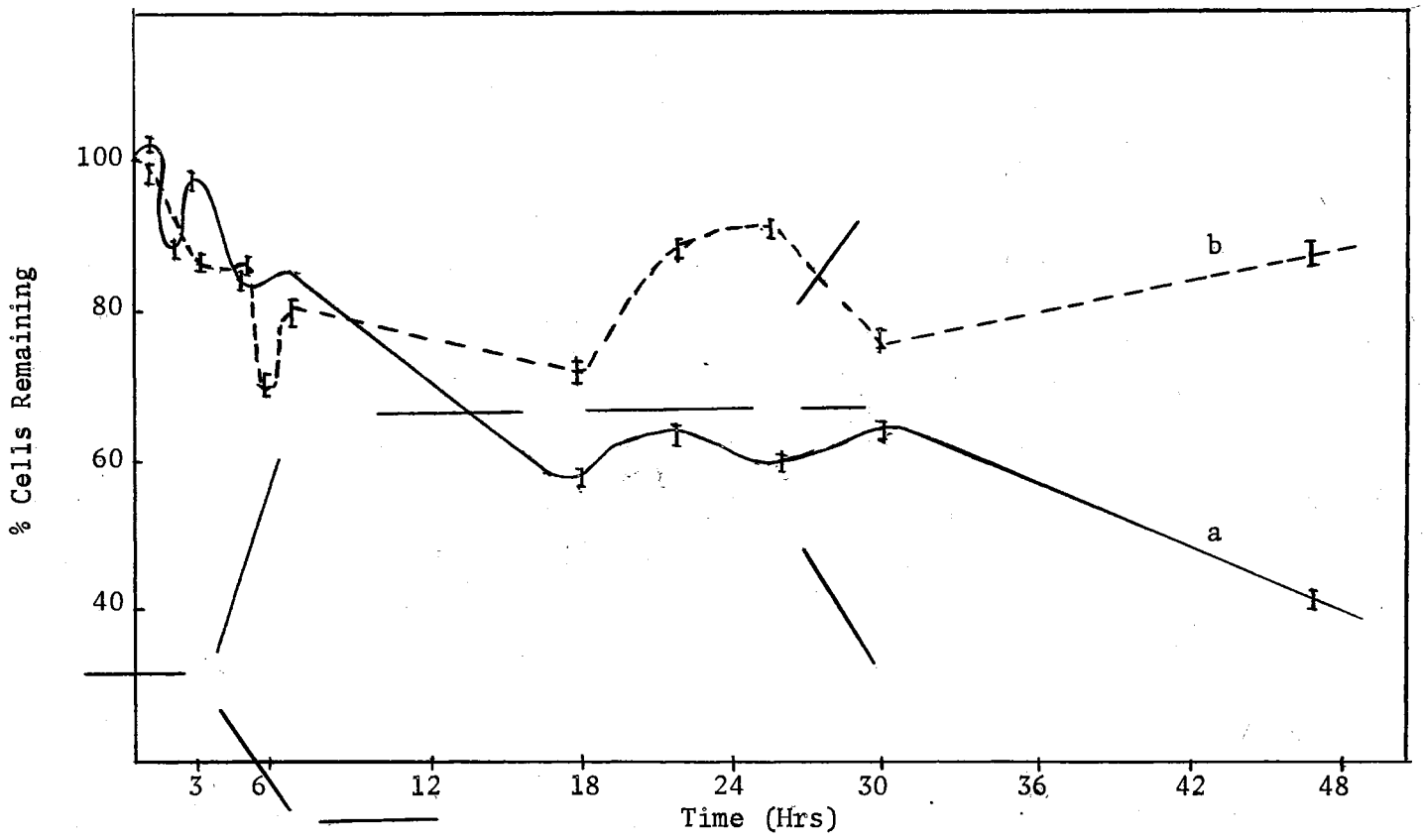

Figure 15. Aggregation of R3230 AC Mammary Tumor Cells in (a) Hanks Minimal Media, 0.1% BSA, (b) Hanks Media With 0.1% BSA, 10% FCS

initial cell concentration and colchichine was determined on the aggregation process.

Results

The experiments on sizing of the R3230 AC mammary tumor cells and the normal mammary cells (Figures 12-14) showed that settings of the current at 1/4 and the amplitude at 2 were optimal for measuring the disappearance of single cells and also effectively eliminated contamination by red blood cells. The rate of aggregation was relatively slow since about 50% of the single cells remained at 24 hours.

When a stock solution of R3230 AC mammary tumor cells was continuously sampled for aggregation and compared to individual samples which were relatively undisturbed, the disappearance of single cells was almost twice as great for the individual samples as for the stock solution. In the remaining experiments, flasks were prepared identically to allow sampling after about eight hours of aggregation. Counting was done every 3-4 hours on alternate flasks.

The rate of aggregation for R3230 AC mammary tumor cells was the same in Hepes minimal media as it was in Medium 199 and this was about 2.3 % $\pm$ 1% per hour over a 20 hour period. Similar results were obtained with normal mammary cells. The rate over a 20 hour period was about 3.0% $\pm$ 1% per hour. Immediate clumping of both normal and R3230 AC mammary tumor cells occurred in media F-10 and F-12. The initial clumping was usually followed by a period of disaggregation and the count of single cells increased over the initial count. It is possible that the initial count was low since the clumping occurred very rapidly. In any case, F-10 and F-12 were not suitable media for aggregation studies.

Table XX shows that there is decreased aggregation as the amount of Bovine serum albumin (BSA) in Medium 199 is increased from 0-5%. Table XXI also shows a decreased aggregation as the amount of Fetal calf serum (FCS) is increased in Medium 199.

A small amount of high molecular weight protein is generally necessary to preserve cellular function in cells when they are cultured over long periods of time (261). This also serves to prevent initial clumping of cells and allows aggregation to be followed by disappearance of single cells. Normal cells, however, aggregated differently from the tumor cells in the presence of BSA or FCS. Normal cells aggregated slowly for four hours from 3-17%. This was followed by a long period of disaggregation from 20-40% which covered about 12 hours in BSA and 40 hours in FCS. The normal cells resumed aggregation slowly after this time, from 20-50% over a period of another 40 hours.

Increasing cell concentration from 1.0 to 6 x 10^6 cells/ml does not seem to have too much effect on the rate of aggregation. There was somewhat less aggregation as concentration of cells was increased but this may have resulted from a general lowering of pH over the period of time they were in suspension. A cell concentration between 1.0-3.0 x 10^6 cells/ml seems to afford optimum aggregation over a 24 hour period. Table XXII shows that aggregation of R3230 AC mammary tumor cells decreased as the pH was increased from 7.3 to 8.0. Table XXIII shows that in normal mammary cell suspensions, aggregation increased as pH was increased from 7.1 to 8.4. This may indicate that the mechanism of aggregation of the two cell types is quite different.

Colchichine was used in an attempt to synchronize cells if they were dividing in suspension. It is known to cause cells to remain in mitosis.

TABLE XX

EFFECT OF INCREASING CONCENTRATION OF BSA IN MEDIUM 199 ON AGGREGATION OF R3230 AC TUMOR CELLS

% BSA Added	Initial Cell Concentration (1×10^6) (100%)	% Single Cells[+] Remaining
0	3.6	50
0.05	3.3	80
0.10	3.2	76
0.50	3.1	72
5.00	4.1	72

[+]Each value is an average of three counts at 20 hrs.

TABLE XXI

EFFECT OF INCREASING CONCENTRATION OF FCS IN MEDIUM 199 ON AGGREGATION OF R3230 AC TUMOR CELLS

% FCS Added	Initial Cell Concentration (1×10^6) (100%)	% Single Cells[+] Remaining
0	4.1	54
0.50	3.6	54
1.00	4.0	56
5.00	3.3	68
10.00	3.1	72

[+]Each value is an average of three counts at 20 hrs.

TABLE XXII

EFFECT OF pH ON AGGREGATION OF R3230 AC MAMMARY TUMOR CELLS

pH	Initial Cell Concentration (1×10^6) (100%)	% Cells[+] Remaining
7.3	3.0	72
7.7	3.0	85
8.0	3.0	90

[+]Each value is an average of three counts after 24 hrs. The media used was Medium 199 + 0.1% BSA.

TABLE XXIII

EFFECT OF pH ON AGGREGATION OF NORMAL MAMMARY CELLS

pH	Initial Cell Concentration (1×10^6) (100%)	% Cells[+] Remaining
7.1	1.0	70
7.7	1.1	45
8.4	1.5	29

[+]Each value is an average of three counts after 19 hrs. The media was Medium 199 + 0.1% BSA.

If cell replication occurred during the aggregation period it should be prevented by the addition of colchichine. Samples of R3230 AC mammary tumor cells were taken at differing time periods, stained for best contrast of chromosomes, and examined under the light microscope for mitotic figures, but none were found. These experiments should be tested by more reliable methods to verify the absence of mitotic figures.

The observed increase of single cells after four hours (2%-10%) and after 24 hours (10-20%) of aggregation time could indicate that some cell replication was taking place. After twenty-four hours, cells cultured in Medium 199 with 0.1% BSA (pH 7.3) had 63% of the cells remaining while in the same media with colchichine added there were 72% of the cells remaining (Table XXIV). At pH 7.7 both cultures had about 85% of the cells remaining. At pH 8.0 the control had 90% of the cells remaining while the colchichine cultured cells had 88% of the cells remaining.

Ficoll, Dextran 100, Dextran 500 and polyvinylpyrrolidone added to suspensions of normal mammary cells cause a gradual increase in the per cent of single cells over periods of 12-24 hours. Figure 16 is representative of the above treatments and shows the effect of Dextran 100 on the aggregation of normal mammary cells. Unfortunately the Medium 199 control did not aggregate appreciably in this series of experiments.

Ficoll, Dextran 100 and Dextran 500 did not affect the rate of aggregation of R3230 AC mammary tumor cells and Figure 17 shows the effects of Dextran 500. Increasing amounts of polyvinylpyrrolidone in Medium 199 caused a progressive increase in the per cent of single R3230 AC tumor cells remaining, though again the controls did not aggregate well (Table XXV). Metabolic activity apparently increased since here was a marked decrease in pH over the period of time the cells

TABLE XXIV

COMPARISON OF AGGREGATION BETWEEN MEDIUM 199 CONTROL[+] SUSPENSION AND MEDIUM 199 WITH COLCHICHINE

Suspension Culture Time Period (Hr)	pH 7.3		pH 7.7		pH 8.0	
	Control	Colchichine	Control	Colchichine	Control	Colchichine
6	17	15	10	5	3	0
24	37	28	15	15	11	12
39	56	47	27	25	18	25

[+]0.1% BSA added both in control and in the colchichine solution.

[++]The values are % of cells aggregated not % of cells disappearing.

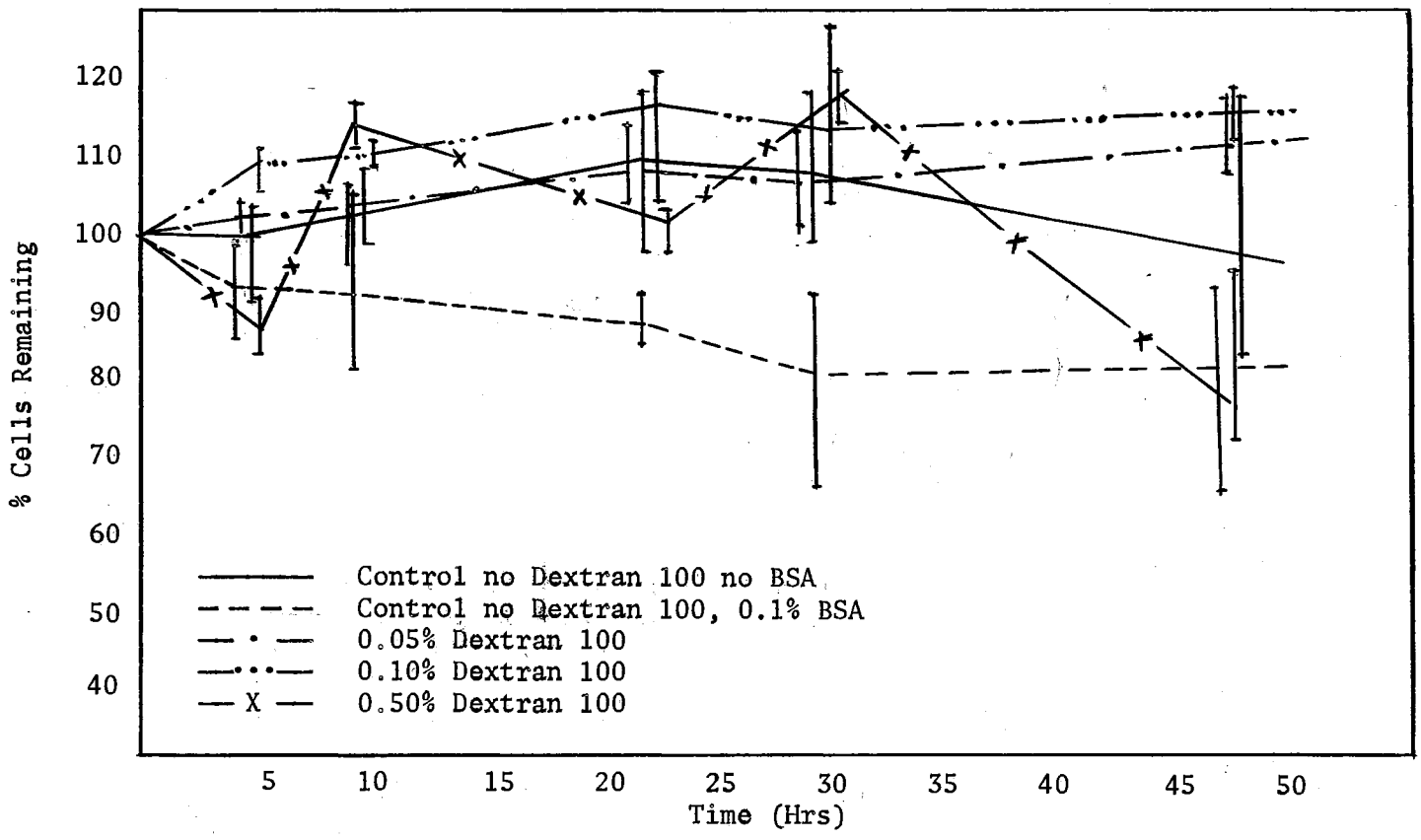

Figure 16. Effect of Dextran 100 on Aggregation of Normal Mammary Cells of the Fischer Rat

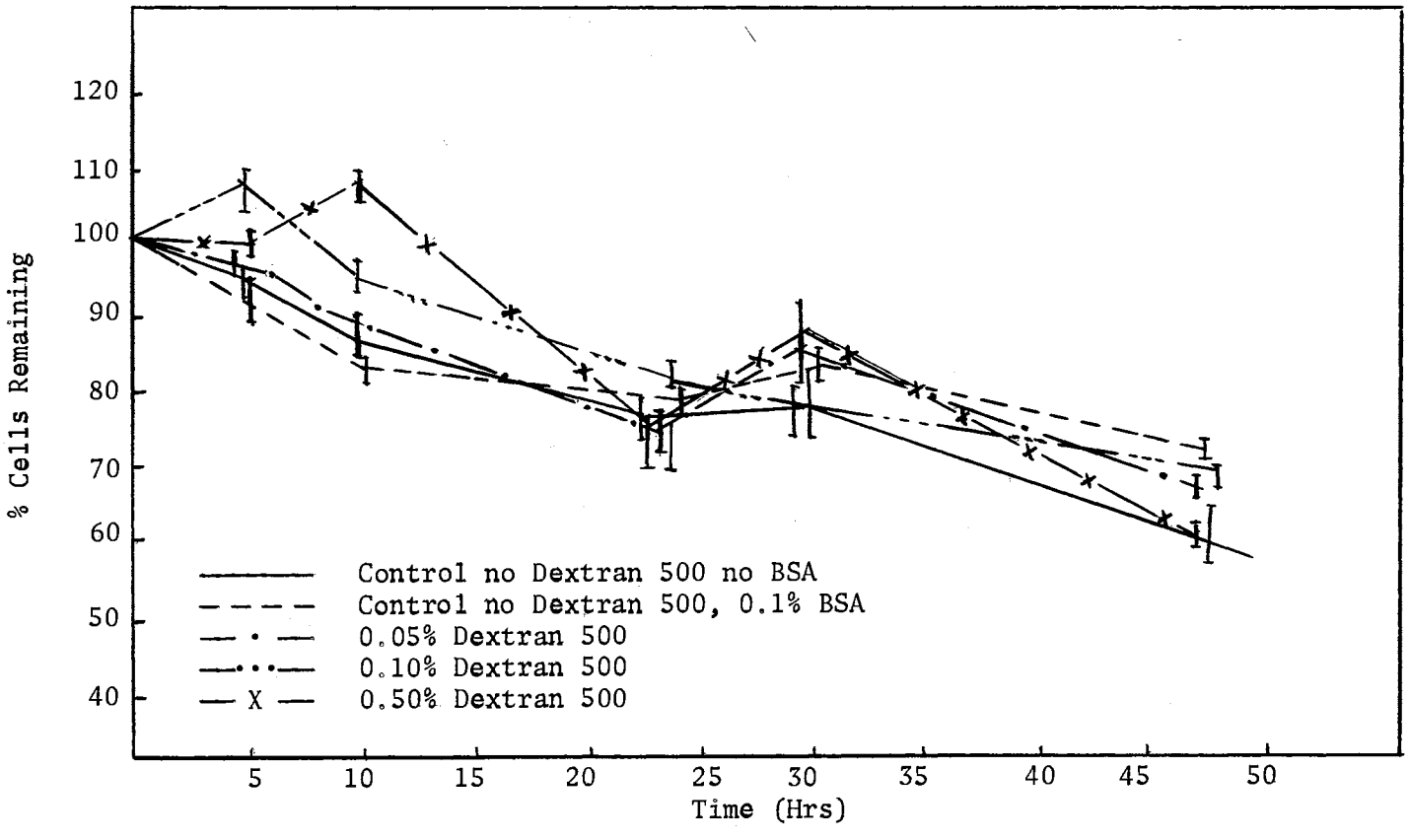

Figure 17. Effect of Dextran 500 on Aggregation of R3230 AC Mammary Tumor Cells of the Fischer Rat

TABLE XXV

EFFECT OF INCREASING CONCENTRATION OF POLYVINYLPYRROLIDONE ON AGGREGATION OF R3230 AC TUMOR CELLS

Polyvinylpyrrolidone	Initial Cell Concentration (1 X 10^6) (100%)	% Cells[+] Remaining
No PVP, No PBS (Control)	0.6	85
0.1% BSA (Control) No PVP	0.6	90
0.05	0.6	76
0.10	0.6	80
0.50	0.6	88

[+]Each value is an average of three counts after 24 hrs. The media used was Medium 199.

were in suspension and undoubtably complicated interpretation of the experiment. Cells and aggregates looked healthy when examined by light microscopy.

A light microscopic examination of cells and aggregates after the aggregation was completed showed that cells were usually as healthy in Ficoll, Dextran 100 and Dextran 500 as in 0.1% BSA. Cells were of normal size and no shrinking was present though there was moderate blebbing on the cell surface. A moderate amount of debris was present but there was no evidence of excessive cell lysis. It would appear that 0.1% BSA could well be replaced by 0.05% Ficoll, 0.05% Dextran 100 or 0.05% Dextran 500.

Summary

The Coulter Counter appears to be satisfactory as an assay for aggregation by measuring the disappearance of single cells. The rate of aggregation with both the tumor and normal cells is a slow process. The sensitivity and reliability of the assay is influenced by pH changes or any factor affecting a pH change.

Initial clumping of cells which would effectively prevent any study of the aggregation mechanism can be controlled by adding small amounts of bovine serum albumin or fetal calf serum or carbohydrate polymers such as dextran, ficoll and polyvinylpyrrolidone to the medium.

Both bovine serum albumin and fetal calf serum seemingly interfere with aggregation of normal mammary cells. Bovine serum albumin, fetal calf serum and polyvinylpyrrolidone retards aggregation of R3230 AC tumor cells.

CHAPTER VI

GENERAL SUMMARY AND DISCUSSION

Mild enzymic treatment of tumor and mammary tissue does isolate cells with apparent intact membranes. This was shown by comparable oxygen uptake values in different media and with added substrates such as glutamine and glutamate in minimal media. Neither substrate significantly increased oxygen uptake. The average oxygen uptake of R3230 AC mammary tumor cells was 8 $\pm$ 2 μl/hr/10^6 cells and of normal mammary cells was 3 μl/hr/10^6 cells. The oxygen consumption rate remained high for 72 hours which gives a wide latitude of time to carry out experiments.

Controlling pH is important in maintaining viability of cells. One important factor in controlling pH is control of the cell population within the range of 2-4 x 10^6 cells/ml. Medium 199 appeared to give the best environment for suspended cells particularly when supplemental with 0.1% BSA or 10% FCS. These two proteins could be replaced by 0.1% Ficoll, Dextran 100, or Dextran 500. High molecular weight substances which take up water are known to protect cells when added in a concentration similar to the concentration of protein (0.4%) found in interstitial fluid (260). Hepes, as a minimal media, appeared suitable for studies of short duration particularly in simple experiments such as determining the effect of the addition of a single substrate.

Oxygen uptake provides a simple method for defining conditions for cell isolation, for determining the initial or preliminary conditions for

short term or long term suspension culture. The most useful purpose of oxygen uptake is to check the integrity of the cell membrane and ultimately the integrity of the soluble enzyme systems in the cytosol.

Three methods were used to isolate the normal and R3230 AC mammary tumor cell membranes. Two methods, Fluorescein mercuric acetate and zinc chloride stabilized the membranes before isolation. In the Tris method, membranes were not stabilized. Gentle homogenization yields large membrane sacs. Membrane sacs have their own sedimentation rate between that of nuclei and other cell organelles. Membranes were isolated by a differential sedimentation procedure. Nuclei and whole cells precipitate at 1,000 x g from the homogenate after one minute of homogenization and were discarded. Centrifugation of the supernatant solution at 7-8,000 x g precipitates plasma membranes. The supernatant mixture containing endoplasmic reticulum, Golgi apparatus, organelles and soluble enzymes was discarded. All three methods were successful in isolating membranes from both the normal mammary and the mammary tumor membrane.

5'-Nucleotidase was used as an aid to identify the plasma membrane after the membrane isolation procedure. 5'-Nucleotidase activity was low in both membranes, 2.6×10^{-3} μmoles/mg/min in the mammary cell and 2.3×10^{-2} μmoles/mg/min in the tumor cell. The activity was ten times higher in the tumor cell than in the normal mammary cell. In cells where 5'-nucleotidase activity is low there is often a high ribosome content and a high rate of thymidine incorporation (259). From electron micrographs of the R3230 AC mammary tumor taken by Dr. William Sanford of the Zoology Department, Oklahoma State University, one can see that there is indeed a high ribosome content in the R3230 AC mammary tumor cell.

The value for galactosyltransferase activity is extremely low in both plasma membranes and may indicate a very minor contaimination of Golgi apparatus in the membrane preparations. Glucose-6-phosphatase was found to be a poor marker for endoplasmic reticulum. No activity was found in the plasma membrane preparations.

Lyophilized tumor cell membranes were difficulty soluble in sodium dodecyl sulfate. They were less soluble than normal cell membranes. Tris prepared membranes from both the normal mammary cell and R3230 AC mammary tumor cell were more soluble than $ZnCl_2$ prepared membranes. This was shown by a conspicuous lack of high molecular weight protein bands in gels from $ZnCl_2$ prepared membranes. No protein bands were obtained with Fluroescein mercuric acetate prepared membranes.

Tris prepared membranes gave the best gel patterns. Approximately 20 protein bands were present after sodium dodecyl sulfate acrylamide gel electrophoresis. The molecular weight range was approximately from 20,000 to 250,000 daltons. There was high background staining of gels which may be due to the enzyme isolation procedure. Proteolytic action of trypsin on red cell membranes showed this same effect in preparations from Dr. Carraway's laboratory, Oklahoma State University.

Very little hydroxylysine and hydroxyproline were present in the membranes. The proline content was lower than the amount found in basement membranes and the glycine content is about four times lower than the amount found in basement membrane and collagen (18). Aspartic acid and glutamic acid content are highest of all amino acids present in the membranes.

The carbohydrate content of Tris and zinc chloride prepared membrane appear to correlate well. Carbohydrate values of the Fluorescein

mercuric acetate prepared membranes were almost twice as high as those found for the zinc and Tris prepared membranes. This may mean that the sulfhydryl reagent, Fluorescein mercuric acetate, preferentially extracted one or more glycoproteins from the membranes. Carbohydrate values of zinc and Tris prepared membrane correlate well with values for other membraines reported in the literature (Table I). Glucose and mannose were the highest carbohydrate values found in the membranes, especially in the R3230 AC mammary tumor cell membrane. When the lipids were extracted with chloroform and methanol, the carbohydrate content was similar for the normal and tumor cell membrane. This may mean that the tumor cell has an abnormal amount of glycolipid present or associated with the membrane.

Xylose is also present in the membrane. This would suggest the presence of connective tissue glycoproteins because in connective tissue the protein carbohydrate linkage is a xylose-serine linkage. Carbohydrates found in connective tissue are largely glucose, galactose and iduronic acid (19) though some forms of collagen have only glucose and galactose present. In collagen, the carbohydrate protein linkage is glucose-galactose-hydroxylysine. Glucose also occurs in glycolipids as the glycosyl residue closest to the lipid moiety. The glucose and mannose levels found in both the normal and tumor membranes were higher than those reported in rat ascites hepatoma AH 7974 H cells (Table II).

Ribose present in the membrane was not of cytoplasmic origin since it was not removed by a one molar solution of NaCl. It was of ribosomal origin and was removed by incubation with ribonuclease over night. The high ribose content correlates with low 5'-nucleotidase activity.

When tumor cells were injected into rats the probability of tumor growth from injected cells at any time within the first 72 hours of

suspension was high indicating that there was no significant loss in this property. Tumor cells were judged to be viable on the basis of oxygen consumption for the full length of time required to make aggregation studies.

Cells varied in size from 10 to 25 μ and were counted with settings of 1/aperture current of 1/4 and 1/amplification of 2. Eighty per cent of the cells were counted with a lower window setting of 20 and an upper window setting of 90.

The criterion of aggregation most easily measured during the aggregation process is the loss of single cells and this was used as a quantitative measure of the rate of aggregation.

After disaggregation of tissue, the single cells frequently clumped immediately together and resisted all methods of mechanical disaggregation. These clumps of cells slowly disaggregated during the period of suspension resulting in counts above the initial counts. It was found that the addition of 0.1% BSA would prevent this initial clumping.

From a study of aggregation of cells as a function of pH, it would appear that the mechanisms of aggregation of the normal mammary cells and cells of the R3230 AC tumor are different since the aggregation of mammary cells is greatest at pH 8.4 and is greatest with tumor cells at pH 7.3. Bovine serum albumin and fetal calf serum prevent aggregation of both cell types. Ficoll, Dextran 100 and Dextran 500 had no effect on the aggregation of these cells. Any of these three compounds in a concentration of 0.05% could be used to replace 0.1% BSA. Polyvinylpyrrolidone caused a progressive increase in the amount of single R3230 AC tumor cells remaining. At the same time there was an increased drop in pH indicating a possible increase in metabolic activity of these cells over

the period of time the cells were in suspension.

A method for assaying aggregation of normal mammary and R3230 AC mammary tumor cells was developed. Aggregation of both systems was quite slow with about 30% aggregation occurring over a period of twelve hours. Membranes of both cell types have little hydroxylysine, hydroxyproline or glycine and it seems improbable that intermolecular bonds such as are found in collagen would form between two opposing cell surfaces. No information is available from this study regarding how these cells recognize one another and aggregate.

When cells held in suspension over 72 hours were injected subdermally, three out of nine rats produced R3230 AC tumors and six did not. Over a period of three weeks additional injections of cells held in suspension approximately 120 hrs without fetal calf serum were made into the six rats which produced no tumors. Again no tumors grew. A transplant was made to each animal and no tumor grew. Four additional rats were injected with one ml of tumor membrane suspension held approximately 120 hrs without fetal calf serum. The same time schedule was used as before and lack of tumor growth was again found when tumor tissue was transplanted. Isolated cells required the presence of fetal calf serum to produce tumors after a period of about twenty-twenty-four hours. Cells which had media changed just before cell injection did not produce tumors.

After disaggregation of the tissue the single cells frequently clumped immediately together and resisted all methods of mechanical disaggregation. These clumps of cells slowly disaggregated during the period of suspension resulting in counts above the initial counts. It was found that the addition of 0.1% BSA would prevent this initial clumping.

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