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MEMBRANE CHANGES IN HeLa CELLS GROWN WITH CORTISOL

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CHAPTER I

INTRODUCTION

Membranes are structurally and functionally complex structures that perform a wide range of physiologic tasks. They serve such diverse functions as: hormone receptor sites, energy production, anchoring sites for enzyme systems, antigenic sites, and sites of active transport. Membranes are generally pictured as a mosaic of lipids, proteins, and carbohydrates. The carbohydrate, in the form of a glycoprotein or glycolipid, is located mainly on the outer membrane.

Interest in cell membrane structure is rapidly growing. Although membrane structure of the intact cell can be profitably studied in situ, invaluable information can often be obtained only after removal of the membrane from the cell. Three general forms of the membrane have been isolated: whole ghosts, fragments in sheets and in vesicular form.

Membranes have been isolated from various tissue culture cells such as; L cells, KB, CHO, CV-1, primary chick and human fibroblasts, MC1M, 3T3, W138, W126, Ehrlich ascites cells, and a variety of cells transformed with viruses. Chemical reagents such as fluorescein

mercuric acetate (FMA), or 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), heavy metal ions such as Zn^{2+}, and Tris (hydroxymethyl) aminomethane (Tris) have been used to stabilize the membrane during isolation. Several investigators have isolated membrane fractions from HeLa 65 cells grown in suspension culture. Three major procedures for the isolation of HeLa cell membranes have been published (1,2, and 3). A modified technique was devised in the present study for isolation of membranes from HeLa cells grown in monolayer culture.

Native complex carbohydrates of higher animal cells are covalently bound to protein or lipid. They are glycoproteins, glycolipids, and glycosaminoglycans (mucopolysaccharides). Detailed structures of some of the carbohydrate complexes associated with proteins or lipids have been reviewed (4). Although complex carbohydrate structures have been implicated in functional roles at the cellular level, none of these compounds have been clearly associated with cellular function (4). Changes in the metabolic state of a cell that permit (and perhaps trigger) mitosis have been suggested from a variety of observations. The premonitory indications of mitosis are: increase in cell volume, loss of cellular water, cortical and endoplasmic viscosity changes (probably due to calcium shifts), and transient availability of protein sulfhydryl groups. The increase of cell volume is coupled with an increase of cell surface area; hence the question of changes in the surface density of carbohydrate complexes arises. Onodera and Sheinin (5) reported that synchronized mouse 3T3 cells show a preference for incorporating glucosamine into trypsin-removable surface material in the late G_1 phase of the cell cycle. Glick et al. (6) showed that the amounts of mannose, galactose, and

fucose decrease during the mitotic phase of the KB-cell cycle, and that the amount of sialic acid also decreases, but later in the cycle. Kraemer (7), on the other hand, found that neuraminidase susceptible sialic acid per unit cell volume remains constant or shows only a small gradual increase throughout the mitotic cycle of Chinese-hamster-ovary cells. However, contrary to the results of Kraemer, Mayhew (8) has reported an increase in the amount of sialic acid which is susceptible to neuraminidase during the mitotic phase. He also noted a transient increase in the electrophoretic mobility of synchronized suspension-cultured human-osteosarcoma cells in the mitotic period (9). Brent and Forrester (10) demonstrated an increased electrophoretic mobility in HeLa cells during the M period. Surface sialic acid has been known to contribute to the electrophoretic mobility of many kinds of cells (11). Kraemer (7) proposed that the transient, neuraminidase-susceptible mobility shift could be a change in the effective in situ pK of the carboxyl groups of the terminal sialic acid molecules, or a change in topology of surface sialic acid molecules in relation to cell's electrokinetic surface. Thus the cellular electrokinetic surface, whose charge density determines the electrophoretic mobility, and the surface that bears sialic acid molecules accessible to neuraminidase are not identical. Recently, Rosenberg and Einstein (12) reported that biosynthesis of sialic acid in synchronized human-lymphoid cells occurs only in the late G_2 phase of the cell cycle, and that the amount and density of surface sialic acid varies considerably throughout the cycle. In view of the literature cited, the relationship between the cell cycle transitions and the changes of the surface complex carbohydrates is still obscure.

The plasma membrane functions as the boundary to the cell, the permeability barrier that separates the internal and external milieu of the cell. It acts as a barrier to allow the passage of ions, of nutrients, and of other chemicals, into and out of the cell. Since most glycoproteins are exported from the parenchymal cells in which they are synthesized, Eylar (13) suggested that the carbohydrate portion of glycoprotein may take part in the process of transport by interacting with the plasma membranes and thus facilitating secretion. This interaction may also help in the reverse process when glycoproteins enter the cells. Cell-surface sialic acid has also been implicated in ion transport. Emmelot and Bos (14) have shown that the activity of K^{+}-Mg^{++}-p-nitrophenyl phosphatase is almost completely abolished by pretreatment of plasma membranes with neuraminidase. This suggested that the carboxyl group of a sialic acid conjugate (sialoglycoprotein or sialoglycolipid) acts as a cation-receptor (or exchanger) in the active cation transport across the plasma membranes of rat liver. Glick and Githens (15) also demonstrated that neuraminidase-sensitive sialic acid participates in the transport of K^{+} across leukemic-cell membranes.

Cell surface sialic acid has been known to be an important part of the receptor sites for all the myxoviruses, as well as polyoma virus, Kilham's rat virus and EMC virus (16, 17, 18 and 19).

Membranes are also involved in hormone receptor sites; they act by changing cyclic AMP levels, functioning in cooperation with calcium concentration (20). Antigenic properties of cells are also closely associated with surface carbohydrate complexes. Treatment of glycoprotein extracted from human B-erythrocytes by α-galactosidase causes the dis-

appearance of its B activity (21). A or B blood group activity has been shown to be mediated through glycolipid of the red cell membrane (22,23). Sialoglycoproteins are generally poor antigens, and antibodies against glycoproteins are not directed toward the sialic acid, even though they are terminal sugars. Actually, it appears that sialic acid may act to conceal or protect more potent antigens. For example, the T antigen in erythrocytes was exposed to react with specific T antibodies by treatment of the erythrocytes with neuraminidase (24). Treatment of erythrocytes with protease also makes certain antigens more available to react with antibodies or other agglutinins. This may be due to a steric protection of the underlying glycolipids by the peptide. Alternatively, removal of sialic acid associated with the peptide may change the configuration of the membrane protein to permit a more ready approach of antibody to the antigen site. Recent studies of alkaline phosphatase indicated a possible three-way relationship of viral receptor, cell-surface antigen and cell-surface enzyme activity (25). Placental alkaline phosphatase exhibits the ability to inhibit haemagglutination of red blood cells by Toolan's H-1 virus. However, this inhibitory activity of the enzyme is completely lost by neuraminidase treatment, which, however, did not alter the enzyme activity (25).

It is generally accepted that the interactions of cancer cells with a host are in part determined by the physicochemical nature of their peripheral regions. Ambrose et al. (26) reported that carcinogen-induced tumor cells have higher electrophoretic mobilities than normal tissue cells. Currie et al. and Sanford indicated that negatively charged sialic acid moieties at tumor-cell surfaces may shield the cells

against immunologic attack by the host and enable them to achieve lethal proportions (27,28). On the other hand, Cook and Jacobson (29) found that mouse acute lymphoblastic leukemic cells have lower electrophoretic mobility than normal lymph node cells. However, Weiss and Hauschka (30) found no correlation between electrophoretic mobility and degree of malignancy of mouse TA3 cells. They indicated that if the surface changes are arranged heterogeneously, then although contact between two cell surface regions is expected to be governed by the density of charged groups within these regions, there may or may not be an observed correlation between electrophoretic mobility and contact.

In recent years, the altered properties of cultured cells transformed by oncogenic virus have been studied in great detail. Permanent genetic changes induced by these viruses include, among others, the acquisition of neoplastic properties and changes in colony morphology, most probably related to the failure of transformed cells to respond to contact inhibition of cell movement and cell replication. In general, the lack of contact inhibition of transformed cells is correlated with their tumorigenic capacity, although exceptions have been found. There are indications that the transformation process alters the surface of the cell. Ohta et al. reported that polyoma-transformed 3T3 cells were deficient in both surface (neuraminidase-removable) and total (acid hydrolysis) sialic acid (31). This result was further confirmed by Wu et al. (32). In contrast, Forrester et al. showed that a clone of polyoma-virus-transformed hamster fibroblasts have slightly higher electrophoretic mobility than the non-transformed cells (33,34). Kalant et al. also indicated an increase in sialic acid in carcinogen-induced-

primary hepatomas compared with tissue from adjacent normal liver (35). Benedetti and Emmelot (36) studied isolated plasma membranes from rat liver and from hepatoma and also found an elevated sialic acid content in the hepatoma membranes. Recently, Buck et al. showed that fucose-containing glycoprotein is decreased in Raus-sarcoma-virus-transformed hamster cells (37).

The incomplete synthesis or shortening of the glycolipid carbohydrate chain in the virally transformed cells has been observed as deletion of the sialosyl residue of hematoside and concomitant increase of lactosyl or glucosylceramide (38,39). More recently, a deletion of the sialosyl residue was observed in higher gangliosides of malignant cells (40,41). It has been indicated that glycolipids are more predominantly present in plasma membrane than other membrane components (34, 42 and 43). Therefore, the qualitative pattern of glycolipids could be one of the direct indicators of the intricate properties of cell membrane. The change of glycolipid pattern could be related to the immunological surface changes of tumor cells; however, immunological specificities of cell surface are governed by more complicated organizational structures (38,44). Markedly enhanced activities of both biosynthetic and degradative enzymes involved in glycoprotein metabolism have been observed in either SV_{40}- or polyoma-virus-transformed 3T3 cells (45,46).

The biosynthesis of glycoproteins and glycolipids have been approached by a number of investigators. Polypeptide chains of glycoproteins are generally considered to be synthesized on ribosomes of rough endoplasmic reticulum of the cells. It also seems well-established

that the oligosaccharide chains are elongated one sugar at a time by a transfer from a nucleotide sugar to the growing oligosaccharide chain. This process occurs primarily in the smooth endoplasmic reticulum and Golgi apparatus (47,48). The sequence of sugars in such an oligosaccharide chain depends on the specificity of the transferase for the glycosyl donor and for the glycosyl acceptors. The genetic control for the specificity of such glycosyltransferases has been elucidated in studies of the genetic control of the enzymes adding the terminal sugars to form the A,B,H and Lewis-active blood group glycoproteins in body secretions (49).

Recently, Caccam et al. (50) have found that GDP-mannose-1-^{14}C incubated with several mammalian tissues is incorporated as such into a lipid having some of the characteristics of a mannosyl-1-phosphoryl polyisoprenol compound (possibly a derivative of Vitamin A). A polyisoprenoid-sugar complex has been shown to be involved in the biosynthesis of cell walls in bacteria. The appearance and loss of radioactivity in rat liver mannolipid have a temporal correlation expected for a precursor of glycoprotein.

An area of considerable interest for studies on the carbohydrates of cell surfaces concerns the mechanisms by which the membrane components are synthesized, the manner in which they are inserted or organized into the cell membrane surface, and how they are removed from the membrane and catabolized, released into the medium or returned to the cells. In this respect, Warren and Glick have shown that the specific activity of radioactive glucosamine decreases at a rate predicted by the growth and consequent dilution in rapidly growing L cells "pulse

labeled" with radioactive glucosamine (51,52). On the other hand, Kraemer (53) and Molnar et al. (54), noted that nongrowing ascites cells continuously release newly synthesized carbohydrates-containing macromolecules into the medium.

Carbohydrates are well suited for formation of stereospecific structures. They are relatively rigid molecules in which there are many groups for hydrogen bonding and for hydrophilic interaction. The shape of the molecule and the position of the reactive groups can be markedly different depending on the sugars involved, the hydroxyl position to which the nonreducing sugar is linked, and the anomeric configuration of the glycosidic linkage. It is, therefore, not surprising to find profound specificity in the carbohydrate-containing antigens and in the reaction of carbohydrates with agglutinins, viruses, antibodies, enzymes, and so forth. It is interesting in this respect that glucose is rarely in the outer portion of sugars in glycoproteins or glycolipids (4). This is consistent with the recognition site hypothesis, since free glucose in the body fluids could otherwise inhibit recognition of specific binding or receptor sites. In conclusion, it is clear that there are complex polysaccharides in cell surface, and that carbohydrates contained therein play a role in cell function--but what role or roles is a matter of speculation.

The corticosteroids have numerous and diversified physiologic functions and pharmacologic actions (55). They influence carbohydrate, protein, fat and purine metabolism; electrolyte and water balance; and the functional capacities of the cardiovascular system, the kidney, skeletal muscle, the nervous system, and other organs and tissues.

Furthermore, the corticosteroids endow the organism with the capacity to resist not a few but all types of noxious stimuli and environmental changes. The adrenal cortex is the organ of homeostasis, being responsible for the relative freedom that higher organisms exhibit in a constantly changing environment.

The means by which the corticosteroids accelerate gluconeogenesis has been the subject of intense experimental study and debate. Evidence has been brought forward to support actions of glucocorticoids at each of the various steps from tissue protein to liver glycogen. There is general agreement that the glucocorticoids are essential for the mobilization of protein and amino acids from tissue store.

The mobilization of protein from different tissues is affected in different ways. Lymphatic tissue evidences such effects dramatically resulting in lysis and pyknosis of the cells, while skeletal muscle and skin show little effect.

Cortisone has been found to increase the rate of de novo synthesis of liver enzymes involved in the conversion of amino acids to glycogen (56).

The mobilization of fat from peripheral fat depots by epinephrine, norepinephrine, growth hormone, or adipokinetic peptides of the adenohypophysis is markedly decreased in the absence of the adrenal cortex.

Cortisol induces sodium retention and potassium excretion, but much less effectively than does aldosterone. The corticosteroids also exert important actions on the various elements of the circulatory system, the capillaries, the arterioles, and the myocardium. The

corticosteroids not only potentiate the pressor response to epinephrine and norepinephrine but, in addition, they exert a protective action against the toxic effects of these catecholamines. The dissolution of lymphoid tissue is under the influence of the adrenal glands.

Cortisol has the capacity to prevent or suppress the development of the local heat, redness, swelling and tenderness by which inflammation is recognized. At the microscopic level, they inhibit not only the early phenomena of the inflammatory process (edema, fibrin deposition, capillary dilatation, migration of phagocytes into the inflamed area, and phagocytic activity) but also the later manifestations (capillary proliferation, fibroblast proliferation, deposition of collagen and cicatrization)(57).

The anti-inflammatory and antiallergic effects of hydrocortisone are considered to be its most striking physiologic functions and the clinical applications of hydrocortisone fall into these two categories. It has also been used with varying degree of success in the treatment of malignant lymphoma and lymphocytic leukemia.

Among the many recent advances in immunology must be counted those in the field of immunosuppressive therapy. The ultimate goal of the therapy is to achieve the ability to suppress the response to a single antigen, or constellation of antigens, while leaving all other immune responses intact. Corticosteroids are among the immunosuppressive agents being used (58,59). Rats, mice and rabbits are extremely sensitive to immunosuppressive activity of the corticosteroids which cause depletion of the small lymphocytes. However, there are other species--among them, man--that are resistant, or at least whose small

lymphocytes are resistant to corticosteroids. However, one cannot ignore the face that in clinical practice, the corticosteroids have proved most important in the treatment of immunologic disorders and in facilitating homograft acceptance. In graft rejection the central biologic event is the interaction between antibody and antigen, which in itself often evokes a strong inflammatory reaction. The possibility therefore exists that corticosteroids may enhance the chances of graft acceptance by suppressing inflammation and thereby improving the host environment for the graft. In contrast, corticosteroids have been known to enhance infections by a number of bacteria, to inhibit various aspects of phagocytic process, including the release of lysosomal enzymes from granulocytes. Thus, the mechanism of immunosuppressive activity from such multifaceted substances such as the corticosteroids still awaits further investigation.

Lin and Knox (60) have shown that rat-liver tyrosine transaminase activity is elevated rapidly in response to glucocorticoids, with a maximum increase of about fivefold occurring within 3-4 hours. There is considerable evidence that alterations of RNA metabolism are involved in enzyme regulation in animal tissue, resulting in an increase of the rate of enzyme synthesis (61,62).

Cortisol and related glucocorticoids have been known to impair bone formation when administered in pharmacological doses to human beings and experimental animals. They markedly inhibit the synthesis of collagen and noncollagen protein in cultured bone cells (63), They also alter the metabolism of bone-cell nucleoside and RNA (64).

In rat-thymocyte cells, cortisol decreases the activity of

RNA polymerase resulting in inhibition of RNA synthesis (65). Cortisol also inhibits the transport and utilization of amino acids and precursors of RNA and DNA in lymphoid cells *in vitro* (66); it does not have differential-inhibitory effect on a specific species of RNA. In contrast, Kidson reported that incubation of lymphoid cells *in vitro* with cortisol resulted in an early inhibition of synthesis of a rapidly turning over RNA species (67,68). Alterations in specific RNA species have also been reported in studies of cortisol action on rat-hepatoma cells (69).

It is extremely difficult to know if the hormonal or nutritional stimulus acts directly or is a consequence of secondary, compensatory responses of the intact animal. To bypass some of these complexities, use has been made of isolated organ and cell culture systems. Two HeLa clones, HeLa 65 and HeLa 71, have been carried continuously in our laboratory for years, and are the experimental systems utilized for these studies. HeLa is a name applied to a human-derived epithelial cell line of cervical origin. The primary adenocarcinoma was adapted to growth in tissue culture by Gey *et al.* in 1951 (70), and the culture has been carried continuously since. Although tissue culture provides us with an excellent system in which to unravel the molecular mechanism and the cell-surface phenomena of hormones, we have to bear in mind that the cultured cells exhibit no characteristic traits of the tissue of origin.

HeLa 65 cells continuously subcultured with 3 μM cortisol (Hcr state) possess a membrane-bound enzyme, alkaline phosphatase, with an increased specific phosphomonoesterase activity (71, 72 and 73). When HeLa 71 cells are continuously subcultured with 3 μM cortisol,

they have an extended cell generation time (35 hours). This is mainly due to an extension of the late G_1 portion of the cell cycle. In contrast, the growth of HeLa 65 cells with cortisol is temporarily retarded in the first generation cycle, but it later returns to the control rate (18-hour doubling time)(74). The amount of sialic acid, a cell-membrane moiety, rises in HeLa 65 cells after short-term cultivation with cortisol (75). Mammalian cell surfaces are reportedly modified by growth with glucocorticoids. For instance, a clone of rat-liver hepatoma cells grown with cortisol changes in cell adhesiveness, electrophoretic mobility, and antigenic properties (76).

These studies were designed to quantitate and characterize such membrane components as sialic acid, hexosamine and their carrier molecules. We also planned experiments to determine the relative content of these components in cell subfractions by the use of cell-membrane fractionation techniques. The sialic acid content of sialoglycoproteins increased in the plasma membrane and the nucleus of HeLa 71 Hcr relative to HeLa 71 control. This increase was paralleled by a rise in glycoprotein CMP-sialic acid transferase activity. The increased sialic acid may have been due to a cortisol-mediated increase in the amount of sialic acid per glycoprotein molecule and/or increased amounts of total cellular sialoglycoproteins.

CHAPTER II

MATERIALS AND METHODS

Cell cultures and media

Two HeLa clones, HeLa 65 and 71, named for their modal chromosome number (77), were grown in monolayer culture either in milk dilution bottles or Blake bottles (E.H. Sargent and Co.) ascertained to be flat on one surface. Growth medium was an autoclavable preparation of Eagle's Minimal Essential Medium (MEM) (78) (Auto Pow, Flow Laboratories) supplemented by 10% calf serum (Colorado Serum Co.), 5 mM glutamine, 50 units/ml of penicillin and 50 µg/ml each of streptomycin and kanamycin. The calf serum was heat-inactivated at 56° for 30 minutes before being added to the medium. The pH of the medium was maintained at about 7.2 with 5 percent CO_2 in balanced air. Cells in the Hcr state were subcultured with cortisol (1.0 µg/ml of medium) obtained from Sigma for at least three weeks before being studied. Techniques for subculturing and harvesting cells have been described elsewhere (79). Each experiment utilized replicate bottle cultures including one which was used for the cell counting. Blake bottles at a confluency of 11-12 x 10^4 cells/cm^2 were used for most of the experiments. This represents an exponentially dividing asynchronous population of cells in late logarithmic growth.

Cell counts

Cell counts were done with a Bright-Line hemocytometer from A.O. Instrument Company, Scientific Instrument Division, Buffalo, N.Y.

Cell-volume measurements

Cells, which had been removed from the monolayer with versene-trypsin (79), were suspended in complete medium. The cell suspension was then sedimented at 400 x g for 15 minutes in a Bauer-Schenck sedimentation tube (Kontes Glass Co., Vineland, N.J.), calibrated in 4 µl increments. After this treatment, no further decrease in pellet volume was obtained. The final reading represents the closest packed volume of the cells since cell deformability excluded essentially all extracellular fluid when packed under these conditions.

Separation of glycoproteins and glycolipids

Cell monolayers were washed twice with isotonic saline in 0.025 M Tris-HCl, pH 7.2, (ST buffer), suspended in this buffer, and sedimented by centrifugation. Cell pellets were extracted with 20 volumes of chloroform:methanol 2:1 (v/v) for 20 minutes at 37°. The insoluble residue was sedimented and reextracted as described above. The chloroform:methanol layers were combined and used for lipid and glycolipid analysis. The precipitate from the chloroform:methanol extract was air-dried and mechanically (Model AAZC170, Precision Scientific Co.) homogenized in water. To this suspension was added an equal volume of 2% phosphotungstic acid in 1.0 N HCl. The precipitate was sedimented by centrifugation, washed by resuspension in 1% phosphotungstic acid in 0.5 N HCl, and resedimented. The pellet was used for

sugar analysis of glycoproteins. Glycoprotein sugars were liberated by hydrolysis in 2 N HCl at 100° for 6 hours. Hexosamine was determined on the acid hydrolysate. Neutral sugars were assayed after column chromatographic separation, as described for glycoprotein neutral sugars (80). Sialic acid was liberated by hydrolysis of the precipitated pellet in 0.1 N H_2SO_4 at 80° for 1 hours and determined as described later.

Sialic acid assay

Sialic acid was assayed as N-acetylneuraminic acid by the method of Warren (81) after this compound had been separated by ion-exchange column chromatography using coupled columns with two resins (Dowex 50W-X8 [H^+], 20 to 50 mesh and Dowex I-X8 [formate], 50 to 100 mesh, from Sigma) (75). Paper chromatography was used to resolve the sialic acids of the purified nuclear fraction into N-acetylneuraminic acid and N-glycolylneuraminic acid. The material was applied to pre-washed Whatman No. I chromatography paper and the chromatogram was developed by descending irrigation with butanol:propanol:0.1 M HCl (1:2:1, v/v/v) (82).

Hexosamine assay

Hexosamine was estimated by the method of Gatt and Berman (83). Hexosamines were further separated into glucosamine and galactosamine on a Bio-Cal Bio-Chrom amino acid analyzer (Rockville Center, N.Y.). The acid hydrolysate of glycoprotein was desiccated over KOH pellets and then dissolved in 0.2 N citrate buffer (pH 2.2). The sample was applied to a standard amino acid analyzing column (50 x 0.9 cm) and eluted with 0.2 N citrate buffer (pH 4.25), to which solid NaCl was added to bring the normality to 0.38.

Neutral sugar assay

The acid hydrolysate of glycoprotein in 2 N HCl was diluted with at least 10 volumes of distilled water and then passed sequentially through Dowex 50W-X8 (H^+) and Dowex I-X8 (formate) columns. The effluent was collected and lyophilized (Virtis Research Equipment, Garden, N.Y.). The sugars were reconstituted in 1 ml of water. An aliquot of the mixture was analyzed for the total neutral sugars by the Park-Johnson ferricyanide method (80). The rest of the sugar mixture was separated and quantitated as ribose, mannose, galactose by gas-liquid chromatography using the technique of Holme et al. (85), with modifications suggested by Dr. A.A. Lindberg. The sugar mixture was reduced to the corresponding alditols with sodium borohydride (15 mg/mg sugar). After sodium borohydride was dissolved (in the ice bath), the mixture was allowed to stand at room temperature for one to two hours and then stored overnight at 4°. Excess borohydride was destroyed by placing the sample on a Dowex 50W-X8 (H^+) column, eluting with 10 ml of distilled water into a 25 ml round-bottom flask, and evaporating the eluate to dryness. Boric acid was removed as methyl borate by distillation from methanol (5 ml) under reduced pressure and the process was repeated for two more times. Toluene (5 ml) was added and the reduced sugar mixture evaporated to dryness. This process was repeated one more time. The mixture was acetylated with acetic anhydride in pyridine (1 ml each) at 80° for 10 min in a glass-stoppered flask. The reaction mixture was cooled in an ice bath and evaporated to dryness under reduced pressure. Excess acetic anhydride and pyridine was removed by adding water and the resulting solution evaporated to dryness. The resulting

mixture of fully acetylated alditols was dissolved in acetone and the individual compounds were separated by gas-liquid chromatography. The solution (1 µl) was injected into a 3% ECNSS (polyester-silicone) (Supelco Inc., Supelco Park, Bellefonte, Pa.) glass column (6 ft x 5 mm internal diameter) fitted in a Barber-Coleman Model 5000 gas chromatograph. The injection temperature was 220°, column temperature 188°, detector temperature 245° and gas flow (argon) 54 ml/min. The areas of the resolved peaks were determined and the amount of sugar was calculated using xylose as internal standard. Sugar standards were obtained from Regis Chemical Co., Chicago, Ill.

Glucose was assayed by the glucose oxidase method (84).

Lipid and phospholipid assays

Total lipid was determined gravimetrically. The chloroform: methanol cell extract was evaporated to dryness under a stream of nitrogen. The residue was redissolved in chloroform, and an aliquot was placed on an aluminum-foil weighing pan and weighed on an analytical Model G Cahn Gram Electromicrobalance (Ventron Instruments Corp., Paramount, Calif.).

Phospholipids were separated by two-dimensional thin-layer chromatography by the method of Rouser et al. (86). The sample was applied on prewashed Silica Gel G plate (20 x 20 cm at 0.5 mm depth, from Brinkman Instruments, Inc. Westbury, New York). The plate was transfered to TLC chambers and was developed in the first dimension with chloroform:methanol:28% aqueous ammonia (65:25:5, v/v/v). After drying for about 10 minutes, the chromatogram was developed in the second dimension with chloroform:acetone:methanol:acetic acid: water

(3:4:1:1:0.5). Lipids were detected by iodine vapor. The spots were scraped from the plates and the lipids extracted with chloroform: methanol:water:formic acid (97:97:4:2) according to Abramson and Blecher (87). The extract was evaporated in a hot water bath and phosphorus was determined (86). The neutral lipids were poorly resolved and were eluted as one fraction and weighed on the microbalance.

Protein assay

Protein was determined by the method of Lowry et al. (88).

RNA assay

RNA was determined spectrophotometrically. The sample was incubated in 0.23 N NaOH at 37° overnight. Protein and DNA were precipitated by the addition of $HClO_4$ to 8% concentration. After the sample had been centrifuged for 30 minutes to remove insoluble material, the optical density of the supernatant fluid was read at 260 nm. Occasionally, this method of alkaline hydrolysis of RNA was checked by hydrolysis with pancreatic ribonuclease (Sigma). Both methods gave similar results.

DNA assay

DNA was determined by the method of Burton (89).

Phosphatase assays

Alkaline phosphatase activity was estimated by the method previously described using 8 mM para-nitrophenyl phosphate (p-NPP) (Sigma) in 0.25 M Tris-HCl, pH 10.0, as substrate (79). 5'-Nucleotidase activity was determined as the amount of phosphate released when aliquots

of various fractions were incubated with 5'-AMP (Sigma) at pH 7.5. Phosphate was measured by the method of King (90).

Colorimetric determinations of protein, DNA, RNA, sialic acid, hexosamine, neutral sugar and phosphorus were read on a Gilford spectrophotometer.

Membrane separation

All operations were performed at 4°. The cell monolayers of replicate Blake bottles (20 to 30) were washed twice with ST buffer and scraped (with rubber policemen) into ST buffer. The cells were sedimented by centrifugation at 200 x g for 10 minutes, pooled in ST buffer, and resedimented as described above. In this way, about 4-5 x 10^8 cells were obtained for each membrane preparation experiment. The cells were suspended in 9 ml of 0.05 M Tris-HCl (pH 7.5), containing 5 mM $MgCl_2$ (TMG buffer). They were allowed to swell for 10 minutes and were then homogenized with 20 complete strokes in a tight glass-to-glass Dounce homogenizer (Kontes Glass Co.). The homogenate was centrifuged at 200 x g for 10 minutes. The pellet was used for the plasma membrane and nuclear preparation.

The supernatant fluid was centrifuged in a Sorvall (Model RC-2) refrigerated centrifuge for 20 minutes at 4000 x g. The pellet, called Fraction VI, contained lysosomes, mitochondria and cytoplasmic membranes was not further resolved. The supernatant fluid from the 4000 x g centrifugation was made 45% with respect to ribonuclease-free sucrose (Schwartz/Mann); the volume was 20 ml. This suspension was successively layered with 13 ml of 35% sucrose, 13 ml of 30% sucrose, and 10 ml of 25% sucrose (all in TMG buffer), in a 60 ml capacity cellulose nitrate

tube. The discontinuous gradient was overlayered with a small volume of TMG buffer. It was then subjected to centrifugation (Beckman Model L-2 ultracentrifuge) at 70,000 x g for 16 hours in an SW-25.2 swinging bucket rotor (Spinco). The supernatant fluid from the 4000 x g centrifugation which was further centrifuged in a discontinuous sucrose gradient was resolved into four distinct fractions (See Figure 1a). The uppermost of these was light and was combined with the fraction below it (Fraction I).

The pellet from centrifugation at 200 x g for 10 minutes was suspended in 20 ml of 45% sucrose and sonified in a Bronson sonifier using the microprobe operating at 70% efficiency with a 14 kcycle output. The sample received three 8-second bursts of sound with thorough cooling in ice during and after each sonification. The sonicate was layered with the other sucrose solutions to form a discontinuous gradient as described above. This fraction was also centrifuged for 16 hours at 70,000 x g in the SW-25.2 rotor. The sonicate from the 200 x g centrifugation, which was centrifuged in a discontinuous sucrose gradient, was separated into three fractions (Figure 1b), one of which was light and was combined with the fraction below it (Fraction IV).

Each fraction obtained by the discontinuous sucrose gradient was diluted at least fourfold with TMG buffer. It was then sedimented by high-speed centrifugation and washed once more with buffer , as described above.

Electron microscopy

Qualitatively, biochemical characteristics were similar for any membrane fraction from one clone to the other. Therefore HeLa 71

Hcr was chosen as the prototype for electron microscopic studies. Membrane fractions were fixed for 2 hours in 6.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 5% sucrose. After fixation, the membrane fractions were washed in phosphate buffer (pH 7.2) for 2 hours and fixed with 1% Dalton's chrome osmium for 2 hours. The material was then washed with buffer, dehydrated in graded ethanol solutions, and embedded in Araldite 6005 (Cargille's Epoxy Resin, Cargille Laboratories, Inc.). Sections (made on a Porter-Blum ultramicrotome) of 800-1000 Å were stained with uranyl acetate and lead citrate and were photographed on a Hitachi HU-11B electron microscope (Perkin-Elmer Corp.).

Glycoprotein sialyltransferase assay

Sialyltransferase activity was measured by modifications of the method of Grimes (91). As exogenous acceptors, both bovine fetuin and bovine submaxillary mucin (Sigma) were stripped of sialic acid by heating at 80° for 1 hour in 0.1 N H_2SO_4. This was followed by neutralization, exhaustive dialysis, and lyophilization to dryness. Cells were washed twice with a buffer composed of 0.8% NaCl, 0.05% KCl, 1 mM EDTA, and 1 mM phosphate (pH 7.4). The cells were scraped from the glass in this buffer and centrifuged. The cell pellet was resuspended in 10 mM phosphate (pH 6.5), homogenized in a tight-fitting Dounce apparatus, and centrifuged at 200 x g for 10 minutes to sediment the nuclear fraction. The pellet was resuspended in the phosphate buffer and centrifuged at 200 x g for 10 minutes, and the supernatant fluid was combined with the previous one. This step was repeated once more. The combined supernatant fluids of the cell homogenate were then centrifuged in an SW-25.2 swinging bucket rotor at 70,000 x g for 3 hours. The resulting pellet was

suspended in 10 mM phosphate buffer (pH 6.5). This served as the enzyme preparation for sialyltransferase assay.

The standard reaction mixture consisted of 250 µg enzyme protein, 500 µg acceptor protein, 50 nC CMP-[^{14}C4,5,6,7,8,9] N-acetyl-neuraminic acid (NANA) (specific activity 223 nC/nmole) (New England Nuclear Corp.), 0.3% Triton X-100 (Rohm and Haas), and 5 mM $MnCl_2$ in a total volume of 0.12 ml. After a period of incubation at 37°, 1 ml of 1% phosphotungstic acid in 0.5 N HCl was added. The reaction mixture was cooled in ice for 30 minutes. The insoluble material was collected by centrifuging the mixture at 500 x g for 15 minutes. The precipitate was washed three times by repeatedly centrifuging it from a 5% trichloro-acetic acid solution (1.5 ml per wash). The pellet was dissolved in 0.3 ml of 0.1 N NaOH; 0.2 ml of 0.1 N HCl was added after solution was attained. The radioactivity of this solution was determined in 15 ml Aquasol (New England Nuclear) in a Mark I scintillation spectrometer. The automatic external standard was used to correct counts to disintegrations per minute. The efficiency varied between 70% and 78% for ^{14}C.

Sialoglycoprotein isolation and electrophoretic analysis

Sialoglycoproteins, such as alkaline phosphatase, can be rendered soluble by extracting an aqueous cell suspension with n-butanol (3 to 1) (72,73). About 80 x 10^6 cells of each clone in each state suspended in 9 ml of ST buffer were extracted with 3 ml of n-butanol by agitating on a vortex mixer run at full speed for a minimum of 3 minutes. The aqueous layer was removed, and the cell butanol layer was reextracted with ST buffer. The first and second aqueous extracts were pooled, dialyzed exhaustively against 25 mM Tris-HCl (pH 7.4), lyophilized and

reconstituted with water to about 1 mg protein per milliliter. The duplicate aliquots of proteins, one of which was treated with Clostridium perfringens neuraminidase (10 µg per 200 µg glycoprotein) (Sigma) for 3 hours at 37°, were analyzed by acrylamide gel electrophoresis by a method previously described (92). The amount of sialic acid in the extract was also determined.

Nuclear fraction isolation

The nuclear fraction of HeLa cells was isolated and purified by the method of Penman et al. (93). About 9 x 10^7 cells of each clone in each state were suspended in 4 ml of RSB buffer (NaCl 10 mM, $MgCl_2$ 1.5 mM, Tris-HCl 10 mM, pH 7.4) and allowed to swell for 15 minutes. The suspension was then homogenized with 20 complete strokes in a Dounce homogenizer. The homogenate was centrifuged at 1800 x g for one minute. The pellet was resuspended in 4 ml of RSB buffer, mixed and centrifuged as described above. The pellet was washed in 0.5% Tween 40 (in RSB buffer) for 15 minutes by mixing constantly, and centrifuged. The pellet was then resuspended in 4 ml of RSB buffer containing 0.6 ml of detergent (10% deoxycholate:10% Tween 40, 1:2, v/v). The suspension was mixed on a vortex for 3 seconds and centrifuged. The nuclear pellet obtained was suspended in water for the determinations of alkaline phosphatase activity, 5'-nucleotidase activity, sialic acid, protein, RNA, and DNA.

CHAPTER III

RESULTS

Glycoprotein carbohydrates of HeLa cells

Tables 1 and 2 show the sialic acid, hexosamine and neutral sugar content of HeLa 65 and 71 in control and cortisol-regulated (Hcr) states. HeLa 71 had a pronounced increase in glycoprotein sialic acid (84%) and hexosamine (71%) in the Hcr state on a cell basis, (Table 1). However, on a per-milligram-of-protein basis, sialic acid increased 51% and hexosamine increased 35% in HeLa 71 Hcr (Table 2). In contrast to HeLa 71, HeLa 65 showed no significant difference in sialic acid and hexosamine content in the Hcr state when expressed either on a cell of protein basis. The average cell diameters, as calculated from the measured cell volume in a Bauer-Schenck sedimentation tube, were 18 μ in HeLa 71 and 19 μ in HeLa 71 Hcr. To calculate the average density of sialic acid per surface area, we calculated the mean surface area of the cell by the formula, area = $4.84\ V^{2/3}$, and assumed 60% of the total sialic acid molecules to be concentrated on the surface of the cell (94). Using these approximations, the average density of sialic acid per surface area of HeLa 71 was 7.1×10^5 molecules/μ^2; for HeLa 71 Hcr, it was 12.1×10^5 molecules/μ^2 (Table 3).

A modified Hale's colloidal iron stain showed that sialic

TABLE 1

CARBOHYDRATE CONTENT OF GLYCOPROTEINS OF CONTROL AND HCR
HeLa CLONES EXPRESSED PER MILLION CELLS

Carbohydrate	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/10^6 cells		nmoles/10^6 cells	
Sialic Acid	2.2 ± 0.5	1.8 ± 0.3	1.9 ± 0.2	3.5 ± 0.3
Hexosamine	6.9 ± 1.5	5.7 ± 0.1	4.9 ± 0.7	8.2 ± 1.4
Neutral Sugars	105 ± 19	199 ± 39	62 ± 15	109 ± 19

Cell counts were performed on one replicate Blake bottle by trypsinization of the cells and the cells from the other three bottles were scraped into ST Buffer and glycoprotein sugar determined as in Methods. The data represent the mean of at least three experiments ± standard error and all determinations were done in duplicate.

TABLE 2

CARBOHYDRATE CONTENT OF GLYCOPROTEINS OF CONTROL AND HCR
HeLa CLONES EXPRESSED PER MILLIGRAM PROTEIN

Carbohydrate	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/mg protein		nmoles/mg protein	
Sialic Acid	9.9 ± 2.4	7.7 ± 1.3	9.9 ± 0.8	14.9 ± 1.3
Hexosamine	31 ± 5.8	24 ± 0.6	26 ± 3.2	35 ± 5.3
Neutral Sugars	471 ± 48	842 ± 174	327 ± 71	462 ± 77

See legend in Table 1.

TABLE 3

SIALIC ACID CONTENT OF HeLa 71 CELLS

	Control	Hcr
Average diameter (μ)	18	19
Mean cell volume (μ^3)	3054	3541
Molecules per cell, total (x 10^8)	12	22.8
Molecules per cell on surface (x 10^8)	7.2	13.8
Mean cell surface area (4.84 $V^{2/3}$)(μ^2)	1016	1133
Molecules/μ^2 area on surface (x 10^5)	7.1	12.1

The average cell diameter is calculated from the measured cell volume in a Bauer-Schenck sedimentation tube. Sialic acid content in nanomoles as shown in Table 1 was converted to molecules by Avogadro's number. Mean surface area of the cell was calculated with the formula, area = 4.84 $V^{2/3}$, and 60% of the total sialic acid molecules were assumed to be concentrated on the surface of the cell (94).

acid was concentrated in the nuclei, the cell periphery, and the membrane junction of the cells. Some of the nuclei stained much more heavily than others, probably reflecting the asynchronous cell population studied.

Hexosamines liberated from glycoprotein by acid hydrolysis were separated into glucosamine and galactosamine peaks by use of an amino acid analyzer, as described in Methods. Galactosamine represented only 2% to 5% of the total hexosamine of either cell clone's glycoprotein in the control or the Hcr state. The preponderance of glucosamine indicated that most of the HeLa cell glycoproteins were the "fetuin" or plasma type.

Both clones doubled their content of neutral sugars in the glycoprotein fraction of the Hcr state (Table 1). Of the neutral sugars, 85% was glucose, as determined by glucose oxidase assay. This glucose occurred in the form of protein-bound glycogen. Partial characterization of this complex will be discussed later. Neutral sugars of HeLa cell glycoproteins were separated and quantitated by gas-liquid chromatography. The results of this analysis are shown in Table 4. Ribose, probably derived from cell RNA, was not significantly altered in either clone in either state. Mannose derived from glycoprotein was not significantly changed in either clone in either state. Galactose increased 49% in HeLa 71 Hcr, but no significant change occurred in HeLa 65 Hcr. Although 0.06 nmoles of fucose per 10^6 cells could have been detected by this method, no fucose was found in these studies.

Lipid composition of HeLa cells

The total lipid was increased by 30% in HeLa 65 Hcr (Table 5). No significant change was found between HeLa 71 control and Hcr (Table 5).

TABLE 4

NEUTRAL SUGARS OF GLYCOPROTEINS OF CONTROL AND HCR
HeLa CLONES EXPRESSED PER MILLION CELLS

Sugar	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/10^6 cells		nmoles/10^6 cells	
Ribose	21 ± 6.3	25 ± 9.2	18 ± 6.3	27 ± 15
Mannose	4.8 ± 1.0	4.3 ± 1.1	4.5 ± 1.5	5.1 ± 0.99
Galactose	2.8 ± 0.44	3.2 ± 1.4	2.7 ± 0.76	4.1 ± 0.73

Neutral sugars were analyzed by gas-liquid chromatography as described in Methods. The numbers represent the mean of three experiments ± standard error.

TABLE 5

TOTAL LIPIDS OF CONTROL AND HCR HeLa CLONES

Clone	Lipids $\mu g/10^6$ cells
HeLa 65 Control	67.3 ± 3.7
HeLa 65 Hcr	86.7 ± 2.5
HeLa 71 Control	83.8 ± 7.8
HeLa 71 Hcr	93.2 ± 8.8

Lipids were obtained by whole cell extraction with chloroform: methanol as described in Methods. The data represent the mean of at least three experiments ± standard error.

The phospholipid composition of both clones in both states was identified as phosphatidylcholine, phosphatidylethanolamine, lyophosphatidylcholine, and phosphatidylserine. Neither the total amount nor the distribution of each class of phospholipid changed significantly in HeLa 71 Hcr state as compared to that in control state (table 6). The total phospholipids decreased slightly in HeLa 65 Hcr compared to those in the control. However, the relative distribution of each class of phospholipids did not change significantly in HeLa 65 in the Hcr state compared to that in the control state. The increased lipids of HeLa 65 Hcr were due to neutral lipids (composed primarily of cholesterol and triglyceride). The lipid distribution within the HeLa cell was investigated by histologic staining of fixed cell monolayers with Sudan black B. No evidence of lipid droplets was observed, but the membrane portions of the cell were stained. This indicates that the increased lipids of HeLa 65 Hcr probably represent increased membrane-localized lipids.

Glycolipid sialic acid increased 40% in HeLa 71 Hcr (Tables 7 and 8). No significant change was shown in any other lipid-bound carbohydrate moiety from control or Hcr cells in either clone.

Separation and partial characterization of HeLa cell membranes

Both cell clones in control and Hcr states had the same membrane banding pattern. A typical membrane separation pattern appears in Figure 1. Six fractions were obtained, and each was analyzed and examined by electron microscopy (Figure 2). Plate I shows smooth endoplasmic reticulum, Golgi and plasma membrane vesicle (Fraction I). Plate II shows smooth endoplasmic reticulum and possibly plasma membrane vesicle (Fraction II). Plate III shows rough endoplasmic reticulum

TABLE 6

NATURE OF THE CLASSES OF PHOSPHOLIPIDS ISOLATED FROM CONTROL AND HCR HeLa CLONES

Phospholipid	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/10^6 cells		nmoles/10^6 cells	
Sphingomyelin and Lysophosphatidylcholine	9.2	5.8	6.1	5.7
Phosphatidylcholine	21	16	15	15
Phosphatidylethanolamine	6.5	7.0	6.3	4.5
Phosphatidylserine	3.4	1.6	1.6	1.7

The methods of separation and determination of phospholipids are described in Methods.

TABLE 7

CARBOHYDRATE CONTENT OF GLYCOLIPIDS OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLION CELLS

Carbohydrate	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/10^6 cells		nmoles/10^6 cells	
Sialic Acid	0.12 ± 0.03	0.15 ± 0.01	0.15 ± 0.02	0.21 ± 0.01
Hexosamine	0.57 ± 0.18	0.71 ± 0.25	0.65 ± 0.23	0.69 ± 0.09
Neutral Sugars	4.0 ± 1.2	4.1 ± 0.4	3.9 ± 0.1	4.4 ± 1.3

Glycolipids were obtained by whole cell extraction with chloroform: methanol as described in Methods. The data represent the mean of at least three experiments ± standard error.

TABLE 8

CARBOHYDRATE CONTENT OF GLYCOLIPIDS OF CONTROL AND HCR
HeLa CLONES EXPRESSED PER MILLIGRAM LIPID

Carbohydrate	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/mg lipid		nmoles/mg lipid	
Sialic Acid	1.8	1.7	1.8	2.2
Hexosamine	8.5	8.2	7.7	7.4
Neutral Sugars	60	47	46	47

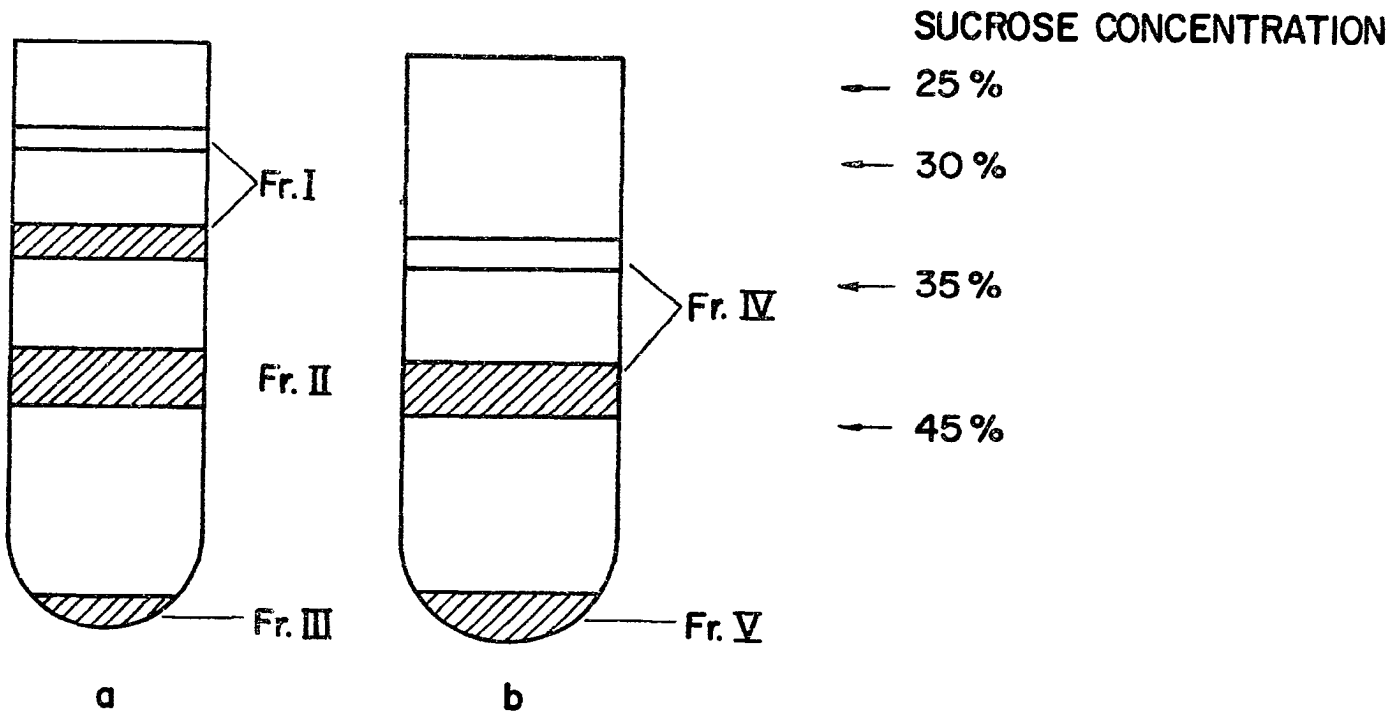

Figure 1--Typical banding pattern of HeLa cell membranes. Preparation and centrifugational flotation of these bands are described in Methods. The characterization and naming of the bands are described in Results. Shaded areas represent heavy opalescent bands and unshaded areas barely visible bands.

Figure 2

Electron Micrographs of HeLa Cell Membrane Fractions

Plate I--Smooth endoplasmic reticulum, Golgi (indicated by ↑) and plasma membrane vesicles (x 27,000).

Plate II--Smooth endoplasmic reticulum (indicated by ↑) and plasma membrane vesicles (x 25,200).

Plate III--Rough endoplasmic reticulum (x 18,750).

Plate IV--Plasma membrane ghost (pm). The presence of inner cytoplasmic membrane such as mitochondria (m) is also noted (x 15,000).

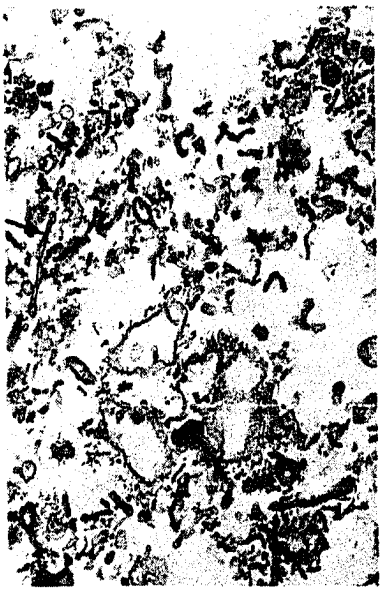

Plate I

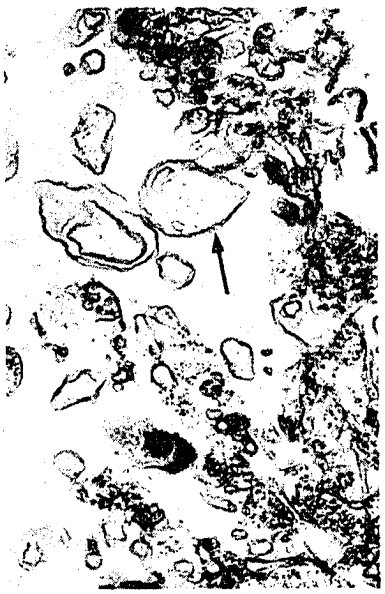

Plate II

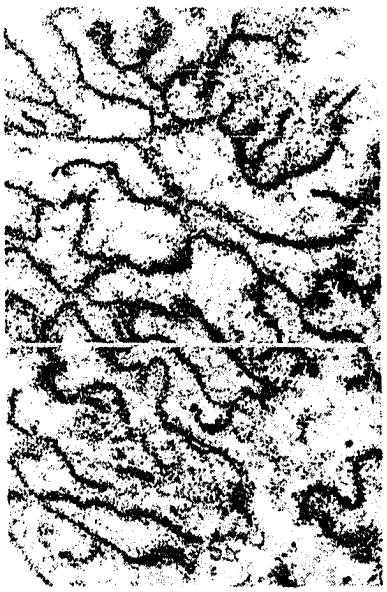

Plate III

Plate IV

Figure 2. Electron Micrographs of HeLa Cell Membrane Fractions

(Fraction III). Plate IV shows whole plasma membrane ghosts (Fraction IV). Fraction V is the nuclear fraction plus contaminating cytoplasm, while fraction VI consists of lysosomes, mitochondria, and contaminating cytoplasm. These membrane preparation experiments were performed at least three times for each clone in each state. The results are representative of any single experiment and are reported from the best experiment of a series.

The membrane fractions were analyzed for protein, RNA, alkaline phosphatase activity, 5'-nucleotidase activity, sialic acid, and hexosamine. These assays were performed to study the effect of hydrocortisone on the cell-membrane system and also to support the nomenclature assigned by electron microscopy for each fraction. Table 9 shows the protein content of isolated membrane fractions. The amount of protein in rough endoplasmic reticulum increased in the Hcr state in both clones (Fraction III, Table 9). In HeLa 65 Hcr, the smooth endoplasmic reticulum (Fraction II, Table 9) was increased in protein content, correlating with the increased amount of lipid found in HeLa 65 Hcr. In HeLa 71 Hcr, the amount of protein in the smooth endoplasmic reticulum was somewhat less than control (Fraction II, Table 9). Plasma membrane protein increased in HeLa 71 Hcr by 52% over control, whereas no significant changes occurred in HeLa 65 Hcr (Fraction IV, Table 9).

RNA was mainly localized in rough endoplasmic reticulum fraction (Fraction III) and nuclear fraction (Fraction V) (Table 10). The amount of RNA found in Fraction VI indicates that a large proportion of the rough endoplasmic reticulum sedimented with lysosomes and mitochondria at 4000 x g for 20 min. DNA determination of these fractions

TABLE 9

PROTEIN DISTRIBUTION IN MEMBRANES OF CONTROL AND HCR HeLa CLONES

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	protein(μg/10^6 cells)		protein(μg/10^6 cells)	
Whole cell	240	220	190	260
Fraction I	0.09	0.03	1.7	1.1
Fraction II	0.40	1.4	2.5	1.9
Fraction III	2.9	5.3	4.4	6.1
Fraction IV	6.1	5.4	2.3	3.5
Fraction V	84	67	78	83
Fraction VI	9.1	16	15	12

Results are expressed on a per cell basis. Cell counts were obtained from a replicate Blake bottle which was trypsinized and counted but not used in the membrane preparation. The numbers are representative of the best single experiment chosen from three separate experiments. The preparation of the cell fractions and the protein assay are described in Methods.

TABLE 10

RNA DISTRIBUTION IN MEMBRANES OF
CONTROL AND HCR HeLa CLONES

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	RNA($\mu g/10^6$ cells)		RNA($\mu g/10^6$ cells)	
Whole cell	33	27	22	17
Fraction I	0.044	0.096	0.32	0.46
Fraction II	0.13	0.38	0.24	0.38
Fraction III	2.0	2.9	2.1	3.0
Fraction IV	0.8	0.64	0.38	0.52
Fraction V	8.5	5.9	6.0	4.7
Fraction VI	4.1	2.9	2.2	2.8

See legend in Table 9. The RNA assay is described in Methods.

showed that over 90% of the total cell DNA was localized in the nuclear fraction (Fraction V) (12.2 μg/10^6 cells in nucleus; 1 μg/10^6 cells total in the other fractions).

Alkaline phosphatase activity for whole cell and Fractions I through VI are shown in Tables 11 and 12. The data are depicted in two ways; (a) on a per-cell basis and (b) on a per-milligram-of-protein basis. HeLa 65 underwent almost a fivefold increase in alkaline phosphatase activity in the cortisol regulated state. This increase in alkaline phosphatase activity was reflected in the plasma membrane (Fraction IV, Table 11 and 12) of HeLa 65 Hcr compared to that of the control. In HeLa 71, cloned for its high constitutive level of alkaline phosphatase, the enzymic activity remained unaltered in the Hcr state. This was reflected in the alkaline phosphatase activity of HeLa 71 plasma membrane fraction from control and Hcr cells (Fraction IV, Tables 11 and 12). 5'-Nucleotidase, another plasma-membrane-localized enzyme, exhibited the same alterations in the Hcr state as alkaline phosphatase (Tables 13 and 14). We believe this to be the first report of cortisol "induction" of 5'-nucleotidase in HeLa 65 cells. Although these two enzymes are considered markers for plasma membrane, and were shown to be relatively concentrated in Fraction IV (supporting the electron microscopic finding), they were also associated with Fractions I and II. Expressed on a per-milligram-of-protein basis, the enzyme activities of Fractions I and II were as high or higher than that of the plasma membrane (Tables 12 and 14). Therefore, we suspect that the smaller amounts of these enzymes in Fractions I and II were actually associated with smooth endoplasmic reticulum, and/or Golgi, and cannot be accounted for simply by the presence of plasma

TABLE 11

SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN MEMBRANES OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLION CELLS

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles p-NPP/min/10^6 cells			
Whole cell	1.5	7.7	190	190
Fraction I	0.011	0.10	14	14
Fraction II	0.017	0.35	14	9.9
Fraction III	0.011	0.084	5.4	1.1
Fraction IV	0.17	0.91	13	19
Fraction V	0.40	1.9	31	36
Fraction VI	0.22	2.3	17	29

TABLE 12

SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN MEMBRANES OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLIGRAM PROTEIN

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles p-NPP/min/mg protein			
Whole cell	6.2	36	1000	720
Fraction I	120	3200	8400	14000
Fraction II	44	260	5700	5200
Fraction III	3.7	16	1200	170
Fraction IV	29	170	5700	5300
Fraction V	4.8	28	400	430
Fraction VI	25	140	1100	2400

TABLE 13

SPECIFIC ACTIVITY OF 5'-NUCLEOTIDASE IN MEMBRANES OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLION CELLS

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles Pi/min/10^6 cells			
Whole cell	0.55	2.3	7.5	4.9
Fraction I	ND	0.018	0.86	0.25
Fraction II	ND	0.096	0.47	0.20
Fraction III	ND	ND	0.14	0.027
Fraction IV	0.15	0.26	0.41	0.40
Fraction V	0.21	0.60	1.8	1.2
Fraction VI	0.14	0.77	2.1	1.0

ND: not detectable

TABLE 14

SPECIFIC ACTIVITY OF 5'-NUCLEOTIDASE IN MEMBRANES OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLIGRAM PROTEIN

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles Pi/min/mg protein			
Whole cell	2.3	11	39	19
Fraction I	ND	560	510	230
Fraction II	ND	71	190	110
Fraction III	ND	ND	30	4.4
Fraction IV	24	48	180	120
Fraction V	2.5	9.0	23	14
Fraction VI	15	48	140	86

ND: not detectable

membrane vesicles in those two fractions.

The changes in sialic acid of the whole cell (Tables 1 and 2) are reflected in the membrane assays shown in Table 15. The plasma membrane fraction (Fraction IV) of HeLa 71 Hcr had a twofold increase in the amount of sialic acid compared to that in the control, while the other cytoplasmic fractions of HeLa 71 Hcr (Fractions I, II and III) showed no significant changes versus control. The sialic acid of the nuclear fraction of HeLa 71 Hcr rose markedly (Fraction V, Table 15). HeLa 65 did not respond to cortisol by significant alteration of sialic acid content in the whole cell glycoprotein; this was reflected in the membrane fractions isolated (Table 15). The data of Table 15, expressed as nmoles of sialic acid per milligram membrane protein is shown in Table 16. The plasma membrane fraction (Fraction IV) of HeLa 71 Hcr had only 29% increase in sialic acid on a per-milligram-of-protein basis, while the Fractions I and II had 100% and 50% increase respectively. Fraction I of HeLa 65 rose markedly in sialic acid content per milligram membrane protein, while Fraction II dropped by 50%. Dissimilarities in the direction of change in Fractions I and II were evident, when one compares the results in Table 15 to those in Table 16. This might indicate that different degrees of glycosylation of membrane proteins occur in these fractions.

Fraction IV (plasma membrane) of HeLa 71 Hcr doubled its hexosamine content compared to control (Fraction IV, Table 17). On a per-milligram-of-protein basis, Fraction IV of HeLa 71 Hcr had only 25% increase in hexosamine compared to that of the control (Fraction IV, Table 18). Although Fractions I, II and III of HeLa 71 Hcr showed no

TABLE 15

SIALIC ACID CONTENT IN MEMBRANES OF
CONTROL AND HCR HeLa CLONES
EXPRESSED PER MILLION CELLS

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	Sialic acid(nmoles/10^6 cells)			
Whole cell	2.2	1.8	1.9	3.5
Fraction I	0.013	0.016	0.10	0.13
Fraction II	0.019	0.031	0.09	0.11
Fraction III	0.008	0.011	0.025	0.023
Fraction IV	0.13	0.10	0.097	0.19
Fraction V	0.30	0.36	0.10	0.17
Fraction VI	0.22	0.31	0.21	0.36

See legend in Table 9. Sialic acid assay is described in Methods.

TABLE 16

SIALIC ACID CONTENT IN MEMBRANES OF CONTROL AND HCR
HeLa CLONES EXPRESSED PER MILLIGRAM PROTEIN

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/mg protein		nmoles/mg protein	
Whole cell	9.9	7.7	9.9	14.9
Fraction I	150	510	61	120
Fraction II	47	23	37	57
Fraction III	2.9	2.0	5.5	3.7
Fraction IV	22	19	42	54
Fraction V	3.5	5.4	1.3	2.1
Fraction VI	24	19	26	30

TABLE 17

HEXOSAMINE CONTENT IN MEMBRANES OF CONTROL AND HCR HeLa CLONES EXPRESSED PER MILLION CELLS

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/10^6 cells		nmoles/10^6 cells	
Whole cell	6.9	5.7	4.9	8.2
Fraction I	ND	0.05	0.12	0.17
Fraction II	0.063	0.15	0.12	0.15
Fraction III	0.25	0.91	0.19	0.15
Fraction IV	0.12	0.16	0.11	0.21
Fraction V	2.90	2.30	1.10	1.30
Fraction VI	0.28	0.69	0.28	0.90

See legend in Table 9. Hexosamine assay is described in Methods. ND: not detectable.

significant changes versus control on a per cell basis (Table 17), two showed marked increase (Fractions I and II) and one showed a decrease (Fraction III) in hexosamine on a per-milligram-of-protein basis (Table 18). Although whole-cell analysis revealed no significant changes in the amount of hexosamine in HeLa 65 Hcr cells compared to that in the control, Fractions I, II, III and VI of HeLa 65 Hcr had more hexosamine than that in the control, whereas Fraction V had less. On a per-milligram-of-protein basis, Fractions I, III, IV and VI increased hexosamine content in HeLa 65 Hcr, while Fraction II decreased slightly. Thus, minor differences existed between the localization of hexosamine and sialic acid in the membrane fractions of the HeLa 65 control and Hcr cells.

Purification of the nuclear fraction and its partial characterization

Cytoplasmic contamination of the nuclear fraction unavoidably attends the simple homogenization method used for preparing membrane fractions. Therefore, the nuclei were isolated by a technique using a concentrated detergent wash (93); the purity of the nuclei was ascertained by electron microscopy. Whole nuclei were the major species present. Over 60% were estimated to have intact nuclear membranes. No contaminating cytoplasm was evident. Interestingly, all nuclear fractions contained significant amounts of sialic acid, alkaline phosphatase, and 5'-nucleotidase activities (Table 19). All these parameters correlated with the alterations in the whole cell and membrane fractions of both clones. Sialic acid increased 2.6-fold in HeLa 71 Hcr, whereas no changes occurred in HeLa 65 Hcr compared to that in the control (Table 19). Paper

TABLE 18

HEXOSAMINE CONTENT OF CONTROL AND HCR HeLa CLONES
EXPRESSED PER MILLIGRAM PROTEIN

Membrane Fraction	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	nmoles/mg protein		nmoles/mg protein	
Whole cell	31	24	26	35
Fraction I	ND	1600	68	170
Fraction II	158	108	46	80
Fraction III	85	170	41	25
Fraction IV	19	30	48	60
Fraction V	34	34	13	16
Fraction VI	31	43	36	74

ND: not detectable

TABLE 19

CHARACTERISTICS OF THE PURIFIED NUCLEAR FRACTION
OF CONTROL AND HCR HeLa CLONES

	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
Protein ($\mu g/10^6$ nuclei)	34	75	47	57
RNA ($\mu g/10^6$ nuclei)	11	15	13	21
Sialic Acid (nmoles/10^6 nuclei)	0.22	0.23	0.086	0.22
Alkaline Phosphatase (nmole p-NPP/min/10^6 nuclei)	0.11	1.1	25	6.4
5'-Nucleotidase (nmole Pi/min/10^6 nuclei)	0.25	0.63	0.93	0.29

Nuclear fraction was prepared as described in Methods. All values have been corrected for the recovery of nuclei based on the average DNA content of a HeLa cell (12 $\mu g/10^6$ cells). Sialic acid was determined in material precipitated by 1% phosphotungstic acid (in 1 N HCl) without extraction of lipid.

chromatography of the acid hydrolysates indicated that all the sialic acid isolated was in the form of N-acetylneuraminic acid. Therefore, the trace of N-glycolylneuraminic acid found in HeLa cells (95) was probably associated with the cell surface. Since the sialic acid recovered in the purified nuclear fraction represented at least 70% of that found in the contaminated nuclear fraction obtained by homogenization (compare Table 15 with Table 19), sialic acid would seem to be an integral part of the HeLa nucleus, as a component of glycoprotein of the membrane and/or chromatin. A 10-fold "induction" of alkaline phosphatase activity occurred, as well as a 2.5-fold "induction" of 5'-nucleotidase activity in purified nuclei from HeLa 65 Hcr compared to those in the control.

Partial characterization of the glycogen protein complex

Electron microscopy and biochemical analysis for neutral sugars and glucose indicated that Fraction III of each clone contained at least 50% of the total recoverable glycogen protein complex of the cell. Since this membrane fraction is enriched in glycogen complex and since HeLa 65 cells have the greatest amount of this complex, Fraction III of HeLa 65 Hcr cells was used to characterize the complex. In addition to the ability to quantitatively precipitate this glycogen complex with phosphotungstic acid, the susceptibility of this material to α-amylase treatment gave additional supporting biochemical evidence for the association of glycogen with protein. When fraction III material was incubated with α-amylase for several hours at 37°, less than 5% of the hydrolyzable glucose was solubilized, as determined by glucose oxidase and reducing sugar assays. If one pre-extracts this complex with chloroform:methanol (2:1), the complex is now enzymatically

hydrolyzed to greater than 90%. This complex was further characterized after pronase digestion and purification. Fraction III was digested with pronase (10 µg pronase/mg protein) for 4 days at 37°. The digestion mixture was applied to a 1 x 60 cm Sephadex G-200 column and glycogen was eluted at the void volume. After concentration by collodion membrane filtration the material was dialyzed against 1 mM phosphate pH 7. This material was 99.6% glucose and 0.4% protein. The complex was then hydrolyzed in 6 N HCl at 110° for 22 hours and amino acid composition of the hydrolysate as determined on an amino acid analyzer gave 15 different types of amino acids. Since a minimum molecular weight of the total glycogen complex was found to be about 4 x 10^6 Daltons as estimated from calibrated electron microscopic photographs, we estimate the molecular weight of the polypeptide associated with the glycogen to be about 17,500. Further centrifugation of Fraction III on a discontinuous sucrose gradient (45%, 50%, and 60% sucrose) failed to separate the glycogen-protein complex from rough endoplasmic reticulum. Treatment of Fraction III with ribonuclease followed by CsCl-gradient centrifugation may provide separation, purification, and characterization of this glycogen-protein (possibly membrane) complex. Experiments such as these are planned for the near future.

Glycoprotein sialyltransferase studies

The activity of HeLa sialyltransferase was measured with endogenous and exogenous acceptors. Using 0.3% Triton X-100, 0.5 mg of desialized fetuin per reaction stimulated endogenous sialyltransferase activity 40-fold. Therefore exogenous acceptors were used in

all further studies. The optimal pH for sialyltransferase activity ranged between 6.5 and 6.8 (Figure 3). It was similar for both clones in both states. Phosphate buffer was optimum at 10 mM concentration, and higher concentrations, such as 100 mM, inhibited the reaction over 90%. Triton X-100 concentration was found to be optimum at 0.3%. Because the initial reaction rate was linear from 0.1 to 0.5 mg of HeLa cell cytoplasmic homogenate per reaction, 0.25 mg per reaction was used thereafter. The reaction rate remained linear up to 30 min. Mn^{++} stimulated this reaction 2.4 times more efficiently at its optimum concentration (5 mM) than Mg^{++} at its optimum concentration (10 mM) (Figure 4). Therefore, Mn^{++} (5 mM) was used throughout the study. When desialized mucin was used as exogenous acceptor, 5 mM of Mn^{++} or Mg^{++} was optimal, producing the same degree of stimulation. Enzyme activity of the cell clones were compared under optimal reaction conditions. With desialized fetuin as exogenous acceptor, HeLa 71 Hcr evidenced a 70% increase in sialyltransferase activity compared to that in HeLa 71 control (Table 20). On a per cell basis, an even greater increase was seen for the HeLa 71 Hcr cell (0.32 pmoles NANA/min/10^6 cells for control compared to 0.90 pmoles/min/10^6 cells for Hcr). However, when desialized mucin was used as acceptor, all cell clones had similar activities (Table 20). The activity was between 30 and 50% of that obtained by using desialized fetuin as acceptor. These data correlate with our previous finding that "plasma"-type glycoproteins such as fetuin are the major constituent of HeLa cell glycoproteins. The results are also consistant with the observation that prednisolone increases the agglutinability of HeLa cells by wheat germ lipase agglutinin (which is specific

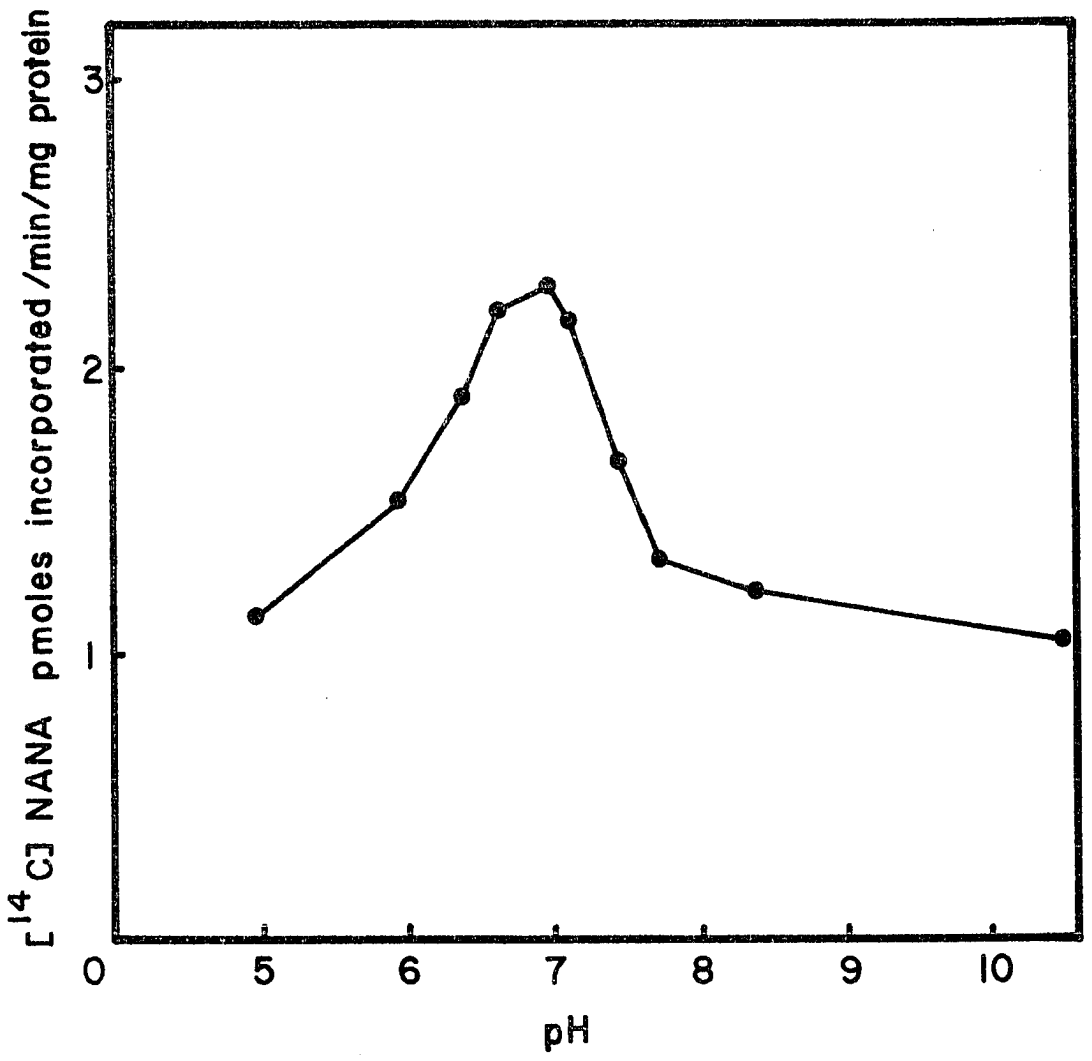

Figure 3--The effect of pH on HeLa CMP-[^{14}C] NANA glycoprotein sialyltransferase activity. The pH of the reaction mixture was adjusted with acid or base without increasing phosphate concentration.

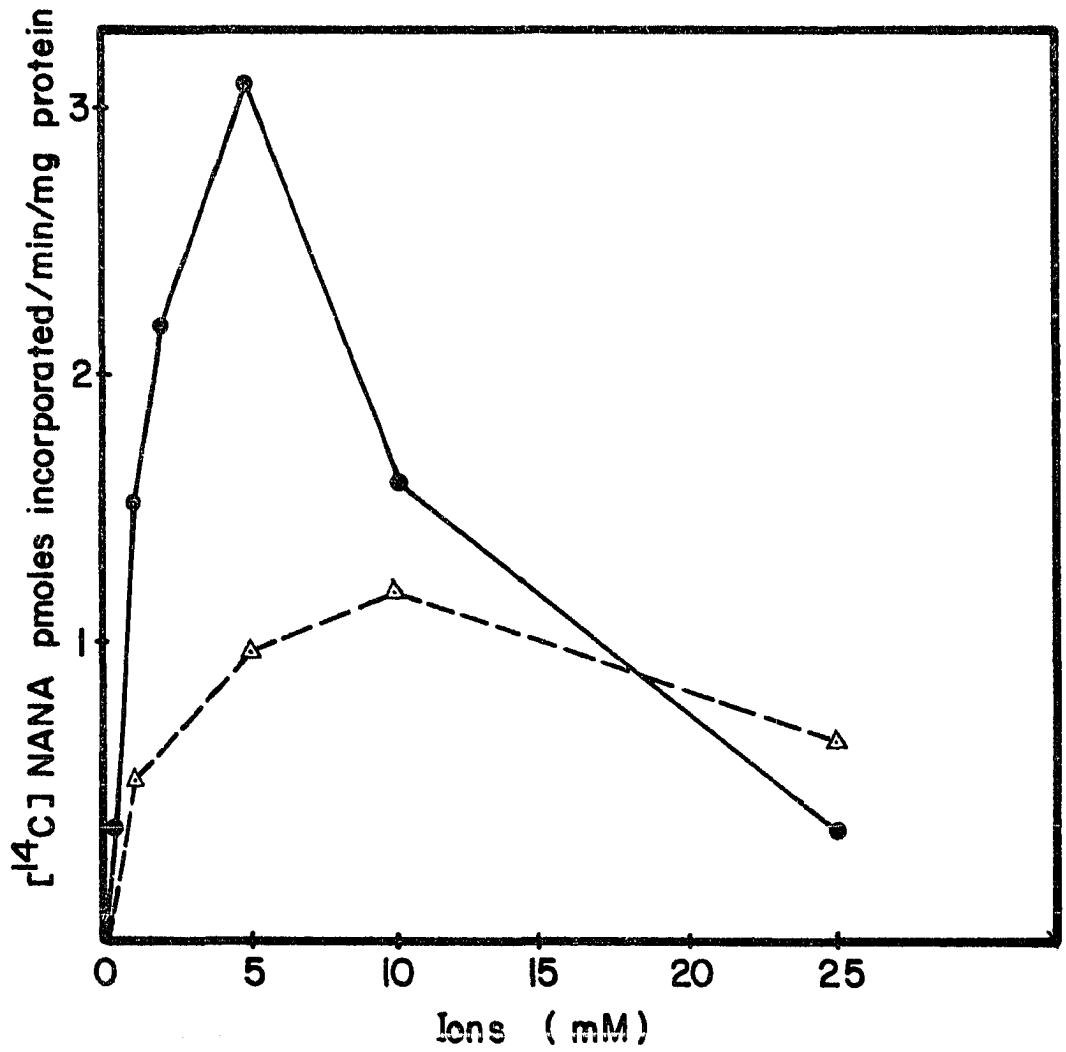

Figure 4--The effect of ions on HeLa CMP-[^{14}C] NANA glycoprotein sialyltransferase activity. See legend in Table 20.

●——●; $MnCl_2$ in mM; △——△; $MgCl_2$ in mM.

TABLE 20

CMP-[^{14}C] NANA GLYCOPROTEIN SIALYLTRANSFERASE ACTIVITY
OF CONTROL AND HCR HeLa CLONES

Acceptor	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	pmoles [^{14}C]NANA/min/mg protein			
Desialized fetuin	3.9	3.1	3.1	5.2
Desialized mucin	1.7	1.7	1.1	1.5

Enzyme and acceptor protein were obtained as described in Methods. Optimum conditions for the assay were also described in Results.

for N-acetyl glucosamine) but not by soybean agglutinin (which is specific for N-acetylgalactosamine)(96). Although cytoplasmic extracts were used for these studies, whole cell homogenates gave very similar results on a per cell basis, indicating the preponderant cytoplasmic localization of sialyltransferase activity.

Sialoglycoprotein studies

Analysis of the aqueous phase of cell-butanol extracts for sialic acid gave between 70% and 90% of the total amount of sialic acid obtainable from glycoprotein of these cells. Analytical polyacrylamide disc gel electrophoresis showed seven major bands in HeLa 71 and HeLa 71 Hcr (Figure 5). Pretreatments of aliquots of these extracts with C. perfringens neuraminidase retarded the movement of most bands on disc-gel analysis, indicating the sialic acid moiety to be a common feature of all these proteins. Alkaline phosphatase was detected in replicate gels histochemically; it appeared to be a major band. When the protein of these sialoglycoproteins were compared on a cell basis, it became evident that the 50% increase in sialoglycoprotein in HeLa 71 Hcr over HeLa 71 control could account for much of the 86% increase of sialic acid in this clone in the Hcr state (Table 21). The amount of sialic acid in the butanol extract was 1.42 nmoles/10^6 cells for HeLa 71 control; 2.68 nmoles/10^6 cells for HeLa 71 Hcr (Table 21).

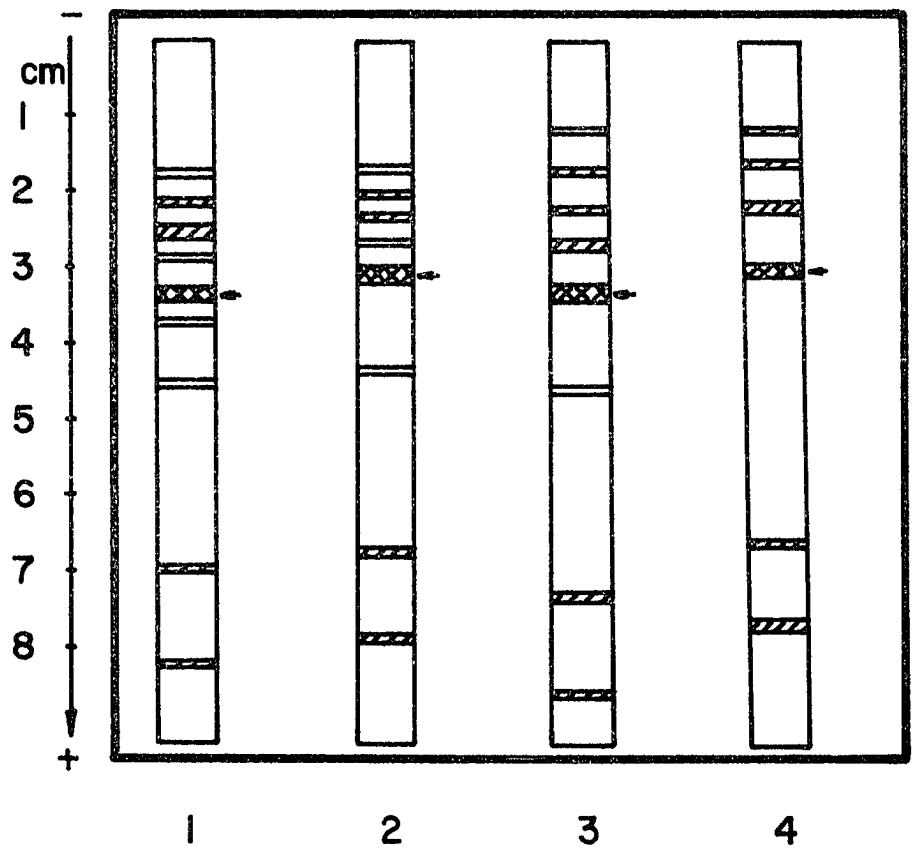

Figure 5--Disc-gel electrophoretogram of HeLa 71 and HeLa 71 Hcr glycoproteins. Electrophoresis was performed as described in Methods. In each sample gel 200 µg of protein was placed and electrophoresis was run for 5.5 hours at 2 mA constant current. Replicate gens were stained for alkaline phosphatase activity. Alkaline phosphatase positive bands are indicated by the horizontal arrows. 1 = HeLa 71 control, 2 = HeLa 71 control incubated with neuraminidase, 3 = HeLa 71 Hcr, and 4 = HeLa 71 Hcr incubated with neuraminidase.

TABLE 21

SIALIC ACID AND PROTEIN CONTENT OF GLYCOPROTEIN SOLUBILIZED BY TREATMENT WITH n-BUTANOL EXTRACTION

	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
Sialic acid (nmoles/10^6 cells)	1.05	2.26	1.42	2.68
Sialic acid (nmoles/mg protein)	12.0	18.5	22.2	31.0
Protein (µg/10^6 cells)	80	88	70	105

The extraction, concentration and assays of these proteins are described in Methods.

CHAPTER IV

DISCUSSION

Two HeLa cell clones have been shown to have marked growth differences in response to cultivation with cortisol. HeLa 71, but not HeLa 65, has a prolonged cell generation time in the Hcr state, mainly at the expense of the G_1 portion of the cell cycle (74). It has been suggested that the effect of hydrocortisone is manifested by changes in the HeLa cell membranes (97). Carubelli and Griffin (75) showed that HeLa 65 responds to short-term growth (72 hours) with cortisol by an increase in cellular sialic acid, more than one half of which was sedimentable at 100,000 x g for 1 hour. In this study, we found that HeLa 65 cells continuously subcultured with cortisol have a similar amount of sialic acid compared to that in the control cells. On the other hand, HeLa 71 Hcr has an 84% increase in sialic acid contained in glycoprotein, and a 40% increase in glycolipid sialic acid, compared to that in the control cells. Sialic acid is one of the terminal sugars found in the prosthetic group of many glycoproteins. It is also found in the peripheral position of some glycolipids. These glycoprotein and glycolipid are normally components of cell membranes. About 60% of proliferating mammalian cell sialic acid is believed to be located on the surface membrane (94,98). Because the average cell

surface areas of HeLa 71 control and Hcr cells do not differ appreciably, the 70% increase in sialic acid per average HeLa 71 Hcr cell surface might either represent a uniformly distributed increase in net negative charge on the cell surface, or increase in negative charge limited to discrete areas. The latter possibility is most probable if this increase is due to greater amounts of terminal sugars on similar numbers of glycoprotein molecules. The fact that HeLa 65 Hcr has an acute, but not chronic, increase in sialic acid content may relate to the increased lipid evident in the Hcr state. It would be interesting to localize these increased amounts of lipids in membrane subfractions in HeLa 65 Hcr. An increase in lipids of HeLa cells grown with cortisol has been reported (99). Although no significant alteration in the distribution of the phospholipid was noted in the HeLa cells grown with cortisol; the possibility that differences in specificity of phospholipid distribution in membrane subfractions might occur is not excluded. Eaglstein et al. have shown that hydrocortisone not only stabilizes the membrane which has been modified by phospholipase C, but also interferes with the hydrolytic activity of the lipase (100). Glycolipids have been implicated as possible intermediates in glycoprotein biosynthesis (47, 101). The activities and distribution of the glycosyltransferases which catalyze the transfer of mannose and N-acetylglucosamine from their glycolipid forms into glycoprotein may be relevant to the fact that the sialic acid content of HeLa 65 cells chronically grown with cortisol returns to the control level.

Hexosamine, another carbohydrate component of glycoprotein and glycolipid, was found to be mainly glucosamine in glycoproteins of

HeLa cells. It increased 71% in HeLa 71 Hcr glycoprotein, but remained unchanged in the glycolipid fraction. Since glucosamine is the characteristic hexosamine of plasma-type glycoprotein (4), we assume that plasma-type glycoprotein predominates in HeLa cell membranes. Galactose, another carbohydrate component of plasma-type glycoprotein, increased 49% in HeLa 71 Hcr glycoproteins over control values. Mannose, on the other hand, did not increase in HeLa 71 Hcr glycoproteins. If we assume that fetuin-like glycoproteins are the major glycoproteins in HeLa cells, two possible polysaccharide structures could explain the disproportionate changes between the amount of mannose and the amounts of sialic acid, hexosamine and galactose in HeLa 71 Hcr compared to those in the control. First, the mannose core [-(mannose)x-], which has been shown to be located in the inner portion of the ordered polysaccharide chain of fetuin (4), seems to be longer in HeLa 71 glycoproteins (mannose/hexose = 0.5 in fetuin; mannose/hexose = 0.9 in HeLa glycoprotein). Thus, in HeLa 71 Hcr, the number of terminal polysaccharide chains branching from this mannose core could be greater than that in the control proteins. The amount of sugars on the branching chains (N-acetylglucosamine, galactose and sialic acid) would then be increased in HeLa 71 Hcr glycoprotein, while the amount of mannose and number of glycoprotein molecules would remain similar in control and Hcr cells. If this was an exclusive mechanism, the major part of increased carbohydrate would be due to greater branching of the polysaccharide chain on similar numbers of proteins in the Hcr cell compared to that in the control. Therefore, the increase of 50% protein in HeLa 71 Hcr n-butanol extract could not be solely glycoprotein. On the other

hand, if the increased amount of protein in HeLa 71 Hcr n-butanol extract is predominantly glycoprotein, the polysaccharide structures of glycoproteins from control and Hcr cells are likely to differ only in the amount of mannose in their core portions (the HeLa Hcr glycoproteins would have less). The increase in more distal sugars could be accounted for by increased glycoprotein content. The results from glycoprotein isolation and electrophoretic analysis indicate that, at least in part, HeLa 71 Hcr increases its cellular sialoglycoprotein. Quantitation of each individual sugar on one major HeLa sialoglycoprotein per cell would be useful in solving this problem. Analysis of glycoproteins solubilized by n-butanol and separated by isoelectric focusing could provide this information. Appropriate experiments are now in progress.

Shen and Ginsburg (102) reported that HeLa S_3 (HeLa 65) suspension cultures contained 0.6 nmoles of fucose per 10^6 cells, contributed mainly by glycoprotein. Bosmann et al. (103) also demonstrated two glycoprotein fucosyltransferases in HeLa cells. These enzymes were responsible for the transfer of L-fucose from GDP-fucose to the specific precursor for fucose-containing glycoproteins. We failed to detect any fucose in HeLa cells from monolayer cultures in both clones. The method we used could detect 0.06 nmoles of fucose per 10^6 cells, and fucose would not have been destroyed under our hydrolytic conditions. Fucose is believed to be an alternative to sialic acid as the terminal sugar in glycoproteins and glycolipids (4). Our data indicate greater amounts of sialic acid in HeLa 65 monolayer culture (2.2 nmoles/10^6 cells) than those reported by Shen and Gingsburg for HeLa S_3 in suspension culture (1.3

nmoles/10^6 cells (102). The difference in sialic acid (0.9 nmoles/10^6 cells) correlates with the amount of fucose they reported (0.6 nmoles/10^6 cells). The amount of fucose in HeLa cell glycoproteins may be limited by a nutritional requirement or culture conditions. Addition of mannose, but not L-fucose, has been reported to alter the morphology of HeLa Ch (HeLa 71)(104). L-fucose, however, was reported to alter the morphology of 3T3 mouse fibroblasts. Moreover, SV40-virus-transformed 3T3 cells resulted in a loss in susceptibility to changes caused by L-fucose (105). The question arises whether the fucosyl transferase is inactivated by virus infection and whether fucosyl transferase for glycoprotein and glycolipid plays a major role in regulation of nonvirus-infected 3T3 cells. Analysis of sialic acid and fucose of glycoproteins and glycolipids of these cells might provide clues to the biochemical changes underlying the morphologic alterations observed. Recently, Inbar et al. (106) failed to detect the fucose-binding sites on the surface membranes of certain cells such as rat-embryo cells, secondary culture of golden hamster, mouse 3T3 cells and all these cells transformed with viruses.

HeLa cell membrane fractions were obtained by Dounce homogenization followed by discontinuous sucrose density flotation of various cellular subfractions. Much of the plasma membrane was sedimented with nuclei at 200 x g for 10 minutes; only a minor part of the plasma membrane in vesicles floated with the other cytoplasmic membranes. Plasma membrane ghosts were separated from nuclei by mild sonication and floated at the interface of 35-45% sucrose. Our method of plasma membrane preparation closely resembled that of Boone et al. (107), who used gentle Dounce homogenization of hypotonically swollen HeLa cells and isolated the ghosts

in a discontinuous sucrose density gradient. Although no single pure membrane fraction could be resolved by our methods, these techniques afforded a relatively high yield of the cell membrane fraction. For instance, sialic acid, which is believed to be primarily a component of membrane-localized molecules, was obtained in yields ranging from 35% to 47% of the amounts obtained from the respective whole cell preparations (from data in Table 15). Emmelot and Bos (108) have shown that alkaline phosphatase and 5'-nucleotidase are localized in plasma membrane or rat-liver hepatocytes. Atkinson and Summers (109) also demonstrated that 50% of 5'-nucleotidase is located on HeLa S_3 plasma membrane. Widnell and Unkeless (110) reported that 5'-nucleotidase occurs in the smooth and rough microsome of rat liver cells, in addition to the plasma membrane. Recently, Widnell (111) presented further support for this statement by cytochemical localization of 5'-nucleotidase in isolated, unfixed rat liver microsomes. This report shows that alkaline phosphatase and 5'-nucleotidase activities are concentrated on the plasma membrane. However, a small amount of these two enzymes was observed in smooth endoplasmic reticulum and/or Golgi and their activities could not be assigned solely to plasma membrane contamination of these fractions. Alkaline phosphatase has been "induced" four- to tenfold in HeLa 65 in the cortisol-regulated state (72,73). Our data show that alkaline phosphatase associated with plasma membrane, endoplasmic reticulum, and Golgi is induced in HeLa 65 Hcr. 5'-Nucleotidase rises about four-fold in HeLa 65 Hcr, but drops about 30% in HeLa 71 Hcr. Thus it seems to be regulated in a manner similar to alkaline phosphatase in these HeLa clones.

Corticosterone has been shown to increase the rate and the extent of polysome binding to smooth microsome in rat liver. It competes with aflatoxin B_1 at a site on the membrane responsible for polysome binding (112,113). Oka and Topper (114) also reported that hydrocortisone and insulin were essential for the accumulation of rough endoplasmic reticulum in mouse mammary epithelial cells in vitro. In their study, hydrocortisone increased membrane-linked NADH-cytochrome C reductase, as well as potentiating the effect of insulin which increased the RNA content of rough endoplasmic reticulum. These results may be quite pertinent in understanding this study. An increase in HeLa 71 rough endoplasmic reticulum in the Hcr state could account for an increased rate of glycoprotein biosynthesis, which, in turn, could result in increased amounts of cell membrane glycoprotein.

From these studies, sialic acid was found to be concentrated on the plasma membrane. Emmelot and Bos (108) reported that mouse liver and hepatoma plasma membrane contained 28 nmoles of sialic acid per milligram of protein; Molnar (115) also showed that plasma membrane of Ehrlich ascites cells had 27 nmoles of sialic acid per milligram of protein. From the data in Table 16, HeLa 65 control and Hcr have 20 nmoles of sialic aicd per milligram of plasma membrane protein, whereas HeLa 71 control has 42 nmoles and HeLa 71 Hcr has 54 nmoles. Marcus et al. (116) showed that the nuclear surface of a HeLa cell clone contained 0.07 nmoles of sialic acid per 10^6 cells. Our purified nuclear preparations, shown by electron microscopy to be relatively free of contamination by cytoplasmic material, also contained significant amounts of sialic acid (Table 19). Robbins and Pederson (117) reported that HeLa

cells contain a large amount of nuclear iron associated with a polysaccharide. Possibly this nuclear iron is associated with sialic-acid-containing polysaccharide(s), since histochemical staining for sialic acid is based on its binding with Fe^{++}. Comings and Kakefuda (118) showed that nuclear membrane is the initiation site of DNA replication in mammalian cells. Fisher and Yeh (119) suggested that the initiation of growth resulting from cell-to-cell contact in monolayer culture could be removed by enzymatic hydrolysis of surface groups such as N-acetylneuraminic acid. Treatment of such cells with neuraminidase was followed by subsequent cell division. Caso (120) also demonstrated that HeLa cells in monolayer culture were susceptible to stimulation of DNA synthesis by incubation with neuraminidase. Moreover, Nordling and Mayhew (121) presented some evidence that neuraminidase was not only active on the cell's surface membrane, it entered the cytoplasm and even the nucleus. Culp et al. (122) suggested that the sialic acid content of plasma membrane might play an essential role in regulating normal cell-to-cell interaction. Pardee (123) speculated that the surface membrane was the target for regulating animal cell division. A decrease in expression of H-2 antigen was noted during the S period, indicating a "masking" or other rearrangement of antigenic determinants on the surface membrane during the cell cycle (124). HeLa 71 Hcr is characterized by an extended G_1 phase of the cell cycle and by an increase of sialoglycoprotein associated with the surface membrane and the nucleus. We propose that surface membrane carries sensor molecules (sialoglycoproteins) which act in cell-to-cell interactions which are important in regulating cell generation transitions such as the passage from G_1 into S phase of

the cell cycle. We also propose that nuclear membrane sialoglycoproteins may play a role as effector molecules which regulate initiation of DNA-mediated DNA polymerase at membrane sites. It would be useful to compare the amounts of sialoglycoprotein in the nucleus and the plasma membrane of a synchronous population of HeLa 71 control and Hcr cells at mid-G_1, and at G_1-S periods of the cell cycle. It would also be interesting to see whether treatment with neuraminidase could potentiate the activity of DNA-mediated DNA polymerase *in vitro*. However, in interpreting these results caution has to be taken, since Pardee indicated that surface membrane changes and DNA replication might not be directly coupled, but rather are related through a sequence of events, some of which might occur in the cytoplasm (123).

Glycoprotein sialyltransferase activity has been demonstrated in various mammalian clones such as mouse 3T3 cells (87) and human erythrocyte membrane (125). In HeLa cells, this enzyme is mainly localized in cytoplasm. It is interesting to know that cytidine 5'-monophosphosialic acid synthetase, the enzyme activating sialic acid for the biosynthesis of glycoprotein and glycolipid, is localized in the nucleus. That a geographic separation within the cell between these two enzymes may play a role in the control of biosynthesis of complex carbohydrates in glycoprotein or glycolipid (126). Kim *et al.* (125) showed that this enzyme activity from erythrocyte membrane is inhibited by Mn^{++}. However, the fetuin-primed sialyltransferase activity of these HeLa clones is optimally stimulated by Mn^{++} (5 mM). Sialyltransferase activity of HeLa cells responds differently to two different exogenous protein acceptors--desialized fetuin and desialized

mucin. HeLa 71 Hcr shows a 70% increase in this activity only with desialized fetuin. This parallels the increased sialic acid in the whole-cell sialoglycoproteins of HeLa 71 Hcr and these proteins are concentrated on the plasma membrane. It would be interesting to see whether sialyltransferase activity is also concentrated in the HeLa plasma membrane, since Roth et al. (127) have reported the presence of UDP-galactose glycosyltransferase on embryonic neural retina cell surface. Pricer and Ashwell (128) have also demonstrated the presence of sialyltransferase in the hepatic plasma membrane. Either two different sialyltransferase species exist in HeLa cells, one specific for each acceptor protein, or one glycoprotein sialyltransferase is present which shows different specificity toward different exogenous acceptors. Further studies are needed to resolve these alternatives.

CHAPTER V

SUMMARY

This study was designed to investigate the biochemical changes in HeLa cell membranes elicited by growth with cortisol. Cortisol prolongs the population-doubling time in HeLa 71 but not in HeLa 65. HeLa 71 and HeLa 65 are named for their major modal chromosomal number. Sialic acid and glucosamine associated with glycoprotein both increase significantly in HeLa 71 Hcr but not in HeLa 65 Hcr compared to controls. ("Hcr" designates the steady state achieved by continuously subculturing HeLa clones with 3 μM cortisol.) HeLa 71 Hcr has an increased amount of glycoprotein galactose, but has the same amount of glycoprotein mannose compared to that in the control. Two possible polysaccharide structures have been suggested to explain the disproportionate changes between the amount of mannose and the amounts of sialic acid, hexosamine and galactose in HeLa 71 Hcr compared to those in the control. Either the amount of mannose in the core portion of the polysaccharide is less in the Hcr glycoprotein or the degree of glycosylation from a uniform mannose core is increased.

Membrane fractions were isolated by flotation on a discontinuous sucrose gradient, were characterized by electron microscopy, and were assayed for the following: 5'-nucleotidase, alkaline phospha-

tase, protein, DNA, RNA, hexosamines, and sialic acid. Both 5'-nucleotidase and alkaline phosphatase activities were increased in HeLa 65 Hcr. In all clones studied, these enzymes were concentrated in a plasma-membrane-enriched fraction. Since sialoglycoproteins containing glucosamine are concentrated predominantly in the plasma membrane fraction, one major site altered by growth with cortisol must be the plasma membrane.

Consistent with the above, plasma-type glycoprotein sialyltransferase activity was increased in HeLa 71 Hcr compared to that in the control. Optimum conditions for the HeLa glycoprotein sialyltransferase were: 0.3% Triton X-100, 5 mM Mn^{++}, 10 mM phosphate, pH 6.5, plus desialized bovine fetuin as exogenous acceptor. This enzyme was localized preponderantly in the cytoplasm of the HeLa cells.

In accord with an increase of sialoglycoproteins on plasma membrane of HeLa 71 Hcr, sialic acid associated with the nuclei also rose markedly compared to that in the control. These two events may represent the major physiological changes in surface and nuclear membrane, accounting for enhanced contact inhibition of cell-doubling in HeLa 71 Hcr.

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