BBA 76130

MEMBRANE CHANGES IN HeLa CELLS GROWN WITH CORTISOL

SHING-HUI TU, ROBERT E NORDQUIST AND MARTIN J GRIFFIN

Oklahoma Medical Research Foundation and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Okla 73104 (U S A)

(Received June 19th, 1972)

SUMMARY

1. This study was designed to investigate the biochemical changes in HeLa cell membranes elicited by growth with cortisol. Cortisol increases the population-doubling time in HeLa 71 but not in HeLa 65. 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 has an increased amount of glycoprotein galactose, but has the same amount of glycoprotein mannose compared to its control.

2 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 phosphatase, 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. This may represent the major physiological change in surface membrane, accounting for enhanced contact inhibition of cell-doubling in HeLa 71 Hcr.

3. Consistent with the above, plasma-type glycoprotein sialyltransferase activity was increased in HeLa 71 Hcr compared to control Optimum conditions for the HeLa glycoprotein sialyltransferase were 0.3 % Triton X-100, 5 mM Mn^{2+}, 10 mM phosphate (pH 6.5), *plus* desialized bovine fetuin as exogenous acceptor.

INTRODUCTION

HeLa 65 cells continuously subcultured with 3 μM cortisol (Hcr state) possess a membrane-bound enzyme, alkaline phosphatase, with an increased specific phosphomonoesterase activity[1,2]. This process is highly specific for glucocorticoids and results in an enzyme with an altered conformation[2]. When HeLa 71 cells are continuously subcultured with 3 μM cortisol, they have an extended cell generation time (35 h). 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 cell generation cycle, but it later returns to the control rate (18-h doubling time)[3] The

amount of sialic acid, a cell-membrane moiety, rises in HeLa 65 cells after short-term cultivation with cortisol[4]. 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[5].

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 isolation 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.

METHODS

Cell cultures and media

Two HeLa clones, HeLa 65 and 71, named for their modal chromosome number[6], were grown in monolayer culture. Growth medium was Eagle's[7] minimal essential medium (Auto Pow, Flow Laboratories) supplemented by 10% calf serum, 2% 250 mM glutamine, 50 units/ml of penicillin and 50 μg/ml each of streptomycin and kanamycin. The calf serum (Colorado Serum Co.) was heat-inactivated at 56 °C for 30 min before being added to the medium. Cells were subcultured by detaching them from the monolayer with isotonic saline containing 0.0025 % trypsin and 0.3 mM EDTA at a pH of about 9 (versene–trypsin). These were diluted into complete medium for inoculation. Cells in the Hcr state were grown with cortisol (3 μM) in the medium at each subculturing (about 3-day intervals) and were assumed at steady state after 3 weeks of subculturing. Blake bottles at a confluency of $11 \cdot 10^4$–$12 \cdot 10^4$ cells/cm^2 were used for most of the experiments. This represents an exponentially dividing asynchronous population of cells in somewhat late logarithmic growth.

Cell-volume measurements

Cells which had been removed from the monolayer with versene–trypsin were suspended in complete medium. The cell suspension was then sedimented at 400 $\times$ g for 15 min in a Bauer–Schenck tube, calibrated in 4-μl increments. After this treatment, no further decrease in pellet volume was obtained.

Separation of glycoproteins and glycolipids

Cell monolayers were washed twice with isotonic saline in 0.025 M Tris–HCl (pH 7.2), scraped into this buffer with a rubber policeman, and sedimented by centrifugation. Cell pellets were extracted with 20 vol. of chloroform–methanol (2·1, v/v) for 20 min at 37 °C. The insoluble residue was sedimented and re-extracted 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 homogenized in water. To this suspension was added an equal volume of 2 % phosphotungstic acid in 1.0 M HCl to precipitate glycoproteins. Phosphotungstic acid precipitates proteins and other organic amines[8], but excludes polysaccharides since we could not precipitate glycogen with this acid. Mucopoly-

saccharide could not be detected in these cells at levels of 0.01 nmole/10^6 cells or above. The precipitate was sedimented by centrifugation, washed by resuspension in 1 % phosphotungstic acid in 0.5 M HCl, and resedimented. The pellet was used for sugar analysis of glycoproteins. Glycoprotein sugars were liberated by hydrolysis in 2 M HCl at 100 °C for 6 h. Hexosamine was determined on the acid hydrolysate. Sialic acid was liberated by hydrolysis in 0.05 M H_2SO_4 at 80 °C for 1 h and determined as described later. The amount of sialic acid was similar to that obtained from direct hydrolysis of whole cells. All sialic acid determinations were corrected for losses that occurred during isolation and purification.

Sialic acid assay

Sialic acid was assayed as *N*-acetyl-neuraminic acid by the method of Warren[9] after this compound has been separated by ion-exchange column chromatography[4]. Paper chromatography was used to resolve the sialic acids of the purified nuclear fraction into *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid[10].

Hexosamine assay

Hexosamine was estimated by the method of Gatt and Berman[11]. Hexosamines were further separated into glucosamine and galactosamine on an amino acid analyzer. The acid hydrolysate of glycoprotein was desiccated over KOH pellets and then dissolved in 0.2 M citrate buffer (pH 2.2). The sample was applied to a standard amino-acid-analyzing column (50 cm × 0.9 cm) and eluted with 0.2 M citrate buffer (pH 4.25), to which solid NaCl was added to bring the molarity to 0.38.

Neutral sugar assay

Neutral sugars were quantitated by converting them to alditol acetate derivatives. They were separated into ribose, mannose, and galactose by gas-liquid chromatography using the technique of Holme *et al.*[12], with modifications suggested by Dr A. A. Lindberg.

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 a Cahn analytical microbalance.

Phospholipids were separated by two-dimensional thin-layer chromatography by the method of Rouser *et al.*[13]. Lipids were detected by iodine vapor. After the spots had been eluted, phosphorus was determined[13]. 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.*[14].

RNA assay

RNA was determined spectrophotometrically. The sample was incubated in 0.23 M NaOH at 37 °C overnight. Protein and DNA were precipitated by the addition of $HClO_4$ to 8 % concentration After the sample had been centrifuged for 30 min

to remove insoluble material, the absorbance of the supernatant fluid was read at 260 nm. Occasionally, this method of alkaline hydrolysis of RNA was checked by hydrolysis with pancreatic ribonuclease. Both methods gave similar results.

Membrane separation

All operations were performed at 4 °C. The cell monolayers of replicate Blake bottles (20–30) were washed twice with saline–Tris–HCl buffer (pH 7.2) and scraped (with rubber policeman) into saline–Tris–HCl buffer. The cells were sedimented by centrifugation at 200 × *g* for 10 min, pooled in saline–Tris–HCl buffer, and resedimented as described above. In this way, about $4 \cdot 10^8$–$5 \cdot 10^8$ 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 $MgCl_2$. They were allowed to swell for 10 min and were then homogenized in two portions in a tight glass-to-glass Dounce homogenizer (length. 80 mm; volume: 7 ml, clearance: 25 μm) with 20 complete strokes. The homogenate was centrifuged at 200 × *g* for 10 min. The pellet was used for the plasma membrane and nuclear preparation.

The supernatant fluid was centrifuged in a Sorvall refrigerated centrifuge for 20 min at 4000 × *g*. The precipitate, called Fraction VI, contained lysosomes, mitochondria, and cytoplasmic membranes, and was not further resolved. The supernatant fluid from the 4000 × *g* centrifugation was made 45 % with respect to sucrose; 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 Tris–HCl–$MgCl_2$ buffer, pH 7.5), in a 60-ml capacity tube of cellulose nitrate. The discontinuous gradient was overlayered with a small volume of Tris–HCl–$MgCl_2$ buffer. It was then subjected to centrifugation at 70000 × *g* for 16 h in an SW-25.2 swinging bucket rotor. The pellet from centrifugation at 200 × *g* for 10 min was suspended in 20 ml of 45 % sucrose and subjected to sonication in a Bronson sonicator using the microprobe operating at 70 % efficiency with a 14-kHz output. The sample received three 8-s bursts of sound, with thorough cooling in ice during and after each sonication. The sonicate was layered with the other sucrose solutions to form a discontinuous gradient as described above. This fraction was also centrifuged for 16 h at 70000 × *g* in the SW-25.2 rotor.

The supernatant fluid from the 4000 × *g* centrifugation which was further centrifuged in a discontinuous sucrose gradient was resolved into four distinct fractions (see Fig. 1a). The uppermost of these was light and was combined with the fraction below it (Fraction I). The pellet from the sonication followed by centrifugation was separated into three fractions (see Fig. 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 4-fold with Tris–HCl–$MgCl_2$ buffer (pH 7.5). It was then sedimented by high speed centrifugation and washed once more with buffer, as described above.

Phosphatase assays

Alkaline phosphatase activity was estimated by the method previously described, using *p*-nitrophenylphosphate as substrate[2]. 5′-Nucleotidase activity was determined as the amount of phosphate released when aliquots of various fractions were incubated with AMP at pH 7.5. Phosphate was measured by the method of King[15].

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 h 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 h and fixed with 1 % chrome osmium for 2 h. The material was then washed with buffer, dehydrated in graded ethanol solutions, and embedded in Araldite 6005 Sections of 800–1000 Å were stained with uranyl acetate and lead citrate and were photographed on a Hitachi HU-11B electron microscope

Glycoprotein sialyltransferase assay

Sialyltransferase activity was measured by modifications of the method of Grimes[16]. As exogenous acceptors, both bovine fetuin and bovine submaxillary mucin were stripped of sialic acid by heating at 80 °C for 1 h in 0.05 M 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 × *g* for 10 min to sediment the nuclei The pellet was resuspended in the phosphate buffer and centrifuged, 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 70000 × *g* for 3 h. 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 nCi CMP-[4,5,6,7,8,9-$^{14}C_6$]-*N*-acetylneuraminic acid (spec. act. 223 Ci/mole, purchased from New England Nuclear Corp), 0 3 % Triton X-100, and 5 mM $MnCl_2$ in a total volume of 0.12 ml. After a period of incubation at 37 °C, 1 ml of 1 % phosphotungstic acid in 0.5 M HCl was added. The reaction mixture was cooled in ice for 30 min. The insoluble material was collected by centrifuging the mixture at 500 × *g* for 15 min. The precipitate was washed three times by repeatedly centrifuging it from a 5 % trichloroacetic acid solution (1.5 ml per wash). The pellet was dissolved in 0.3 ml of 0.1 M NaOH, 0 2 ml of 0.1 M 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 cpm to dpm. 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)[1,2]. About $80 \cdot 10^6$ cells of each clone in each state were extracted with *n*-butanol, the aqueous layer was removed, and the cell butanol layer was re-extracted 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 ml The duplicate aliquots of proteins, one of which was treated with *Clostridium perfringens* neuramini-

dase (10 μg per 200 μg glycoprotein) for 3 h at 37 °C, were analyzed by acrylamide gel electrophoresis by a method previously described[17].

Nuclear fraction isolation

The nuclear fraction of HeLa cells was isolated and purified by the method of Penman *et al.*[18].

RESULTS

Glycoprotein carbohydrates of HeLa cells

Table I shows the carbohydrate 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. In contrast to HeLa 71, HeLa 65 showed no significant difference in sialic acid and hexosamine content in the Hcr state The average cell diameters, as calculated from the measured cell volume in a Bauer–Schenck sedimentation tube, were 18 μm in HeLa 71 and 19 μm in HeLa 71 Hcr. To obtain 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[19]. Using these approximations, the average density of sialic acid per surface area of HeLa 71 was $7.1 \cdot 10^5$ molecules/μm², for HeLa 71 Hcr, it was $12.1 \cdot 10^5$ molecules/μm². A modified Hale's colloidal iron stain showed that sialic 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.

TABLE I

CARBOHYDRATE CONTENT OF GLYCOPROTEIN OF HeLa CLONES

Cell counts were performed on one replicate Blake bottle by trypsinization of the cells The cells from the three bottles were scraped into saline–Tris–HCl buffer (pH 7 2), and glycoprotein sugars were determined as described in Methods Each value represents the mean of at least three experiments ± S E All determinations were done in duplicate.

Compound	*HeLa 65 (nmoles/10⁶ cells)*		*HeLa 71 (nmoles/10⁶ cells)*	
	Control	*Hcr*	*Control*	*Hcr*
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
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

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 2–5 % 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.

Table I also shows the content of neutral sugar of HeLa cell glycoproteins. Ribose, probably derived from cell RNA, was not significantly altered in either clone in

either state. The high variability in ribose values probably results from incomplete precipitation of RNA by phosphotungstic acid. Mannose of 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 nmole of fucose per $1 \cdot 10^6$ 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 in HeLa 65 Hcr by 30% (from 67.3 ± 3.7 to 86.7 ± 2.5 $\mu g/10^6$ cells). No significant change was found in HeLa 71 Hcr (control, 83.8 ± 7.8 $\mu g/10^6$ cells; Hcr, 93.2 ± 8.8 $\mu g/10^6$ cells). The phospholipid composition of both clones in both states was identified as phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and phosphatidylserine. Neither the total amount nor the distribution of each class of phospholipid changed significantly between either clone or between each clone in the control and Hcr states. 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, from 0.15 ± 0.02 nmole/10^6 cells for control to 0.21 ± 0.01 nmole/10^6 cells for Hcr. No significant change was shown in any other lipid-bound carbohydrate moiety from control or Hcr cells in either clone.

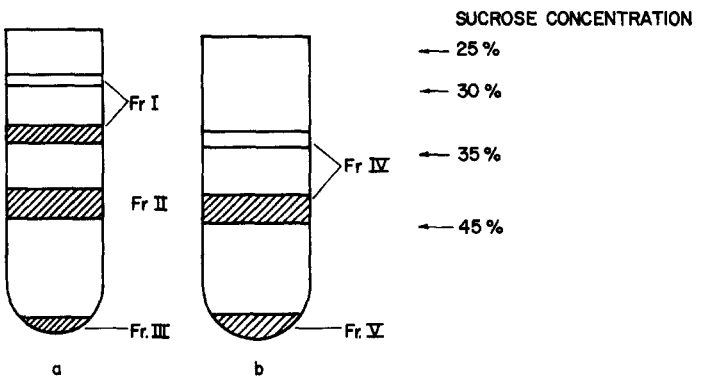

Fig. 1. Typical banding pattern of HeLa cell membranes. Preparation and centrifugational flotation of these bands are described in Methods. The characterization and naming of the bands are described in Results. Shaded areas represent heavy opalescent bands and unshaded areas barely visible bands.

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 Fig. 1. Six fractions were obtained, and each was analyzed and examined by electron microscopy. Fig. 2a shows smooth endoplasmic reticulum, Golgi, and plasma membrane vesicle (Fraction

I). Fig. 2b shows smooth endoplasmic reticulum and possibly plasma membrane vesicles (Fraction II). Fig. 2c shows rough endoplasmic reticulum (Fraction III). Fig. 2d 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 experi-

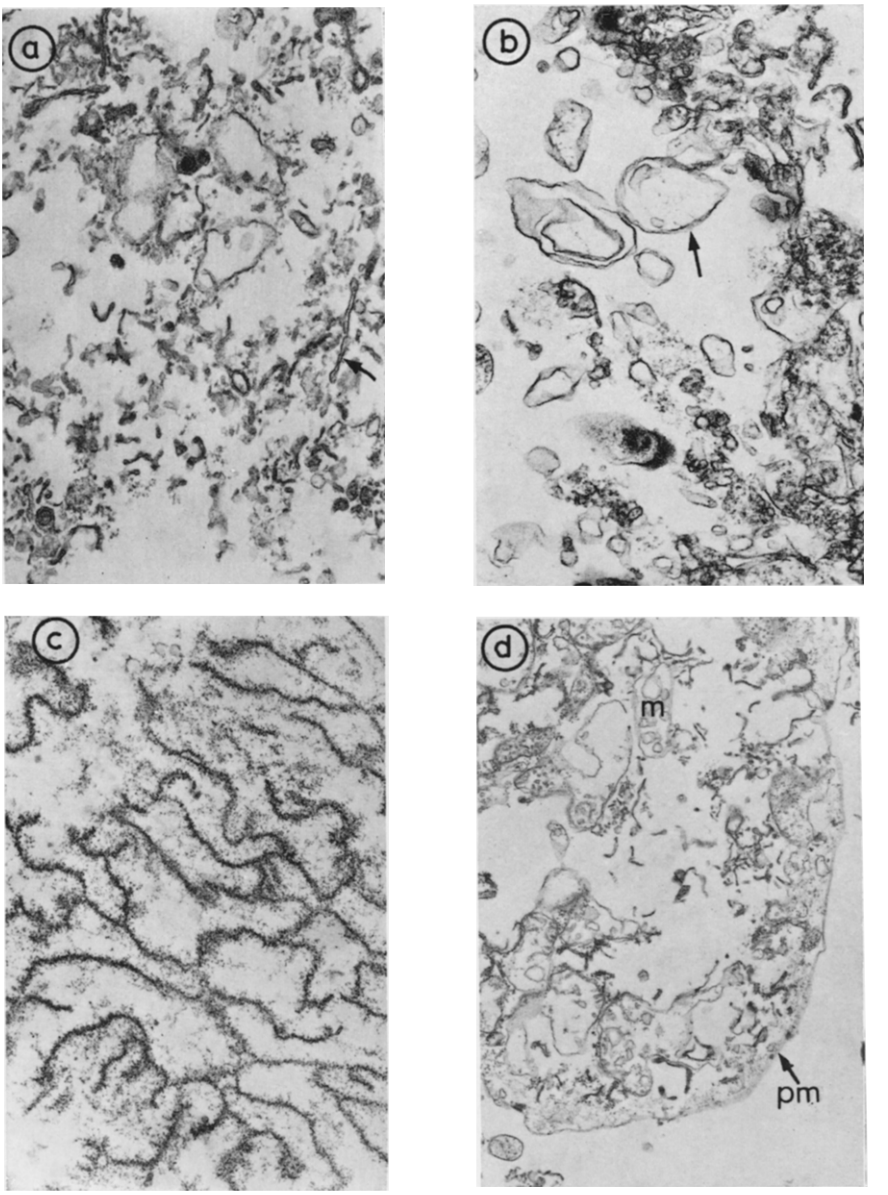

Fig 2. (a) Smooth endoplasmic reticulum, Golgi (indicated by ↑) and plasma membrane vesicles (27000 ×) (b) Smooth endoplasmic reticulum (indicated by ↑) and plasma membrane vesicles (25200 ×) (c) Rough endoplasmic reticulum (18750 ×) (d) Plasma membrane ghost (pm). The presence of cytoplasmic membrane such as mitochrondria (m) is also noted (15 000 ×).

ments were performed at least three times for each clone in each state, and results are reported from one experiment. The amount of any given molecular species for an individual membrane fraction was similar (± 10 %) from one experiment to the next. The yield obtained by summation of membrane fraction constituents was also rather uniform between experiments with standard deviation no greater than 15 %. Thus, the results are representative of amounts of chemical species from the particular clone described.

The membrane fractions were analyzed for protein, RNA, alkaline phosphatase activity, 5'-nucleotidase activity, sialic acid, and hexosamine. The 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 II shows the protein and RNA content of isolated membrane fractions. The amount of protein in rough endoplasmic reticulum increased in the Hcr state in both clones (Fraction III, Table II) In HeLa 65 Hcr, the smooth endoplasmic reticulum (Fraction II, Table II) 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 II). Plasma membrane protein increased in HeLa 71 Hcr by 52 % over control, whereas no significant changes occurred in HeLa 65 Hcr (Fraction IV, Table II).

TABLE II

DISTRIBUTION OF PROTEIN AND RNA IN MEMBRANES OF HeLa CLONES

Results are expressed on a per-cell basis Cell count was obtained from a replicate Blake bottle which was trypsinized and counted but not used in the membrane preparation. The preparation of the cell fractions, the protein, and the RNA assays are described in Methods

Membrane fraction	*Protein ($\mu g/10^6$ cells)*				*RNA ($\mu g/10^6$ cells)*			
	HeLa 65		*HeLa 71*		*HeLa 65*		*HeLa 71*	
	Control	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*
Whole cell	240	220	190	260	33	27	22	17
Fraction I	0 09	0 03	1 7	1 1	0 044	0 096	0.32	0 46
Fraction II	0 40	1 4	2 5	1 9	0 13	0 38	0 24	0.38
Fraction III	2 9	5 3	4 4	6 1	2 0	2 9	2 1	3 0
Fraction IV	6 1	5 4	2 3	3 5	0 8	0 64	0 38	0 52
Fraction V	84	67	78	83	8 5	5 9	6 0	4 7
Fraction VI	9 1	16	15	12	4 1	2 9	2 2	2 8

RNA was mainly localized in rough endoplasmic reticulum fraction (Fraction III, Table II) and nuclear fraction (Fraction V, Table II). The amount of RNA found in Fraction VI indicates that part of the rough endoplasmic reticulum sedimented with lysosomes and mitochondria at 4000 × *g* for 20 min. DNA determination of these fractions by the method of Burton[20] showed that over 90 % of the total cell DNA was localized in the nuclear fraction (Fraction V) (12.2 $\mu g/10^6$ cells in nucleus, 1 $\mu g/10^6$ cells total in the other fractions).

5'-Nucelotidase and alkaline phosphatase activities for whole cell and Fractions I through VI are shown in Table III. HeLa 65 underwent a 5-fold increase in alkaline phosphatase activitiy in the cortisol-regulated state. This increase in alkaline phos-

TABLE III

ALKALINE PHOSPHATASE AND 5′-NUCLEOTIDASE ACTIVITIES OF HeLa CELL MEMBRANE FRACTIONS

Alkaline phosphatase activity is expressed as nmoles *p*-nitrophenylphosphate/min per 10^6 cells 5′-Nucleotidase activity is expressed as nmoles of phosphate released from AMP at pH 7 5 as described in Methods n d , not detected

Membrane fraction	*Alkaline phosphatase (nmoles p-nitrophenylphosphate/min per 10^6 cells)*				*5′-Nucleotidase (nmoles P_i/min per 10^6 cells)*			
	HeLa 65		*HeLa 71*		*HeLa 65*		*HeLa 71*	
	Control	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*
Whole cell	1 5	7 7	190	190	0 55	2 3	7 5	4 9
Fraction I	0 011	0 10	14	14	n d	0 018	0 86	0 25
Fraction II	0 017	0 35	14	9 9	n d	0 096	0 47	0 20
Fraction III	0 011	0 084	5 4	1 1	n d	n d	0 14	0 027
Fraction IV	0 17	0 91	13	19	0 15	0 26	0 41	0 40
Fraction V	0 40	1 9	31	36	0 21	0 60	1 8	1 2
Fraction VI	0 22	2 3	17	29	0 14	0 77	2 1	1 0

phatase activity was reflected in the plasma membrane (Fraction IV, Table III) of HeLa 65 Hcr compared to control. In HeLa 71, cloned for its high constitutive level of alkaline phosphatase, phosphatase 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, Table III). 5′-Nucleotidase, another plasma-membrane-localized enzyme, exhibited the same alterations in the Hcr state as alkaline phosphatase (Table III). 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-mg-of-protein basis, the enzyme activities of Fractions I and II were as high as or higher than those of the plasma membrane (For comparison, take the ratio of enzyme activity per million cells [Table III]divided by the amount of protein per million cells [Table II] for each fraction). 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 membrane vesicles in those two fractions.

The changes in sialic acid of the whole cell (Table I) are reflected in the membrane assays shown in Table IV. The plasma membrane fraction (Fraction IV) of HeLa 71 Hcr had a twofold increase in the amount of sialic acid compared to 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 IV). 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 IV).

Fraction IV (plasma membrane) of HeLa 71 Hcr doubled its hexosamine content compared to control (Fraction IV, Table IV). Although whole-cell analysis revealed

TABLE IV

SIALIC ACID AND HEXOSAMINE CONTENT OF HeLa CELL MEMBRANE FRACTIONS

See legend in Table II Sialic acid and hexosamine assays are described in Methods n d , not detected

Membrane fraction	*Sialic acid (nmoles/10⁶ cells)*				*Hexosamine (nmoles/10⁶ cells)*			
	Hela 65		*HeLa 71*		*HeLa 65*		*HeLa 71*	
	Control	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*	*Control*	*Hcr*
Whole cell	2 2	1 8	1 9	3 5	6 9	5 7	4 9	8 2
Fraction I	0 013	0 016	0 10	0 13	n d	0 05	0 12	0 17
Fraction II	0 019	0 031	0 09	0 11	0 063	0 15	0 12	0 15
Fraction III	0 008	0 011	0 025	0 023	0 25	0 91	0 19	0 15
Fraction IV	0 13	0 10	0 097	0 19	0 12	0 16	0 11	0 21
Fraction V	0 30	0 36	0 10	0 17	2 90	2 30	1 10	1 30
Fraction VI	0 22	0 31	0 21	0 36	0 28	0 69	0 28	0 90

no significant changes in the amount of hexosamine in HeLa 65 Hcr cells compared to controls, Fractions I, II, III and VI of HeLa 65 Hcr had more hexosamine than controls, whereas Fraction V had less. 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[18]; 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.

TABLE V

CHARACTERISTICS OF THE PURIFIED NUCLEAR FRACTION OF HeLa CLONES

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 μg/10⁶ cells) Sialic acid was determined in material precipitated by 1% phosphotungstic acid (in 0 5 M HCl) without extraction of lipid

Characteristic	*HeLa 65*		*HeLa 71*	
	Control	*Hcr*	*Control*	*Hcr*
Protein (μg/10⁶ nuclei)	34	75	47	57
RNA (μg/10⁶ nuclei)	11	15	13	21
Sialic acid (nmoles/10⁶ nuclei)	0 22	0 23	0 086	0 22
Alkaline phosphatase (nmole *p*-nitrophenylphosphate/min per 10⁶ nuclei)	0.11	1.1	25	6 4
5′-Nucleotidase (nmole P_i/min per 10⁶ nuclei)	0 25	0 63	0 93	0 29

No contaminating cytoplasm was evident. Interestingly, all nuclear fractions contained significant amounts of sialic acid, alkaline phosphatase, and 5'-nucleotidase activities (Table V). 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 control (Table V). Paper 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[21] was probably associated with the cell surface. Since the sialic acid recovered in the purified nuclear fraction represented at least 70 % of the contaminated nuclear fraction obtained by homogenization (compare Table IV with Table V), 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 occurred, as well as a 2.5 fold "induction" of 5'-nucleotidase activity in purified nuclei from HeLa 65 Hcr compared to control.

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. 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 % as did isotonic saline. 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^{2+} stimulated this reaction 2.4 times more efficiently at its optimum concentration (5 mM) than Mg^{2+} at its optimum concentration (10 mM). Therefore, Mn^{2+} (5 mM) was used throughout the study. When desialized mucin was used as exogenous acceptor, 5 mM of Mn^{2+} or Mg^{2+} was optimal, producing the same degree of stimulation. Enzyme activities of the cell clones were compared under optimal reaction conditions.

TABLE VI

CMP-*N*-ACETYL[^{14}C]NEURAMINIC ACID GLYCOPROTEIN SIALYLTRANSFERASE ACTIVITY OF HeLa CLONES

The sialyltransferase activities are expressed as pmoles of *N*-acetyl[^{14}C]neuraminic acid incorporated per min per mg of protein Enzyme and acceptor protein were obtained as described in Methods. Optimum conditions for the assay are also described in Results

Acceptor	*pmoles N-acetyl[^{14}C]neuraminic acid per min per mg protein*			
	HeLa 65		*HeLa 71*	
	Control	*Hcr*	*Control*	*Hcr*
Desialized fetuin	3 9	3 1	3.1	5 2
Desialized mucin	1 7	1 7	1 1	1 5

With desialized fetuin as exogenous acceptor, HeLa 71 Hcr evidenced a 70 % increase in sialyltransferase activity compared to HeLa 71 control (Table VI). On a per-cell basis, an even greater increase was seen for the HeLa 71 Hcr cell (0.32 pmole *N*-acetylneuraminic acid/min per 10^6 cells for control compared to 0.90 pmole *N*-acetylneuraminic acid/min per 10^6 cells for Hcr). However, when desialized mucin was used as acceptor, all cell clones had similar activities (Table VI). 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. 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 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 (Fig. 3). Pretreatment 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 representative sialoglycoprotein. The amount of sialoglycoprotein was 70 μg/10^6 cells for HeLa 71 control and 105 μg/10^6 cells for HeLa 71 Hcr. Therefore, the 50 % increase in protein obtained by butanol extract for HeLa 71 Hcr compared to HeLa 71 control could account for much of the 86 % increase of sialic acid associated with protein in this clone in the Hcr state. 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.

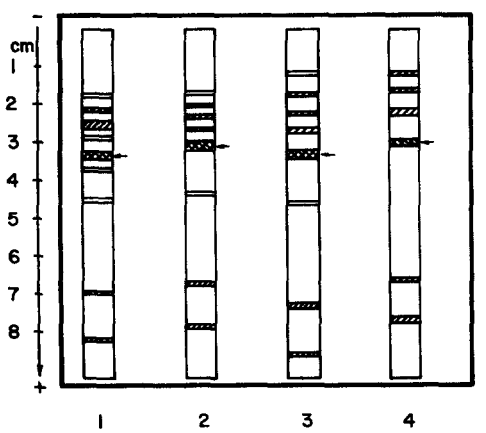

Fig 3 Disc-gel electrophoretogram of HeLa 71 and HeLa 71 Hcr glycoproteins Electrophoresis was performed as described in Methods In each sample gel 200 μg of protein were placed and electrophoresis was run for 5.5 h at 2 mA constant current Replicate gels were stained for alkaline phosphatase activity 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

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[3]. It has been suggested that the effect of hydrocortisone is manifested by changes in the HeLa cell membranes[22]. Carubelli and Griffin[4] showed that HeLa 65 responds to short-term growth (72 h) with cortisol by an increase in cellular sialic acid, more than one half of which was sedimentable at 100000 × *g* for 1 h. In this study, we found that HeLa 65 cells continuously subcultured with cortisol have a similar amount of sialic acid compared to control. 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 control. 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[19,23]. 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[24].

Hexosamine, another carbohydrate component of glycoprotein and glycolipid, was found to be mainly glucosamine in glycoproteins of these HeLa cells. It increased 71% in HeLa 71 Hcr glycoprotein, but remained unchanged in HeLa 71 Hcr glycolipid. Since glucosamine is the characteristic hexosamine of plasma-type glycoprotein[25], 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 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[25], seems to be longer in HeLa 71 glycoproteins (mannose/hexosamine = 0.5 in fetuin; mannose/hexosamine = 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 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 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 and the amount of this protein 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 Gingsburg[26] reported that HeLa S_3 suspension cultures contained 0.6 nmole of fucose per 10^6 cells, contributed mainly by glycoprotein. Bosmann *et al.*[27] also demonstrated two glycoprotein fucosyltransferases in HeLa cells. These enzymes were responsible for the transfer of L-fucose from GDPfucose to the effective precursor for fucose-containing glycoproteins. We failed to detect any fucose in HeLa monolayer cultures in both clones. The method we used could detect 0.06 nmole of fucose per 10^6 cells, and fucose was not destroyed under our hydrolytic conditions. Fucose is believed to be an alternative to sialic acid as the terminal sugar in glycoproteins and glycolipids[25] 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[26] (1.3 nmoles/10^6 cells). The difference in sialic acid (0.9 nmole/10^6 cells) correlates with the amount of fucose they reported (0.6 nmole/10^6 cells). The amount of fucose in HeLa cell glycoproteins may be limited by a nutritional requirement or culture conditions.

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 $\times$ *g* for 10 min; only a minor part of the plasma membrane in vesicles floated with the other cytoplasmic membrane. 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 resembles that of Boone *et al.*[28], 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 fractions. For instance, sialic acid, which is believed to be primarily a component of membrane localized molecules, was obtained in yields ranging from 30% to 46% compared to amounts obtained from the respective whole cell (from data in Table IV). Emmelot and Bos[29] have shown that alkaline phosphatase and 5′-nucleotidase are localized in plasma membrane of rat liver hepatocytes. Atkinson and Summers[30] also demonstrated that 50% of 5′-nucleotidase is located on HeLa S_3 plasma membrane. Widnell and Unkeless[31] reported that 5′-nucleotidase occurs in the smooth and rough microsome of rat liver cells, in addition to the plasma membrane. Recently, Widnell[32] 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" 4–10-fold in HeLa 65 in the cortisol-regulated state[1,2]. Our data show that alkaline phosphatase associated with plasma membrane, endoplasmic reticulum, and Golgi, is induced in HeLa 65 Hcr. 5'-Nucleotidase rises about 4-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[33,34]. Oka and Topper[35] 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.

In our studies, sialic acid was found to be concentrated on the plasma membrane. Emmelot and Bos[29] reported that mouse liver and hepatoma plasma membrane contained 28 nmoles of sialic acid per mg of protein; Molnar[36] also showed that plasma membrane of Ehrlich ascites cells had 27 nmoles of sialic acid per mg of protein. From the data in Tables II and IV, one can calculate that HeLa 65 control and Hcr have 20 nmoles of sialic acid per mg of plasma membrane protein; whereas, HeLa 71 control has 42 nmoles and HeLa 71 Hcr has 54 nmoles. Marcus *et al.*[37] showed that the nuclear surface of a HeLa cell clone contains 0.07 nmole 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 V).

The cell surface has been shown to be the target for regulating animal cell division[38]. Polyma-transformed 3T3 cells had somewhat less sialic acid than that of the untransformed cells, although no significant difference was found for the activity of L-glutamine:D-fructose-6-phosphate aminotransferase (EC 2.6.1.16), the first enzyme of sialic acid biosynthesis, in these cell lines[39]. SV 40-transformed 3T3 cells had only 60% as much sialic acid and only 55% the sialyltransferase activity as controls[16]. The virus-transformed cells show considerably less contact inhibition of growth than do controls. Sialic acid-containing membrane components have been implicated in regulation of cell growth. Fisher and Yeh[40] suggested that the inhibition 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[41] also demonstrated that HeLa cells in monolayer culture were susceptible to stimulation of DNA synthesis by incubation with neuraminidase. HeLa 71 Hcr has increased contact inhibition of growth characterized by an extended G_1 phase of the

cell cycle[3]. These cells have an increase of sialoglycoprotein associated with the surface membrane and the nucleus. We propose that surface membrane carries sensor molecules which are sialoglycoproteins and which act in cell-to-cell interactions that are important in regulating cell generation transitions such as the passage from G_1 into S phase of the cell cycle. It would be useful to compare the amounts of sialoglycoprotein in 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

Glycoprotein sialyltransferase activity has been demonstrated in various mammalian clones such as mouse 3T3 cells[16] and human erythrocyte membrane[42]. Kim *et al.*[42] showed that this enzyme activity from erythrocyte membrane is inhibited by Mn^{2+}. However, the fetuin-primed sialyltransferase activity of these HeLa clones is optimally stimulated by Mn^{2+} (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.*[43] have reported the presence of UDP-galactose glycosyltransferase on embryonic neural retina cell surface. Pricer and Ashwell[44] 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.

ACKNOWLEDGMENTS

This investigation was supported by National Institutes of Health Grant CA 10614. M.J.G. is recipient of a Research Career Development Award (5-K4-GM-18171) from the U.S. Public Health Service.

We wish to acknowledge the technical assistance of Dr R. Delaney for the quantitation of glucosamine and galactosamine, Dr W. A Joel for histochemical staining of sialic acid and lipid, Mr R. K. Burns for neutral sugar assay, and Mrs J. A. Alcott for her technical assistance. We are also grateful to Dr C. M. MacLeod for his helpful discussions with us.

REFERENCES

1 M J Griffin and R P Cox, *J Cell Biol*, 29 (1966) 1
2 M J Griffin and R P Cox, *Proc Natl Acad Sci U S*, 56 (1966) 946
3 G M Kollmorgen and M J Griffin, *Cell Tissue Kinetics*, 2 (1969) 111.
4 R Carubelli and M J Griffin, *Science*, 157 (1967) 693
5 P L Ballard and G M Tomkins, *Nature*, 224 (1969) 344
6 R H Bottomley, A L Trainer and M J Griffin, *J Cell Biol*, 41 (1969) 806.
7 H Eagle, *Science*, 130 (1959) 432
8 E Drechsel, *Ber Dent Chem Ges*, 20 (1887) 1452
9 L Warren, *J Biol Chem*, 234 (1959) 1971
10 E Svennerholm and L Svennerholm, *Nature*, 181 (1958) 1154
11 R Gatt and E R Berman, *Anal Biochem*, 15 (1966) 167
12 T Holme, A A Lindberg, P T Garegg and T J. Onn, *J Gen Microbiol*, 52 (1968) 45.

13 G Rouser, S Fleisher and A Yamamoto, *Lipids*, 5 (1970) 494.
14 O. H. Lowry, N J Rosebrough, A L. Farr and F J Randall, *J. Biol Chem*, 193 (1951) 265.
15 E J King, *Biochem. J*, 26 (1932) 292.
16 W J Grimes, *Biochemistry*, 9 (1970) 5083
17 R. P Cox, N A Elson, S H Tu and M J Griffin, *J Mol Biol*, 58 (1971) 197
18 S Penman, I Smith and E. Holtzman, *Science*, 154 (1966) 786
19 P M Kraemer, *J Cell Physiol*, 67 (1966) 24
20 K Burton, *Biochem J*, 62 (1956) 315
21 R Carubelli and M J Griffin, *Biochim Biophys Acta*, 170 (1968) 446
22 N Keller, R E Nordquist and M J Griffin, in R L Clark *et al*, *Oncology*, Vol 1, 1st edn, Year Book Medical Publishers, Chicago, 1971, p 440
23 M C Glick, C A Comstock, M A Cohen and L Warren, *Biochim Biophys. Acta*, 233 (1971) 247
24 R T Dellórco and G. Melnykovych, *J Cell Physiol*, 76 (1970) 101
25 P M Kraemer, in L A Manson, *Biomembrane*, Vol 1, 1st edn, Plenum Press, New York, 1971, p 67
26 L Shen and V Ginsburg, *Arch Biochem Biophys*, 122 (1967) 474
27 H B Bosmann, A Hagopian and E H Eylar, *Arch Biochem Biophys*, 128 (1968) 470.
28 C W Boone, L E Ford, H E. Bond, C. D. Stuart and D Lorenz, *J Cell Biol*, 41 (1969) 378.
29 P. Emmelot and C J Bos, *Biochim Biophys Acta*, 99 (1965) 578
30 P H Atkinson and D F Summers, *J Biol Chem*, 246 (1971) 5162
31 C C Widnell and J C Unkeless, *Proc Natl Acad Sci U S*, 61 (1968) 1050
32 C C Widnell, *J Cell Biol*, 52 (1972) 542
33 D J Williams and B R Rabin, *FEBS Lett*, 4 (1969) 103
34 D W James, B R Rabin and D J Williams, *Nature*, 224 (1969) 371
35 T Oka and Y J Topper, *J Biol Chem*, 246 (1971) 7701
36 J Molnar, *Biochemistry*, 7 (1967) 3064
37 P I Marcus, J M Salb and V G Schwartze, *Nature*, 208 (1965) 1122.
38 T O Fox, J R Sheppard and M M Burger, *Proc Natl Acad Sci U S.*, 68 (1971) 244.
39 N Ohta, A B Pardee, B R. McAusland and M M Burger, *Biochim Biophys Acta*, 158 (1968) 98
40 H W Fisher and J Yeh, *Science*, 155 (1967) 581
41 L V Caso, *Anat Rec*, 172 (1971) 551.
42 Y S Kim, J Perdomo, A Bella Jr and J Nordberg, *Biochim Biophys. Acta*, 244 (1971) 505.
43 S Roth, E J McGuire and S Roseman, *J Cell Biol*, 51 (1971) 536
44 W E. Pricer and G. Ashwell, *J Biol Chem*, 246 (1971) 4825