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EFFECT OF CORTISOL ON HeLa CELL METABOLISM AND MORPHOLOGY

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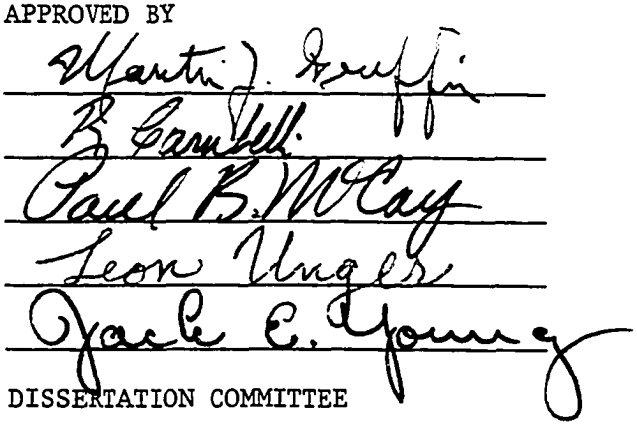
EFFECT OF CORTISOL ON HeLa CELL

METABOLISM AND MORPHOLOGY

APPROVED BY



DISSERTATION COMMITTEE

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PART I

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CHAPTER I

INTRODUCTION

These studies were conducted using the HeLa cell in monolayer culture. HeLa is a name applied to a human derived epithelial cell line of cervical origin. This primary carcinoma was adapted to growth in tissue culture by Gey, Coffman and Kubicek in 1951 (1), and has been carried continuously since. Through the years there have been many different variants developed by cloning from this primary culture. In these studies we have utilized two of these strains, HeLa 65 and HeLa 71. The numbers refer to the major modal chromosome number of each of these two strains, and represent 62 percent of the aneuploid population, i.e., 62 percent of all chromosome counts taken result in a 65 or 71, respectively.

HeLa 65 was developed originally as a suspension or spinner culture and it was later adapted to monolayer growth. This strain has been used extensively to support the growth of various viruses, e.g., polio virus and the polyoma virus. HeLa 71 was originally cloned for its high constitutive level of alkaline phosphatase activity.

These two strains have been carried continuously in our

laboratory for four years, and are the experimental system for these studies. They have both been monitored at various times, and have been found to be free of PPLO (pleural pneumonia-like organisms). The modal chromosome number has also been checked several times throughout the course of these studies and has remained constant with regard to number and percentage.

The steroid hormone, cortisol, has been used with varying degrees of success in the treatment of malignancies, especially lymphocytic leukemia. Since this gluconeogenic hormone has differential effects on human white cell populations, we have chosen to study its mechanism of action in HeLa clones as a model system. Both HeLa 65 and HeLa 71 respond to growth with cortisol by alterations in biochemical composition and morphology, but the response is qualitatively and quantitatively unique for each cell clone. These differing responses are the subject of this study.

Previous studies have listed increases in cell water, RNA and protein (2) as well as DNA, RNA, and protein (3) and in sialic acid (4) after short term cultivation of certain HeLa strains with glucocorticoids such as cortisol. A recent study shows that one HeLa strain, HeLa 71, has an increased G-1 portion of the cell generation cycle, resulting in an increased population doubling time, when grown and subcultured continuously with cortisol (5). Another strain, HeLa 65, exhibits no long-term growth deterrent with this steroid (5).

The present study shows that these two HeLa strains have different biochemical, biophysical and morphological responses to continuous cultivation with cortisol. Some of the observed biochemical differences

may underlie the different growth pattern established in HeLa 71 when cultured with this glucocorticoid. The data compiled to date would appear to indict membranes as one target site of action of cortisol.

PART I

CHAPTER II

MATERIALS AND METHODS

Materials

The cells utilized in this study were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 50 units per ml penicillin, 50 micrograms per ml each of streptomycin and kanamycin (all supplied by Grand Island Biological Company, Grand Island, New York), and ten percent calf serum (Colorado Serum Company, Denver, Colorado).

The cortisol for the cells in the hydrocortisone regulated state (Hcr) was obtained from Sigma Chemical Company, St. Louis, Missouri.

Also used in cell culture were trypsin (0.25%) and 7% sodium bicarbonate from Gibco.

Cell counts were done routinely on a Coulter Counter (Model B) from Coulter Electronics, Hialeah, Florida and were checked with a Bright-Line hemocytometer from A. O. Instrument Company, Scientific Instrument Division, Buffalo, New York.

Doubling time curves were drawn with cells grown in T-25 flasks from Falcon Plastics, Division of BioQuest, Los Angeles, California.

Cells for all experiments were grown either in milk dilution bottles or Blake bottles, ascertained to be flat on one surface, obtained

from E. H. Sargent and Company, Dallas, Texas.

Cell volume measurements were obtained with a Bauer-Schenck sedimentation tube from Kontes Glass Company, Vineland, New Jersey, calibrated in 4 µl increments.

Conical centrifuge tubes (7 ml) from Ace Glass Company, Vineland, New Jersey were utilized for cell water measurements.

Ca^{2+} and Mg^{2+} were measured on a Model 290 B Atomic Absorption Spectrometer at 459.0 mµ and 209.5 mµ, respectively, and Zn^{2+} on a Model 303 Atomic Absorption Spectrometer at 83.5 mµ. Both instruments were products of Perkin Elmer Corporation, Norwalk, Connecticut. Na^{+} and K^{+} were estimated by a Flame Photometer Model 143 from Instrumentation Laboratory, Inc., Boston, Massachusetts.

Thin layer chromatography utilized 20 x 20 cm. glass plates supplied by Brinkman Instruments, Inc., Westbury, New York, and Silica Gel G from E. Merck Ag., Darmstadt, Germany.

The determination of ATP utilized the reconstituted luciferase enzyme preparation from Sigma Chemical Company, St. Louis, Missouri, and the Mark I Scintillation Counter from Nuclear Chicago, Houston, Texas.

The glycogen assay was done with horseradish peroxidase and glucose oxidase from Sigma Chemical Company, St. Louis, Missouri.

Colorimetric determinations of glycogen, protein, DNA, RNA, and phosphorous were read on a Spectrophotometer from Gilford Instrument Laboratories, Inc., Oberlin, Ohio equipped with both a tungsten and a hydrogen light source.

Electron Microscopic work was done on a Hitachi HU-11B microscope distributed by Perkin Elmer Corporation, Norwalk, Connecticut.

Samples were embedded in Cargille's Epoxy Resin (Araldite 6005) from Cargille Laboratories, Inc., Cedar Grove, New Jersey and sectioned on a Porter-Blum ultramicrotome from Ivan Sorvall, Inc., Norwalk, Connecticut.

All other common laboratory reagents were obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey and were reagent grade.

All chemicals were used as obtained unless otherwise specified.

Methods

The HeLa cells were grown at 37° C. in MEM supplemented as described above. The pH was maintained at about 7.2 with 5 percent CO_2 in balanced air. Cells were transferred for serial subculturing after short exposure to EDTA-trypsin (0.7 mM EDTA and 0.025% trypsin) in Puck's buffer, as previously described (7).

The cells in the hydrocortisone regulated state were grown and subcultured for a minimum of three weeks with hydrocortisone at a concentration of 3 μM present in the growth medium.

Each experiment utilized replicate Blake cultures including one which was used for cell counting. All cultures were sacrificed at no more than 70 percent confluency and not less than 50 percent confluency and represent asynchronous, exponentially growing cell populations. Doubling time determinations utilized T-25 flasks (Falcon) containing 4 ml of growth medium.

Assays

For cell volume measurements the cells were scraped from the monolayer in 15 ml of medium at room temperature, sedimented in a large tube, resuspended in 0.3 ml and finally packed in the Bauer-Schenck tube

at 1000g until the volume was constant (usually 15 minutes sufficed). The final reading represents the closest packed volume of the cells, since cell deformability excluded essentially all extracellular fluid when packed under these conditions.

For cell water determination, the cells were packed as above, using preweighed conical centrifuge tubes. The last drop of medium was removed with a Pasteur pipette drawn to a fine point, and the weight thus achieved was taken as the wet weight of the cells. The cells in the tube were desiccated in vacuo over P_2O_5 until constant dry weight was obtained.

Ions were determined on cells scraped from the substrate in medium and isolated as described for water determinations. The cell pellet was suspended in 1.5 ml of a solution 3N in nitric acid and 4N in perchloric acid and mineralized at 100° C for one hour. The solution was filtered and the resulting filtrate used for measuring ion content of the cells as described above.

Total lipid was determined gravimetrically on cells washed with cold saline buffered with tris-HCl(5mM), pH 7.4. After extraction of the freshly washed cells with a total of 6 ml of chloroform: methanol (2:1), the extract was evaporated under a stream of nitrogen and the resulting lipid desiccated over P_2O_5 to constant weight.

Thin layer chromatography to determine classes of lipid in the two cell strains was performed on glass plates coated with a 250μ layer of activated Silica gel G, and developed in hexane: ether (95:5). Spots were detected by spraying with sulfuric acid and charring. Phospholipid phosphorous was determined by the method of Fiske and SubbaRow (8).

ATP was determined by the luciferin-luciferase method of

Addanki, et al., (9). The cells were washed with, and scraped into cold buffered saline and sedimented at 200g. The cell pellet was mixed with 1 ml of 1N perchloric acid at 0° C, and insoluble cell material removed by centrifugation at 300g. The acid supernatant liquid was exactly neutralized with 1N potassium hydroxide, and the insoluble potassium perchlorate sedimented and discarded. The supernatant liquid was repeatedly frozen and thawed (at least three times) to remove the last traces of perchlorate ions, which inhibit the luciferase activity. Filtration of the reconstituted luciferase enzyme preparation prior to ATP determination is essential in order to remove interfering fluorescent particulate material.

For glycogen determination, the cell monolayer was washed with buffered saline followed by cell lysis with 2 ml of 15 percent trichloroacetic acid. After sedimentation of insoluble components (used for RNA and DNA analysis), glycogen was precipitated by addition of ethanol to the supernatant liquid to a final concentration of 80 percent and storing for a minimum of 15 hours at -20° C. The glycogen precipitate was sedimented, hydrolyzed in 1 ml of 2N HCl for 30 minutes in a boiling water bath, neutralized with NaOH, and glucose determined by the glucose oxidase method (10, 11).

RNA and DNA estimations were done after hydrolyzing the 15 percent trichloroacetic acid-insoluble fraction in 1.2 ml 1N perchloric acid at 90° C for 30 minutes. DNA was determined by the diphenylamine method of Burton (12) while RNA was estimated by the orcinol technique (13). Cell protein was determined by the Lowry method (14) on cells which were washed with Tris-saline on the monolayer and lysed in a total

of 1 ml of 0.5 percent sodium deoxycholate.

The half-lysis index was performed by counting the number of cells lysed, as a function of time, among those growing exponentially in a monolayer on cover slips in Leighton tubes. Isotonic saline was used as a medium for the lysing agents. Lysis is defined as penetration of the cell by a 0.05 percent solution of trypan blue.

All values reported represent the mean obtained from at least three experiments with a range no greater than 12 percent.

Electron Microscopy

Thin sections were prepared from pelleted cells quickly fixed in 2 percent glutaraldehyde in 0.1M cacodylate buffer containing 5 percent sucrose. Some cells were fixed directly on the glass by pouring glutaraldehyde into the tissue culture bottle and incubating at 4° C for two hours. Following fixation the cells were rinsed in 0.1M cacodylate buffer overnight. The cells were then transferred to 1 percent osmic acid in 0.1M cacodylate buffer plus 5 percent sucrose and incubated at 4° C for two hours. Following fixation the cells were dehydrated in graded alcohols, embedded in Cargille's Epoxy Resin (Araldite 6005) or maraglas and polymerized at 60° C for 24 hours. All thin sections were stained with uranyl acetate and lead citrate and photographed at various magnifications on a Hitachi HU-11B. Replicas for electron microscopy were prepared by a special procedure (15). Whole cells grown on slides were fresh frozen in liquid nitrogen, and placed on blocks of dry ice. A large block of brass was then cooled with liquid nitrogen and placed in a Hitachi vacuum evaporator. The slides were transferred to the top of the brass block and the chamber was evacuated to a sufficient vacuum

for evaporation. The cells were coated with carbon and palladium *in vacuo* by a probe set at a 45° angle to the specimen. The replica was removed from the slide by immersion in 10 percent NaOH at 4° C overnight, which also removes most of the organic material. The replica was then rinsed in 3 percent acetic acid and placed on a copper grid for viewing.

PART I

CHAPTER III

RESULTS

The two cell strains showed marked growth differences in response to cultivation with cortisol. Figure 1 shows the growth curves of exponentially distributed asynchronously growing cells of both strains grown without (control state) and with (Hcr state) cortisol. HeLa 65 exhibits no appreciable difference in doubling time between control (18.0 ± 1.7 hours) and Hcr (21.0 ± 2.3 hours) states. When HeLa 71 is cultured with cortisol, its population doubling time is increased 50 percent (28 ± one hours for Hcr, 18 ± one hours for control).

Cell size of HeLa 71 and HeLa 65 in both steady-state conditions is expressed (Table 1) in three ways: (a) the average volume measured as described above; (b) the average diameter of the living cell as measured from phase contrast photographs of the monolayers using a Unitron 10B inverted microscope; and (c) the average diameter calculated from the measured volume. It is apparent from these data that the HeLa 71 Hcr cell is much larger than its corresponding control cell, whereas HeLa 65 shows no significant increase in size when continuously cultivated with this steroid. By comparing the calculated diameter with the measured diameter of each of these cells, it can be seen that a HeLa 71

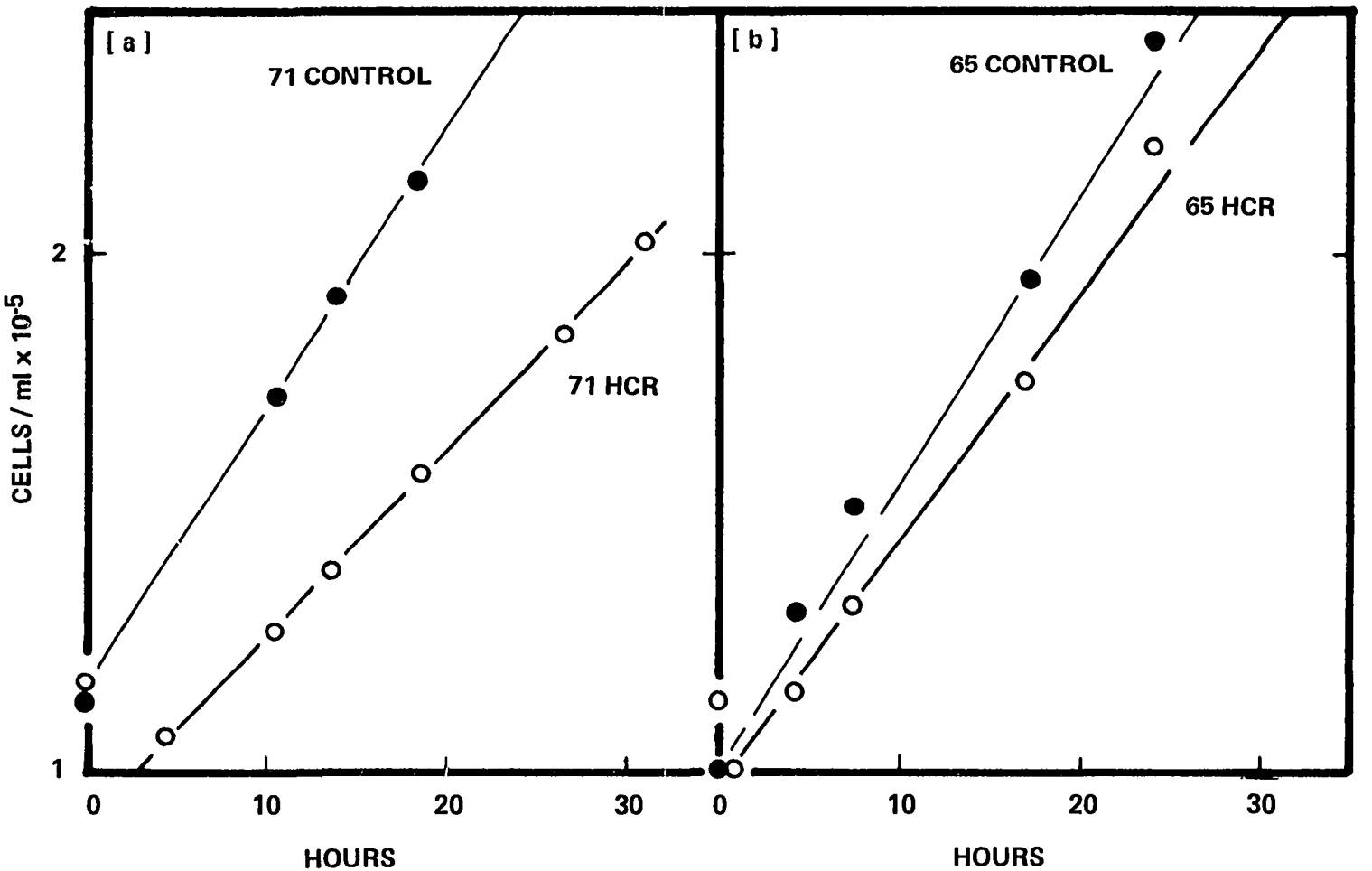


Figure 1--Growth curves for HeLa 71 and HeLa 65 cells with and without cortisol. Lines are plotted on semi-logarithmic paper and were calculated from a linear regression analysis.

TABLE 1

CELL SIZE MEASUREMENTS AND WATER CONTENT FOR HeLa 71 AND HeLa 65 CELLS IN CONTROL AND Hcr STATES

Cell Strain	Average Measured Volume μμl/cell	Monolayer Average Diameter ± S.D. μ	Average Diameter Calculated from Average Volume μ	Water Content μμg/cell
HeLa 71 Control	3.7	23.1 ± 1.5	19	3,600
HeLa 71 Hcr	5.9	30.7 ± 1.6	22	5,700
HeLa 65 Control	3.3	22.3 ± 1.8	19	3,200
HeLa 65 Hcr	4.0	23.8 ± 0.2	20	3,700

Cell volume and monolayer diameter measurements were made as described under Materials and Methods. Calculated diameters were computed from the measured average cell volume. Water content was measured as described under Materials and Methods.

Hcr cell grows on the substrate in a more flattened manner compared to its suspended state, than do any of the other three cell types.

The results of measurements of cell water are also recorded in Table 1. HeLa 71 Hcr has 50 percent more water on a per cell basis than its corresponding control cell. This stands in contrast to HeLa 65 which shows only about a 10 percent increase in the Hcr state. The dry weights are as follows: HeLa 71, 550 μμg/cell; HeLa 71 Hcr, 1000 μμg/cell; HeLa 65, 530 μμg/cell, HeLa 65 Hcr, 540 μμg/cell. From the above data, we estimated water to represent from 83 to 85 percent of the measured cell volume for either strain in either state.

An increase in the water content of HeLa 71 Hcr is particularly significant when the cell molar concentration of divalent and monovalent metal ions is examined (Table 2). Both strains have increased levels of sodium in the Hcr steady state. Whereas little change was seen in K^+ in HeLa 71, a 20 percent increase was observed in the Hcr state in HeLa 65.

Calcium ions increase in HeLa 71 Hcr by 30 percent whereas it decreases in HeLa 65 Hcr by about 30 percent. Magnesium ions increase in HeLa 65 Hcr by 23 percent, but show no significant change in HeLa 71 from one state to the other. Both strains have about a 50 percent increase in levels of Zn^{2+} in asynchronous exponentially growing cell populations in their Hcr states.

Another component of HeLa 71 cells which is increased significantly in the Hcr state is total lipid (Table 3). Whereas the cell volume increases only 50 percent in HeLa 71 Hcr, the lipid increase is 80 percent (170 μμg/cell versus 310 μμg/cell). Thin layer chromatography

TABLE 2

INTRACELLULAR CONCENTRATION (mM) OF IONS FOR HeLa 71 AND HeLa 65 CELLS IN THE CONTROL AND Hcr STATES

Lines	Na^+	K^+	Ca^{2+}	Mg^{2+}	Zn^{2+}
HeLa 71	100	38	3.4	7.2	0.21
HeLa 71 Hcr	130	35	4.5	7.6	0.35
HeLa 65	150	54	3.0	6.1	0.22
HeLa 65 Hcr	190	63	2.1	7.5	0.32
MEM	130	6	1.7	0.8	0.01

The bottom line shows the concentration of these ions in complete growth medium.

TABLE 3

LIPID CONTENT OF HeLa 71 AND HeLa 65 CELLS IN CONTROL AND Hcr STATES

Lines	Total Lipid μμg/cell	Total Phospholipid Phosphorous μμmoles/cell	Phospholipid* μμg/cell / Total Lipid μμg/cell
HeLa 71	170	0.08	0.34
HeLa 71 Hcr	310	0.13	0.28
HeLa 65	200	0.09	0.28
HeLa 65 Hcr	220	0.12	0.33

*Based on an estimated average molecular weight of 750 for phospholipid.

of these lipids suggest cholesterol and phospholipid as the major components, with minor amounts of diglycerides and cholesterol esters. An increase in phospholipid phosphorous was observed in the Hcr state of both cell strains; it is more pronounced in HeLa 71 (Table 3). Phospholipid to total lipid ratios are not significantly different for either clone in either state.

Results of the DNA assay show no difference for either clone in either state. The DNA content is 11 ± 2 μμg/cell. RNA and protein, on the other hand, show a 30 percent increase in HeLa 71 Hcr, whereas no significant change is exhibited in HeLa 65 Hcr (Table 4).

The amount of energy available to an Hcr cell, in the form of ATP, was shown to be more than twice that present in a control cell in both strains (Table 4). Although the absolute amounts of ATP differ widely between the cell strains, the relative increase from the control to the Hcr state is quite similar, with HeLa 71 showing a proportionally larger increase.

The glycogen assay showed a 100 percent increase in this compound in the Hcr state of both strains (Table 4).

The difference in the effect of lipolytic lysing agents on both strains in both states is illustrated in Table 5. HeLa 65 Hcr is more resistant to lysis with both sodium deoxycholate (DOC) and saponin than HeLa 65 control. In contrast, little increase in resistance to either agent is evidenced by HeLa 71 Hcr versus its control, although a slight protection against the uncharged saponin appears to exist for this strain in the Hcr state.

Histochemical results also substantiate biochemical (Table 4)

TABLE 4

QUANTITATION OF RNA, PROTEIN, ATP, AND GLYCOGEN IN HeLa 71 AND HeLa 65 CELLS IN CONTROL AND Hcr STATES

Cell Strain	RNA μμg/cell	Protein μμg/cell	ATP μμmoles/cell	Glycogen μμg/cell
HeLa 71 Control	25.2	640	0.003	109
HeLa 71 Hcr	33.8	880	0.008	210
HeLa 65 Control	25.5	790	0.024	157
HeLa 65 Hcr	23.6	730	0.052	320

These values represent a mean of three determinations with a range no greater than 12 percent.

TABLE 5

HALF LYSIS INDEX OF HeLa 71 AND HeLa 65 CELLS
IN CONTROL AND Hcr STATES

Concentration of lysing agent	HeLa 71		HeLa 65	
	Control	Hcr	Control	Hcr
		Minutes*		
0.050% DOC†	20	10	10	10
0.040% DOC	20	20	12	40
0.030% DOC	30	30	22	>120
0.020% DOC	>120	>120	>120	>120
0.020% Saponin	3	3	2	2
0.010% Saponin	25	75	36	>120
0.005% Saponin	>120	>120	>120	>120

*Results represent the time in minutes required to lyse 50 percent of the cells, as measured by admittance of trypan blue.

†Sodium deoxycholate.

and electron microscopic (Figures 2 through 7) results. Very little difference can be demonstrated between the two states of HeLa 71 and HeLa 65 with regard to RNA and protein (see Table 4). Conversely, the enhanced glycogen content in the Hcr state of both strains is readily apparent. One interesting result from the histochemical studies was the demonstration of two types of glycogen-containing cells in HeLa 71. In the control culture there are cells containing some glycogen in large granules, and also cells which contain very little glycogen. HeLa 71 Hcr exhibits about 15 percent of its population as giant cells which are nearly filled with glycogen. Also present in this culture are large cells which contain fine granules of glycogen and small cells with large granules. These results suggest that glycogen varies qualitatively and quantitatively throughout the cell cycle.

Moreover, it was demonstrated histochemically that there are no pools of cholesterol or triglycerides within lipid droplets in the two strains studied biochemically. Hence, it is inferred that the lipid in the HeLa 65 and HeLa 71 cells is membrane bound and that the increase in lipid found in HeLa 71 Hcr represents an increase in membrane lipid.

Electron microscopic studies showed the cytoplasm of HeLa 65 to contain relatively fewer organelles of all types than HeLa 71. For example, the mitochondria are fewer in number and lysosomes are relatively smaller and fewer compared to the other clone (compare Figures 2 and 5). The golgi zones are adjacent to the nucleus and are small in size. There are short lengths of rough endoplasmic reticulum, but no discernible organization. Smooth endoplasmic reticulum is present, but it is very sparse and located near the periphery of the cell. HeLa 65 has a

Figure 2--Electron micrograph of a HeLa 65 cell in the Control state. Nucleus (N), mitochondria (M), lysosome (Ly), endoplasmic reticulum (ER), and golgi (Go). X 11,500.


ER
2

Figure 3--Electron micrograph of a HeLa 65 cell in the Hcr state demonstrating blebbing of peripheral membrane. Nucleus (N), mitochondria (M), lysosome (Ly) and endoplasmic reticulum (ER). X 14,500.

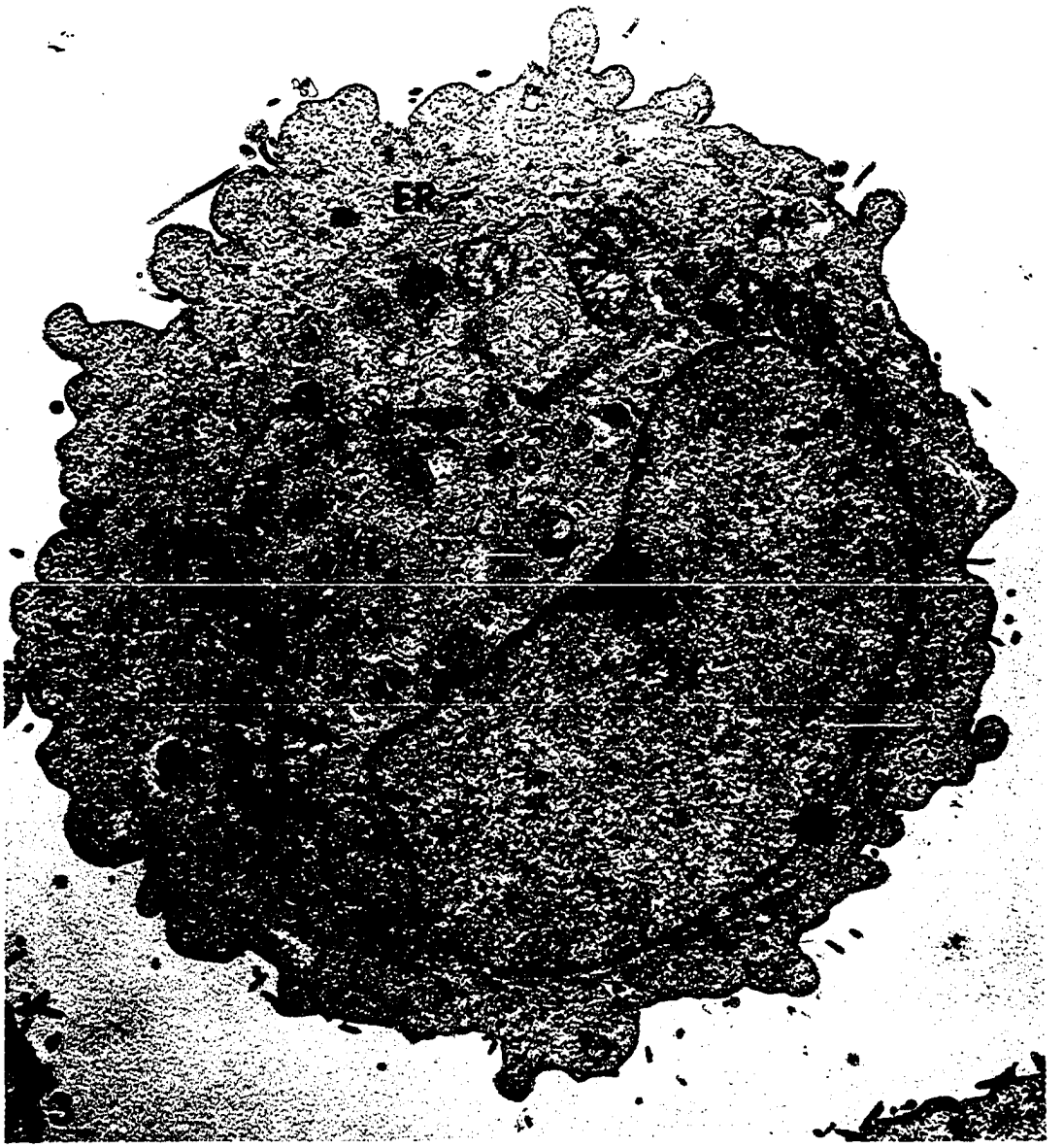
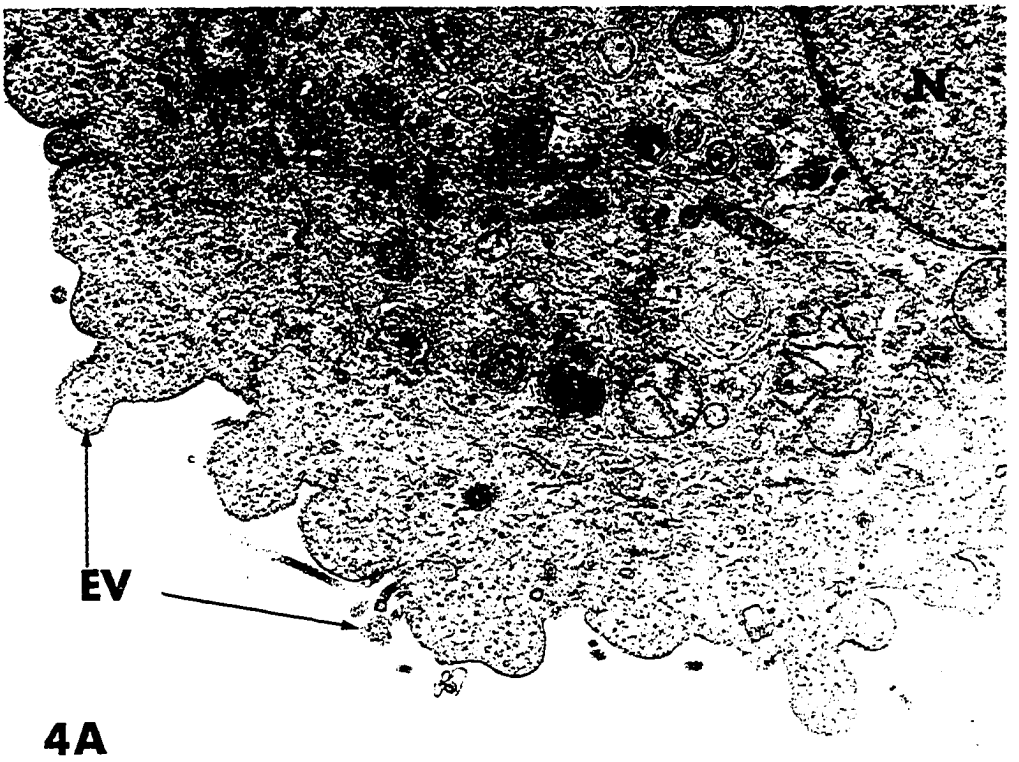
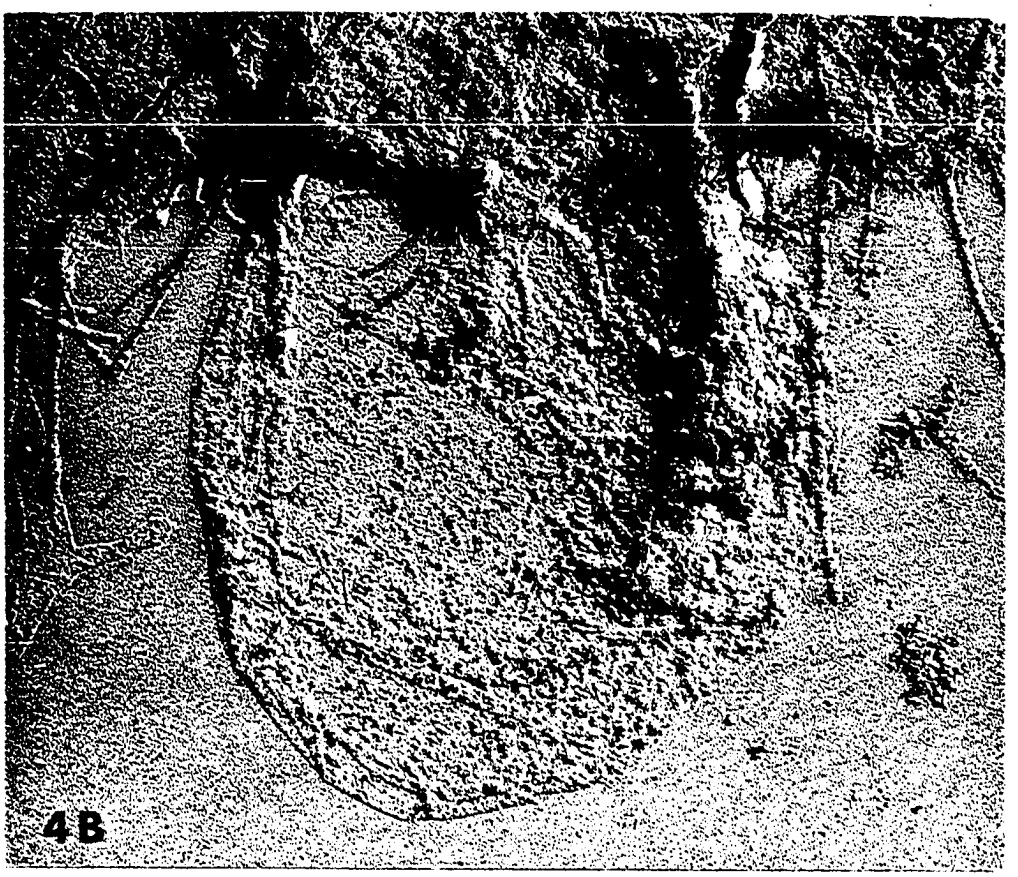

ER

Figure 4--Electron micrographs of a portion of the plasma membrane from a HeLa 65 cell in the Hcr state. (A.) High magnification showing evaginations (blebs) of the plasma membrane. Nucleus (N), and evaginations (EV). X 22,000. (B.) Carbon-palladium replica of bleb on the plasma membrane. X 71,000.



4A



4B

Figure 5--Electron micrograph of a HeLa 71 cell in the Control state. Nucleus (N), endoplasmic reticulum (ER), mitochondria (M), lysosome (Ly) and glycogen (G). X 16,000.

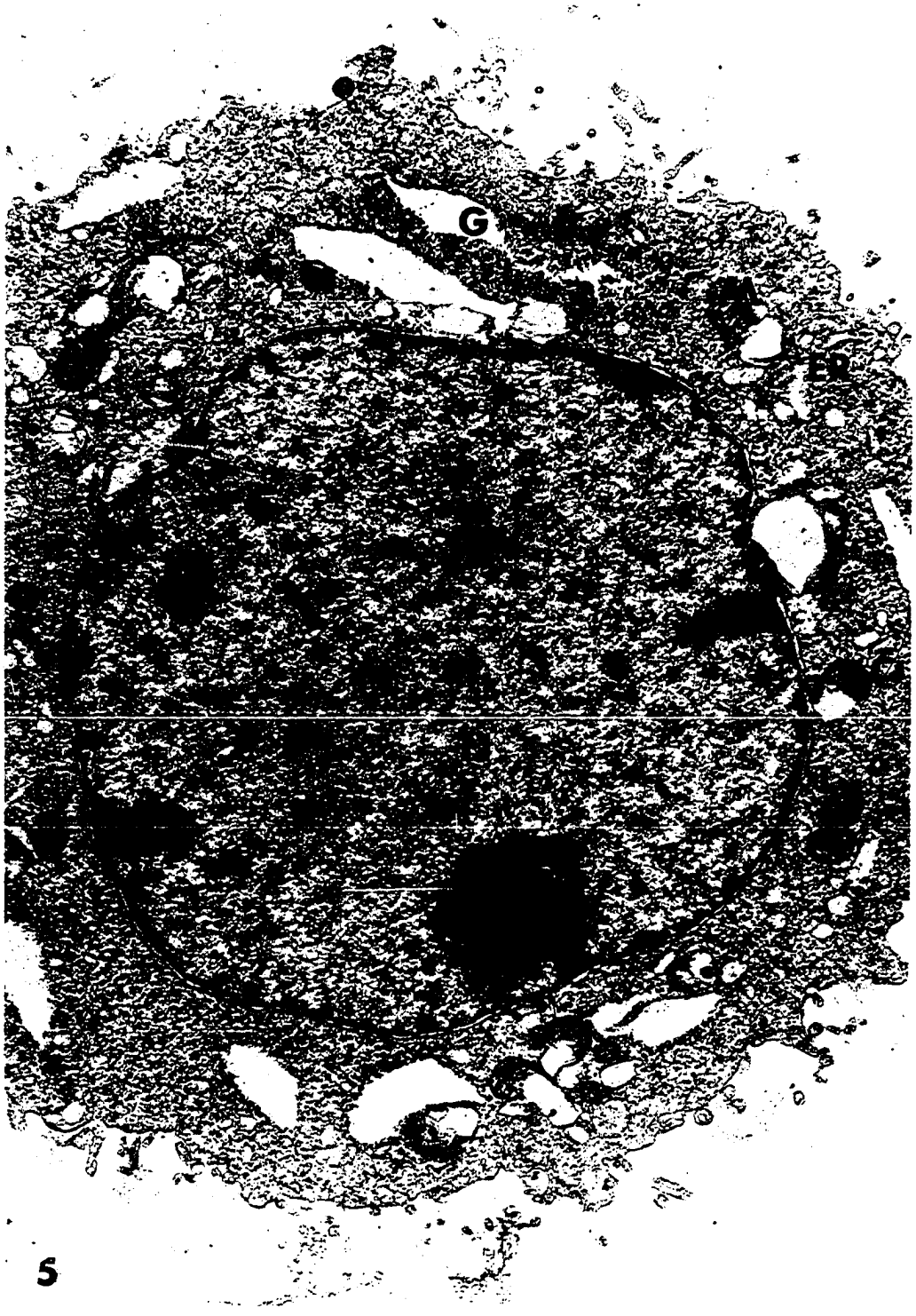
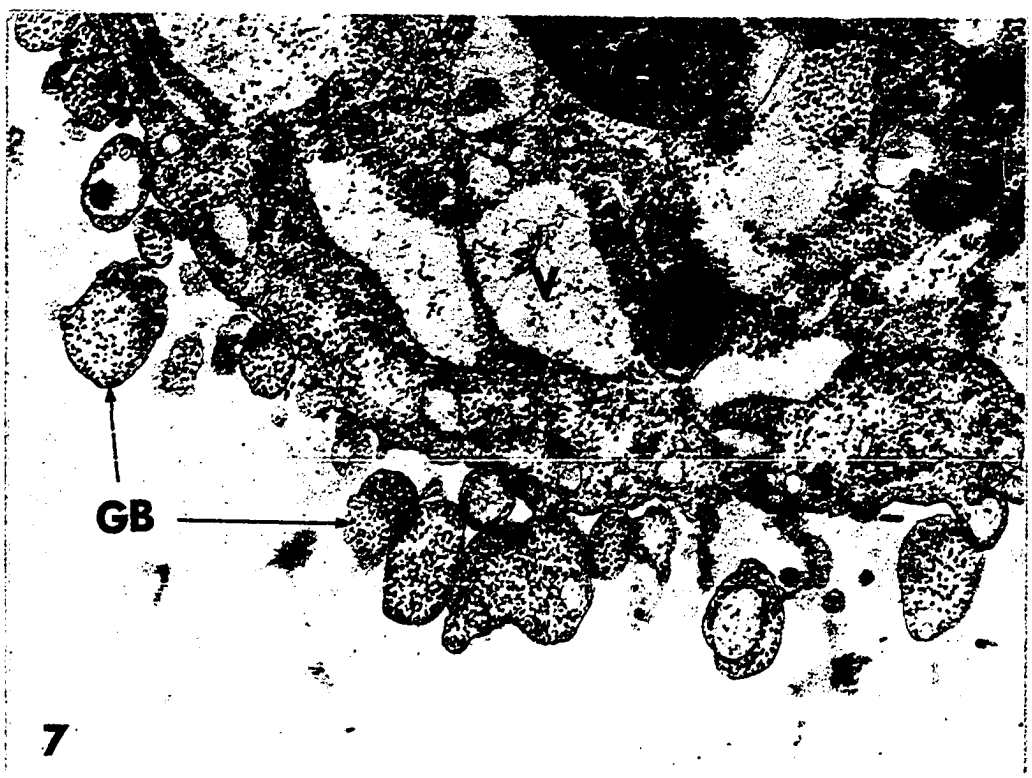

G
ER
S

Figure 6--Electron micrograph of HeLa 71 cell in the Hcr state. Nucleus (N), mitochondria (M), endoplasmic reticulum (ER) and glycogen (G). X 10,000.


6

Figure 7--Electron micrograph of a portion of the plasma membrane from a HeLa 71 cell in the Hcr state. High magnification showing glycogen in cytoplasmic vacuoles (V) and extra cellular membrane bound bodies (GB). X 23,000.


GB
7

relatively smooth cytoplasmic periphery, in the control state, though occasional cells exhibit blebs or evaginations of the plasma membrane, which contain ribosomes or other cytoplasmic organelles. The nuclear membrane of this clone provides a nuclear silhouette that is continuous, smooth and shows relatively few micro invaginations compared to HeLa 71 (Figures 2 and 5). In the Hcr state the plasma membranes of HeLa 65 form large evaginations (blebs), some of which contain organelles (Figures 3 and 4A). Figure 4B shows a carbon-palladium replica of one bleb.

In the control state HeLa 71 has a highly crenated outer periphery (Figure 5). The cytoplasm contains moderate amounts of glycogen, which are dispersed or are found in large vacuoles that are associated with the rough endoplasmic reticulum. Numerous mitochondria are seen in this strain. Endoplasmic reticulum is abundant but apparently somewhat dilated, and in some cells, the lumen is filled with a moderate amount of glycogen (Figure 5). In a small number of cells the plasma membrane has evaginations, which contain glycogen.

HeLa 71 has a large increase in glycogen in the Hcr state (Figure 6). The major part of this glycogen is found within large membrane bound vacuoles some of which are studded with ribosomes. In many fields, topographically separate membrane bound glycogen bodies appear adjacent to the outer plasma membrane. Similar bodies are seen attached to the plasma membrane (Figure 7).

PART I

CHAPTER IV

DISCUSSION

The unbalanced growth of HeLa S_3 cells (herein referred to as HeLa 65) described by Kim, et al., (16) is an experimental situation in which synchronized HeLa cells were exposed to DNA inhibitors for varying lengths of time. The resulting loss of cell viability (defined as colony-forming ability) was reversible for up to sixteen hours, but prolonged exposure of the cells to these drugs led to their death. Presumably during this initial sixteen hours, in which colony-forming ability is recoverable, there are other physiological alterations which are reparable to varying degrees. These alterations still allow cell viability, but recovery processes are uncertain. Lambert, et al., (17) describe experiments utilizing HeLa cells which have been treated with excess thymidine. The end result of prolonged treatment with this compound was again cell death. This type of metabolism is also referred to as unbalanced growth. In contrast to inhibitors of cell maturation which are ultimately lethal, the system described in this dissertation affords a continued, although altered, proliferation cycle. Growth of HeLa 71 in the presence of cortisol results in cells capable of continued subculture, but with an increased generation time. This new steady-state growth

condition shall be called rebalanced growth (to differentiate between the types of drug-elicited effects on cells). HeLa 65 grown in the presence of cortisol shows only an acute growth response (5), after which control doubling-time is achieved and retained. Because of the biochemical alterations seen in these cells, this is again rebalanced growth with no alteration in the growth cycle.

The increase in size of the HeLa 71 Hcr cell correlates well with the increases found in cell water in this strain. The predicted cell diameter from measured cell volume is in close agreement with average measured diameter of the living cell. Cell volume increase has also been reported (2, 3) in HeLa S_3 cells treated for 72 hours with cortisol. We have also studied the acute response of HeLa 65 to growth with cortisol, but this acute response is reversed in the Hcr steady-state condition (after three weeks of growth with cortisol) in this strain. Thus, in HeLa 65, the Hcr cells have a cell volume very similar to their corresponding control cells, and also nearly the same water content.

The results of the ion studies become more meaningful when viewed in conjunction with the results of cell water measurements. In both cell strains intracellular Na^+ concentration is increased when grown in the presence of cortisol, while K^+ concentration is increased only in HeLa 65 Hcr. The increased Na^+ concentration above MEM Na^+ in HeLa 65 cells may represent ions attached at absorptive sites within this cell clone. In HeLa 71 Hcr the sodium ion content is increased to nearly that of the surrounding medium. No single mechanism of cortisol mediated inhibition or stimulation of the Na^+, K^+-activated ATPase pump is sufficient to explain all these observations. Increases in Na^+ and

K^+ have been reported in rat liver after a 2-4 day treatment *in vivo* with cortisol (18).

Of the divalent cations examined, only Zn^{2+} increases significantly in both HeLa 65 and HeLa 71 in the Hcr state. The increase is about 50 percent for both strains. An increase in Zn^{2+} uptake by both cell strains in the presence of steroid hormones with glucocorticoid activity has been reported (19) in studies done with cells grown in the presence of the steroid for a total of 72 hours. Our steady-state results correlate well with these acute responses. While Mg^{2+} concentration was increased by 23 percent in HeLa 65 Hcr, there was no significant change in HeLa 71 from one state to the other. In HeLa 65 Hcr the Ca^{2+} concentration is decreased about 30 percent, while this ion is increased by 30 percent in HeLa 71 Hcr. These results for monolayer cultures of HeLa cells contrast with those found by Morrill and Robbins (20) in their studies of HeLa cells in suspension culture. They found a correlation between Ca^{2+} levels and the steady-state level of Na^+ and K^+, but K^+ levels were higher than Na^+ levels. It is apparent that total amounts of anions must be considered in cation studies since suspension culture medium contains ten times as much phosphate or bicarbonate ion as the medium used for monolayer culture.

The magnitude of increase in lipid in HeLa 71 Hcr is of interest. Since the surface: volume ratio is a function of the radius squared to the radius cubed, a 50 percent increase in volume would demand less than 50 percent total increase in surface membranes of the cytoplasm and the nucleus. The 80 percent increase seen in lipids in the Hcr state of HeLa 71 cells may indicate that this cell strain responds to cortisol by

producing more lipids than would be required just for surface membrane production. The disproportional increase in lipid may represent an increase in total membrane production in the Hcr cells, with more membranes per cell. On the other hand, it could also represent an increased uptake of lipid in the already existing membrane mosaic. Quantitation of these compounds from membrane fractions will determine which of these alternatives is correct. Rosenberg (21) has reported that changes in the gross composition of lipid in membranes may well affect both the structure and the function of those membranes. The possibility of membrane changes becomes particularly important when it is remembered that Jacob, Brenner and Cuzin (22) suggested that initiation of DNA replication in bacteria required attachment of the chromosome to the cell membrane, while Comings and Kakefuda (23) suggested that DNA replication in mammalian cells may be initiated at the nuclear membrane. Alteration in the nuclear membrane with altered DNA attachment sites is one possible explanation for the extended generation cycle in HeLa 71 Hcr, since it is believed that the initiation of DNA synthesis is being delayed.

The base line differences in ATP content between the two cell strains may reflect the increase in alkaline pyrophosphatase activity found in HeLa 71 (24), since HeLa 71 possesses high constitutive activity of this enzyme.

A large increase in glycogen was observed in the Hcr cells of both strains. Results also showed more of this material in HeLa 65 than in HeLa 71. This stands in contrast to electron microscopic results of HeLa 65 which showed no particulate glycogen. One possible explanation for the apparent discrepancy between biochemical and electron microscopic

results is a difference in glycogen particle size. If the glycogen in the HeLa 65 clones was in the size range of the γ particles (3 mμ) mentioned by Barber, et al., (25), then it would appear ultrastructurally, as diffuse background. By contrast the particles of glycogen in HeLa 71 must be similar to α particles (200 mμ) these authors have described, and thereby detectable by the electron microscope. Moreover, the biochemical results agree well with reported amounts of glycogen found in liver tissue and muscle (26, 27).

The increase in glycogen content of these cells in the presence of cortisol suggest a uniform gluconeogenic response much like that exhibited by other organs such as liver, spleen, kidney, etc. In any case, the changes seen in those components involved in energy production for the cell do not seem to be the basis of the growth cycle response of HeLa 71 Hcr, since they are elevated from control levels and do not represent a starvation condition.

Melnykovych (3) and Cox (2) have reported increases in RNA and protein in HeLa 65 in the acute state of cortisol treatment. The data presented here for the hydrocortisone regulated state agrees very well with their observations.

The data with the lysing agents provide further suggestive evidence for different physiological membrane responses of HeLa 71 and HeLa 65 to growth with cortisol. The clone without any growth alterations in the Hcr steady-state, HeLa 65, shows an increased resistance to lipolytic lysing agents. This clone has been shown to gain resistance to deoxycholate lysis by short periods of growth with prednisolone (28). The cell clone with a permanent growth alteration in the Hcr steady-state, shows

little or no resistance to these lysing agents. The inability to gain resistance when grown with cortisol might result from alterations in membrane lipids to increase the number of lipophilic absorptive sites in HeLa 71 Hcr.

Electron microscopic findings indicate that cellular complexity increases as chromosomal numbers increase. HeLa 65 is a relatively simple cell type which contains few organelles and little if any storage products. HeLa 71 contrasts greatly with HeLa 65 in that the cytoplasm contains many mitochondria, organized golgi zones, extensive lengths of endoplasmic reticulum and large glycogen particles.

PART I

CHAPTER V

SUMMARY

Two HeLa cell strains, HeLa 65 and HeLa 71, identified by their major modal chromosome numbers, were studied by biochemical and electron microscopic techniques in two steady-state conditions. Cells cultured continuously with 3×10^{-6} M cortisol were called Hcr, while those cultured in medium without added cortisol served as controls. Both strains showed a variety of responses to growth with cortisol. Exponentially growing, asychronous populations of HeLa 71 show increased amounts of water, lipid, sodium, calcium, zinc, RNA, protein, glycogen and ATP in the Hcr state. This clone undergoes an increase in doubling time when grown with cortisol. In contrast, HeLa 65, the morphologically more primitive cell by electron microscopy, shows increases in sodium, potassium, zinc, glycogen and ATP when grown with cortisol. No permanent increase in doubling time is evidenced by this clone in the Hcr state. Ultrastructural studies corroborate some of the biochemical changes elicited in cells grown continuously with cortisol, and suggest alterations in cellular membranes. A study with two lipolytic lysing agents provided further support for this postulation. Physiological differences which may be related to altered growth of HeLa 71 Hcr cells are the

increases in water, lipid, protein, RNA and calcium ions, and lack of increase of potassium ions.

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PART II

ISOLATION AND PARTIAL CHARACTERIZATION OF THE MEMBRANE SYSTEMS OF HeLa CELLS

CHAPTER I

INTRODUCTION

In the previous section it has been suggested that the effect of hydrocortisone is manifested by changes in the membrane system of the HeLa cell. As a result, it was decided that much could be learned if asynchronous cells were fractionated and the membranes isolated to determine if continued growth with hydrocortisone does, in fact alter the membranes, and if so, which membrane system is altered to the greatest extent by growth with this steroid.

Other investigators have isolated membrane fractions using HeLa 65 cells in suspension culture, and the literature disclosed three major modifications (1, 2, and 3) of the same basic technique for isolation of HeLa cell membranes. None of these techniques were entirely satisfactory when applied to our cells from monolayer culture. The most satisfactory separation was achieved by a combination of techniques which utilized portions from each of the previously published methods.

The results reported here represent only a beginning in the proposed comprehensive study of the membrane systems of the HeLa cell.

This study is to include data on these membranes from several cell types both in the presence and absence of hydrocortisone and as a function of the cell cycle. The three components quantitated here for each separate membrane fraction are sialic acid, alkaline phosphatase and protein, all of which are known components of HeLa membrane systems. Initial data is also presented on separation and quantitation of whole cell phospholipid classes, and cholesterol which are also primarily membrane constituents.

PART II

CHAPTER II

MATERIALS AND METHODS

Materials

The sucrose for the gradients came from Merck and Company, Inc., Rahway, New Jersey, and was dissolved in water to achieve the specified w/w percentage.

Centrifugation was usually done in an International Refrigerated Centrifuge, International Equipment Company, Boston, Massachusetts. However, mitochondria and lysosomes were separated in the Servall Model RC-2 from Ivan Sorvall, Inc., Norwalk, Connecticut. The gradients were all spun in either the Model L or Model L-2 ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California, utilizing swinging bucket heads SW 39L or the SW 25.1., also from Spinco.

Column chromatography was done with two resins supplied by Sigma Chemical Company, St. Louis, Missouri; Dowex 50 W(H^+), 25 to 50 mesh and Dowex I(Cl^-), 50 to 100 mesh.

Reagents for the thiobarbituric acid method of quantitating sialic acids were acquired from J. T. Baker Chemical Company, Phillipsburg, New Jersey except for 2-thiobarbituric acid which was obtained from Eastman Organic Chemicals, Rochester, New York. The thiobarbituric acid

was recrystallized from boiling water (25 gm in 600 ml), and dried in a vacuum dessicator over NaOH pellets until complete dehydration was achieved.

The substrate for the alkaline phosphatase assay was p-nitrophenyl phosphate (8mM) in 0.125N Tris-HCl, pH 10.0, both reagents were obtained from Sigma Chemical Company, St. Louis, Missouri.

Saline-Tris (S-T) Buffer which is 0.15N NaCl containing 0.005M Tris-HCl pH 7.4, was prepared from Trizma Base from Sigma Chemical Company, St. Louis, Missouri, and NaCl from J. T. Baker Chemical Company, Phillipsburg, New Jersey. All buffers were made with glass distilled water and the pH adjusted with hydrochloric acid from Fisher Scientific Company, Philadelphia, Pennsylvania.

The protein quantitations for this section were done utilizing the same reagents reported in PART I, CHAPTER II.

Lyophilization was accomplished using a lyophilizer from Virtis Research Equipment, Gardner, New York.

The cells were ruptured for membrane preparations with a Dounce homogenizer from Kontes Glass Company, Vineland, New Jersey, and a Biosonic Sonicator, from Bronwill Scientific, Division of Will Scientific, Inc., Rochester, New York.

Thin layer chromatography equipment utilized was from DeSaga/Brinkman Equipment, Brinkman Instruments, Inc., Westbury, New York; Anacil H gel from Analytical Engineering Laboratories, Inc., Hamden, Connecticut for preparative plates; and Silica Gel HR for analytical plates from E. M. Reagents Division, Brinkman Instruments, Inc., Westbury, New York.

The media, Auto Pow, used for these experiments was obtained from Flow Laboratories, Rockville, Maryland, and is an autoclavable preparation of Eagle's MEM. The other tissue culture reagents, and the bottles used in growing the vast number of cells required for these experiments were obtained from the same sources listed in PART I, CHAPTER II.

Common reagents, i.e., acids, bases, etc., are not listed separately but were generally supplied by J. T. Baker Chemical Company, Phillipsburg, New Jersey and were reagent grade.

Methods

The method for culturing the cells for these experiments was essentially the same as reported in PART I, CHAPTER II except that the growth media was Auto Pow, which required the addition of sterile glutamine along with the antibiotics and calf serum normally added to all commercial media. The cells were transferred from feeder bottles into seventeen replicate Blake bottles for each strain and each state. One bottle from each set was trypsinized and diluted to 50 ml with media for counting. All counts represent at least four separate estimations made on the Bright-Line hemocytometer.

All cells were sacrificed at mid-log growth and therefore represent asynchronous populations of exponentially growing cells. Sacrifice was accomplished by two gentle washings of the cell monolayer *in situ* with 10 ml of S-T Buffer and scraping the monolayer, with a rubber policeman, into 10 ml of the same buffer. The sacrificed cells were pelleted by centrifugation in the refrigerated centrifuge at 200g for 15 minutes. The cell pellet was then resuspended in a total of 7 ml of

0.05M Tris-HCl, pH 7.5, which is 0.005M in MgCl, and homogenized with a Dounce homogenizer. The minimum number of strokes required to achieve what appeared to be complete rupture of the plasma membrane under the microscope varied for each cell strain and from the control to the Hcr state; fourteen strokes for 65 Control, twelve strokes for 65 Hcr, twenty strokes for 71 Control and eighteen strokes for 71 Hcr. The nuclei were then separated from the cytoplasm by centrifugation at 200g for ten minutes. The cytoplasm was separated from the nuclear pellet and centrifuged again in the Servall for 10 minutes at 4000g to remove the mitochondria and lysosome fraction.

The supernatant fluid from this spin was made 45 percent with respect to sucrose and transferred to the tubes for the SW-25.1 swinging bucket head. Successive layers of 35 percent sucrose, 30 percent sucrose, 25 percent sucrose and finally 0.05M Tris-HCl were carefully added so that little or no mixing of the gradient resulted. These tubes were then spun for 16 hours at 70,000g (22,000 rpm) in the Model L-2 ultracentrifuge, to separate the cytoplasmic membrane fractions by flotation.

The nuclear pellet in the ultracentrifuge tubes, was made 45 percent with respect to sucrose and sonicated, at 70 percent efficiency for two 5-second bursts. This gradient was then made by successive layers of 30 percent sucrose and 0.05M Tris-HCl. The discontinuous gradient was then spun for sixteen hours in the SW-39 L head at 70,000g (32,500 rpm) in the Model L ultracentrifuge, to separate the nuclear membrane from the chromatin.

The membrane suspensions were drawn off from the bottom of each band with a syringe and needle appropriately bent to facilitate easy

collection. Each of these fractions was washed by a dilution of at least 1:4 with 0.05M Tris-HCl containing 5mM $MgCl_2$ and recentrifuged at 70,000g for 1 hour. The membrane pellets were resuspended in 2.0 ml of water and the alkaline phosphatase activity, protein and sialic acid content of each determined.

Sialic acid quantitation was done by the thiobarbituric acid method of Warren (4), after separation of these compounds by the column chromatographic technique reported by Carubelli and Griffin (5), with modifications suggested by Mayron and Tokes (6). For sialic acid determination each sample was made 0.1N with respect to sulfuric acid, the tubes were capped with a marble and the sample hydrolyzed in an 80° C water bath for one hour. After cooling, the samples were neutralized with 0.1N $Ba(OH)_2$ and the slight excess of $Ba(OH)_2$ removed as $Ba(CO_3)_2$ by addition of dry ice followed by centrifugation. The supernatant fluid containing the sialic acid was passed through a 4 by 40 mm Dowex 50 $W(H^+)$ column followed by 2 ml distilled water. This mixture was passed through a Dowex $I(Cl^-)$ column of the same size which was prepared by washing the packed column with three column volumes of 3N NaOH, and then three column volumes of 3N HCl, and finally with distilled water until free of chloride ions. The sialic acids were eluted from the column with 15 ml of 0.2M NaCl (6). The eluent was fast-frozen in acetone and dry ice and lyophilized overnight. The resultant dried material was redissolved in 1 ml distilled water and duplicate 0.4 ml aliquots made for the TBA quantitation of sialic acid. A standard containing a known amount of N-acetyl neuraminic acid was subjected to the same treatment as the experimental samples, and values obtained were then corrected according to the losses

incurred through hydrolysis and column separation of the standard.

Histochemical studies utilized cells grown on cover slips in Leighton tubes. These were fixed with phosphate buffered formalin (10%), pH 7.0, and stained with Hale's colloidal iron stain for acid mucopolysaccharides and sialic acids (15). Since these cells contain no measurable amounts of acid mucopolysaccharide (S. Tu, private communication), the positive staining reaction is believed to be due to the presence of sialic acids.

Pictures of the histochemical slides were taken with Kodachrome II, Type A professional film, with a film speed of 40, a magnification of 400 X, using an 8282 C filter. The exposure times were 1/4 second except for 3A which had a 1/2 second exposure time.

The alkaline phosphatase activity of each membrane fraction was estimated by the previously reported method (7) using para-nitrophenyl phosphate for a substrate, and is expressed as international units (μmoles p-nitrophenol released per minute).

The protein values for each membrane fraction were obtained by the method of Lowry, et al., (8).

For lipid analysis, the cells were sacrificed as described above for membrane isolation. After the scraped cells were centrifuged, the lipid was extracted by the Folch method (9), in a total of 6 ml of chloroform:methanol (2:1) per Blake bottle. The extractant was evaporated to dryness under a stream of nitrogen and redissolved in 0.5 ml chloroform for spotting. Preparative thin layer plates were spread to a thickness of 750μ with an emulsion of 40 gm of Anacil H and 120 ml of 1mM $(Na)_2CO_3$. The solvent system for the preparative plates was chloroform:methanol:

water: acetic acid (60:35:5:0.5), and the spots were detected in an iodine chamber. Following detection the spots were scraped from the plates and the lipid extracted with chloroform: methanol: water: formic acid (97:97:4:2) according to Abramson and Blecher (10). The extractant was evaporated in a hot water bath and phosphorous content determined by the Fiske-SubbaRow method (11). Analytical plates were spread to a thickness of 250μ with an emulsion of 35 gm of Silica Gel HR, sifted through a 100 mesh copper screen, and 87.5 ml of water. The solvent system (12) for the analytical plates was chloroform: methanol: ammonium hydroxide (70:30:4) and spots were detected by successive spraying with rhodamine-6-G (13), ninhydrin reagent (0.5% in n-butanol) and modified zinzadze molybdenum reagent (14) which allowed identification of each phospholipid component.

All values reported represent a mean of three experiments which resulted in a range in a few cases of as much as 20 percent, but most determinations fall within a range of 10 percent of the mean.

PART II

CHAPTER III

RESULTS

The discontinuous sucrose gradient proved to be a useful method of separating the various membrane systems of the two HeLa cell strains. With this type of gradient, the various membranes separate into distinct bands which are easily recovered. The following membrane preparations are obtained from the cytoplasmic fraction: the smooth endoplasmic reticulum at the top of the gradient; the rough endoplasmic reticulum separates into two fractions at the 30-35 percent sucrose interface and at the 35-45 percent sucrose interface; and the plasma membrane at the bottom of the 45 percent sucrose layer. The nuclear membrane fraction floats to the 25-30 percent sucrose interface while chromatin sediments to the bottom of the 45 percent sucrose layer. A typical pattern of separation is shown in Figure 1 for both the cytoplasmic and nuclear fractions.

Both strains and both states of each strain show essentially the same pattern. The only difference is seen in the amount of the two rough endoplasmic reticulum bands which have varying amounts of material by visual inspection from experiment to experiment, i.e., sometimes the top band appears to contain more material than the bottom band, whereas

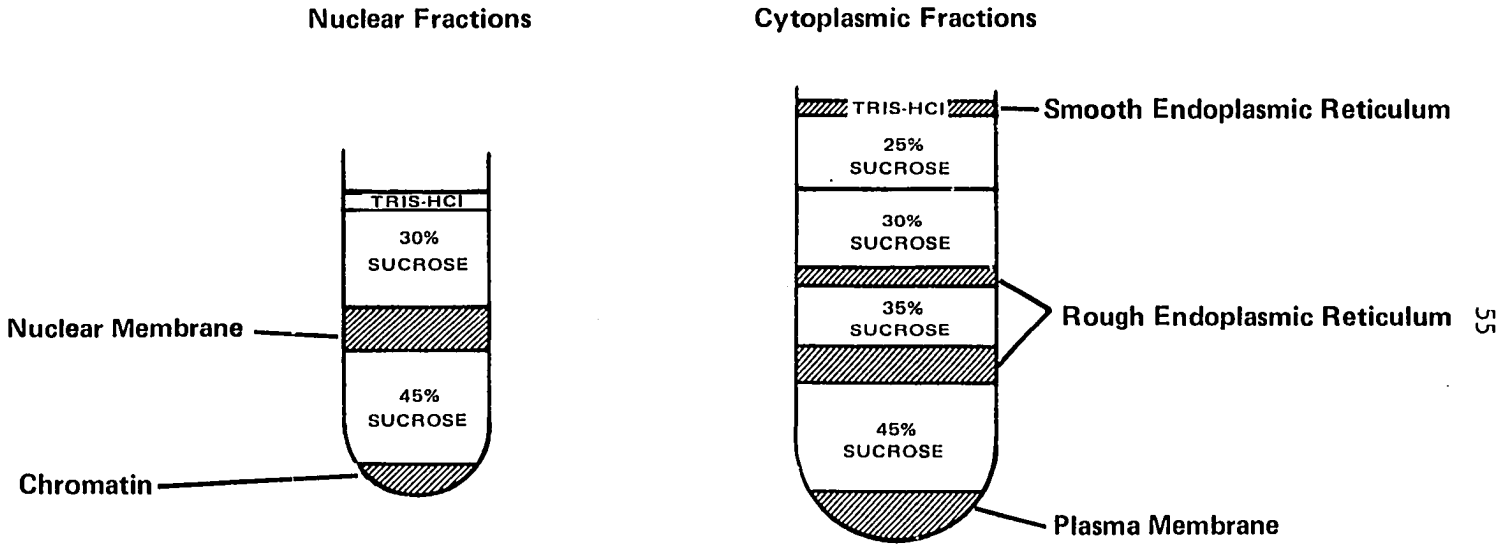


Figure 1--Schematic drawing showing a typical pattern of HeLa membranes separated by discontinuous sucrose gradient ultracentrifugation.

at other times the bottom band seems to have the greater amount. This appears to be an artifact of preparation which is dependent on the efficiency of the Dounce homogenization. However, the minor differences in the gross appearance of these bands did not affect the quantitative results since these two bands were combined for the purpose of reporting total content, in the rough endoplasmic reticulum, of the three components assayed.

The data from whole cell lysates indicates that in HeLa 65, on a per cell basis, the sialic acid content decreases by 30 percent in the hydrocortisone regulated state compared to the control (Table 1). This is in direct contrast to HeLa 71 which shows the hydrocortisone regulated value to be twice that of its corresponding control (Table 2).

The direction of change in both of these clones, exhibited on a whole cell basis, is also reflected in the component membrane fractions. Quantitation of sialic acid in the various membrane fractions of HeLa 65 control and HeLa 65 Hcr is shown in Table 1. It appears that only one fraction increases appreciably in sialic acid content in the Hcr state: the smooth endoplasmic reticulum shows a five-fold increase (0.0003 nmoles/10^6 cells as compared to 0.0017 nmoles/10^6 cells). Chromatin appears to have a slightly enhanced content of sialic acid, but this increase is small compared to that seen in the smooth endoplasmic reticulum. The decrease in sialic acid in the whole cell lysate (0.310 nmoles/10^6 cells as compared to 0.20 nmoles/10^6 cells) is most prominently reflected in the rough endoplasmic reticulum (0.10 nmoles/10^6 cells as compared to 0.070 nmoles/10^6 cells), the mitochondria-lysosome fraction (0.06 nmoles/10^6 cells as compared to 0.05 nmoles/10^6 cells) and in the nuclear

TABLE 1

SIALIC ACID CONTENT OF SUB-CELLULAR FRACTIONS OF HeLa 65 CELLS IN CONTROL AND Hcr STATES

	65 Control		65 Hcr	
	nmoles/10^6 cells	nmoles/mg protein	nmoles/10^6 cells	nmoles/mg protein
Whole cell	0.310	1.5	0.200	0.73
Mitochondria and lysosomes	0.060	3.4	0.050	3.0
Smooth E. R.	0.0003	0.35	0.0017	1.4
Rough E. R.	0.100	14.2	0.070	12.7
Plasma membrane	0.008	2.0	0.006	1.4
Nuclear membrane	0.024	7.7	0.020	5.7
Chromatin	0.050	1.8	0.057	2.6

TABLE 2

SIALIC ACID CONTENT OF SUB-CELLULAR FRACTIONS OF HeLa 71 CELLS IN CONTROL AND Hcr STATES

	71 Control		71 Hcr	
	nmoles/10^6 cells	nmoles/mg protein	nmoles/10^6 cells	nmoles/mg protein
Whole cell	0.210	1.0	0.450	1.6
Mitochondria and lysosomes	0.060	2.3	0.090	2.6
Smooth E. R.	0.005	1.7	0.070	23.3
Rough E. R.	0.080	10.3	0.100	16.0
Plasma membrane	0.004	1.3	0.008	1.6
Nuclear membrane	0.027	6.0	0.045	6.0
Chromatin	0.057	2.2	0.071	2.7

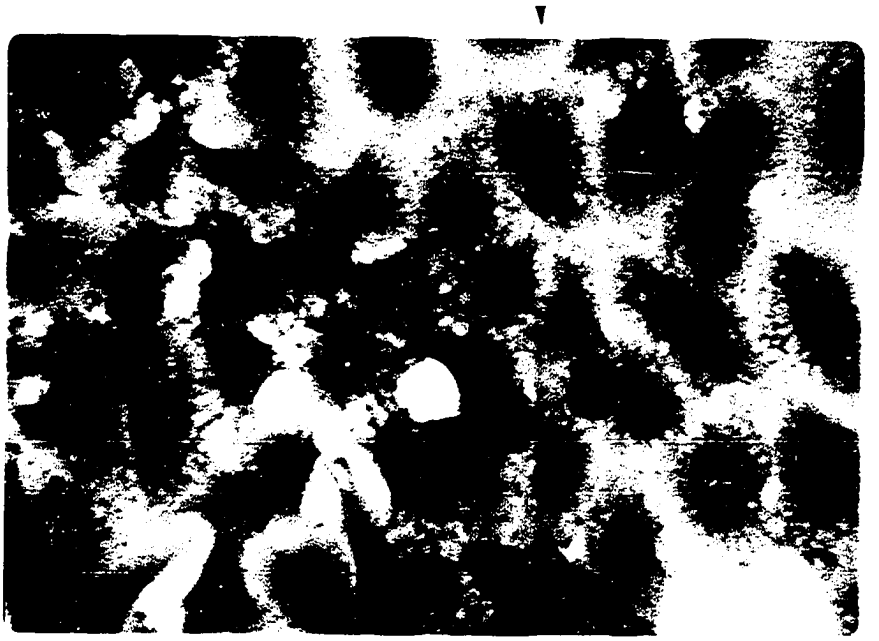
membrane fraction (0.024 nmoles/10^6 cells as compared to 0.020 nmoles/10^6 cells).

A similar compilation of data showing quantitative sialic acid results for fractions from HeLa 71 control and HeLa 71 Hcr is seen in Table 2. The whole cell results are again reflected in the values found for the membrane fractions. In this table all values for the hydrocortisone regulated state are higher than those for the control cells. However, the largest percent increase is again seen in the smooth endoplasmic reticulum, this time the increase is by a factor of fourteen (0.005 nmoles/10^6 cells as compared to 0.070 nmoles/10^6 cells). The mitochondria-lysosome fraction has a 50 percent increase in sialic acids, the plasma membrane and the nuclear membrane fractions are twice as high in this component, and chromatin appears to be increased about 25 percent. These increases however, are not nearly of the magnitude observed in the smooth endoplasmic reticulum.

All sialic acid results for both strains and both states are depicted in two ways; (a) on a per cell basis and (b) on a per mg of protein basis. Though the percentage of change is slightly different on a per mg of protein basis, still the trend is in the same direction and the relative magnitudes are comparable.

Histochemical findings corroborate the biochemical results with regard to sialic acid content of both clones. Figures 2 and 3 are photomicrographs of both clones in both states stained with a modification of Hale's colloidal iron stain (15). This modification was designed by Dr. Walter Joel, Cancer Section, Oklahoma Medical Research Foundation. HeLa 71, in the hydrocortisone regulated state, demonstrates the presence

Figure 2--Photomicrographs of (A.) HeLa 71 Control and (B.) HeLa 71 Hcr cells stained for sialic acid with a modified Hale's colloidal iron stain.



A. HeLa 71 Control

B. HeLa 71 Hcr

Figure 3--Photomicrographs of (A.) HeLa 65 Control and (B.) HeLa 65 Hcr stained for sialic acid with a modified Hale's colloidal iron stain.

A. HeLa 65 Control

B. HeLa 65 Hcr

of more sialic acid than does its corresponding control (compare Figures 2A and 2B), while HeLa 65 shows little qualitative difference from one state to the other (compare Figures 3A and 3B). There does appear to be a concentration of color around the nuclear periphery of both clones which correlates with the greater percentage of sialic acids present in the endoplasmic reticulum, the nuclear membrane and chromatin of both clones in both states.

Since alkaline phosphatase is known to be a membrane component in HeLa cells, the activity of this enzyme was determined for each membrane fraction, and these results for HeLa 65 in both steady-state conditions are shown in Table 3. Results are expressed in both international units per 10^6 cells and specific activities (international units/mg protein of the fraction).

It is interesting to note that the direction of change for this enzyme (units/10^6 cells) is different from that of the sialic acid results. The examination of a whole cell lysate of HeLa 65 shows an increase by a factor of fifteen in the hydrocortisone regulated state over its control. This increase is again reflected in the membrane components of this strain with the single exception of the plasma membrane. The membrane fraction showing the largest increase in this enzyme is the rough endoplasmic reticulum which is increased by a factor of fifteen. The nuclear membrane and chromatin fractions exhibit a small increase compared to the large increase in the rough endoplasmic reticulum. In contrast to the other components, the plasma membrane shows a slight decrease in alkaline phosphatase activity in the hydrocortisone regulated state. Although there are minor differences in the magnitude of change

TABLE 3

ALKALINE PHOSPHATASE ACTIVITY OF SUB-CELLULAR FRACTIONS
OF HeLa 65 CELLS IN CONTROL AND Hcr STATES

	65 Control		65 Hcr	
	International units/10^6 cells	Specific activity	International units/10^6 cells	Specific activity
Whole cell	0.40	1.9	6.00	22.0
Mitochondria and lysosomes	0.05	2.5	1.10	55.0
Smooth E. R.	0.005	5.0	0.006	6.0
Rough E. R.	0.02	6.7	0.34	110.0
Plasma membrane	0.06	15.0	0.05	13.0
Nuclear membrane	0.13	43.0	0.51	130.0
Chromatin	0.18	6.7	0.36	16.0

in this enzyme when viewed on a per mg protein basis, the direction of change is the same.

Table 4 shows the results of these same measurements when applied to HeLa 71 cells in both the control and hydrocortisone regulated states. This clone, which is inducible in the transition state, shows a decrease in alkaline phosphatase activity in the hydrocortisone regulated state. The magnitude of decrease in the whole cell lysate is sixty-six percent. The decrease is mirrored in all of the cell fractions, with considerable decreases in activity found in the smooth endoplasmic reticulum, the rough endoplasmic reticulum, chromatin and the nuclear membrane. The plasma membrane fraction exhibits decreased phosphatase specific activity, but has a small increase in this enzyme on a per cell basis.

The protein values for the cell membrane fractions were essentially the same for both states of HeLa 65; but HeLa 71 showed increases in the rough endoplasmic reticulum, plasma membrane and nuclear membrane fractions in the hydrocortisone regulated state (Table 5). This observation allows a selection between alternative explanations of the magnitude of increases in lipids observed in the previous section. The increase in lipid may be partially due to an increase in all cellular membranes, but is probably predominately due to an increased percentage of lipid per membrane since the increase in protein in the cell membrane fractions of HeLa 71 Hcr is not sufficient to explain the much larger increase in lipid in this cell.

The phospholipid composition studies of both clones in both states utilized whole cell extracts. The identification of the major

TABLE 4

ALKALINE PHOSPHATASE ACTIVITY OF SUB-CELLULAR FRACTIONS
OF HeLa 71 CELLS IN CONTROL AND Hcr STATES

	71 Control		71 Hcr	
	International units/10^6 cells	Specific activity	International units/10^6 cells	Specific activity
Whole cell	200.0	920.0	69.0	260.0
Mitochondria and lysosomes	58.0	2900.0	13.0	430.0
Smooth E. R.	0.55	180.0	0.12	40.0
Rough E. R.	11.0	2800.0	4.40	880.0
Plasma membrane	0.21	70.0	0.30	60.0
Nuclear membrane	15.0	3800.0	8.5	1200.0
Chromatin	35.0	1400.0	11.0	420.0

TABLE 5

PROTEIN CONTENT OF SUB-CELLULAR FRACTIONS OF HeLa 65
AND HeLa 71 CELLS IN CONTROL AND Hcr STATES

	65 Control mg/10^6 cells	65 Hcr mg/10^6 cells	71 Control mg/10^6 cells	71 Hcr mg/10^6 cells
Mitochondria and lysosomes	0.020	0.020	0.020	0.030
Smooth E. R.	0.001	0.001	0.003	0.003
Rough E. R.	0.003	0.003	0.004	0.005
Plasma Membrane	0.004	0.004	0.003	0.005
Nuclear Membrane	0.003	0.004	0.004	0.007
Chromatin	0.027	0.022	0.026	0.026

phospholipids found in these two HeLa cell strains was done by thin layer chromatography on a 250μ layer of Silica Gel HR, with both standards and experimental material on the same plate. A schematic drawing of the results after successive sprayings with rhodamine-6-G, ninhydrin reagent, and modified zinzadze reagent for identification are shown in Figure 4.

Quantitation of the three major phospholipids, phosphatidyl-choline, phosphatidyl-ethanolamine, and phosphatidyl-serine, found on the analytical plate, was done on preparative plates with a 750μ layer of Anacil H gel. A schematic drawing of this type of plate as it appeared after detection in an iodine chamber is shown in Figure 5. The spots were scraped, the phospholipids eluted and phosphate determined as described in Methods.

The data compiled from these quantitative assays is reported in Table 6. This table shows the whole cell content of all three of these phospholipids from both clones in both states, on a per cell basis. All the phospholipid components are increased in the hydrocortisone regulated state of both HeLa cell clones. The magnitude of increase is about the same for phosphatidyl-ethanolamine and phosphatidyl-serine in both clones (two to three fold). Phosphatidyl-choline, however, is appreciably increased only in HeLa 71 Hcr.

Table 7 compares the phospholipid phosphorous from the whole cell extract (from the previous section) and the sum of all three phospholipid fractions eluted from the thin layer plate. Recovery from the plate was better for both clones in the hydrocortisone regulated state, but was best for HeLa 71 Hcr. It is uncertain whether these losses

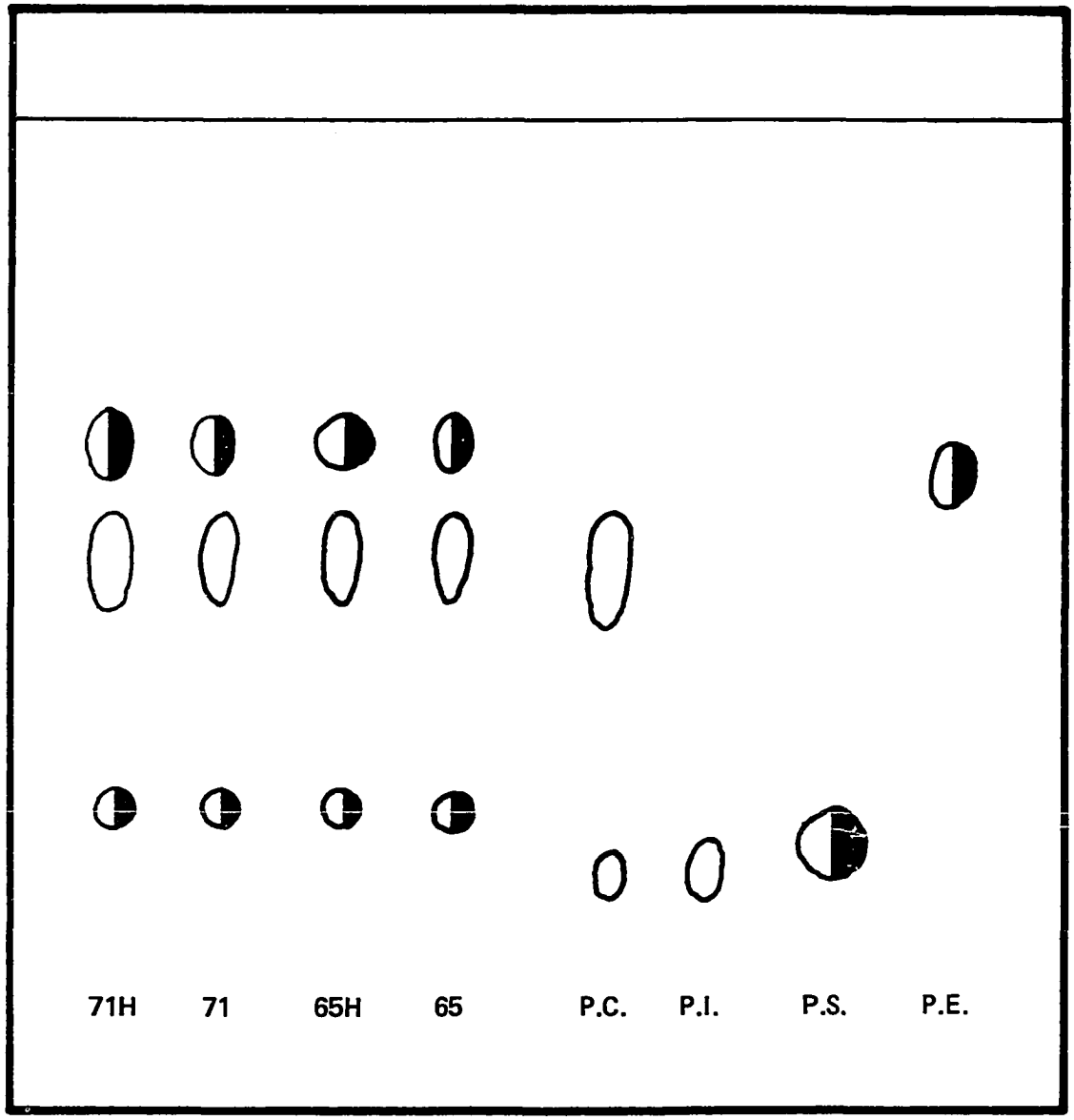


Figure 4--Schematic drawing of an analytical thin layer chromatogram of lipids from whole cell extracts of HeLa 71 and HeLa 65 cells in the control and Hcr states. The plate was sprayed successively with rhodamine-6-G, ninhydrin reagent and modified zinzadze molybdenum reagent. All spots were phosphorous positive, black indicates ninhydrin positive. Abbreviations are phosphatidyl-choline (P.C.), phosphatidyl-inositol (P.I.), phosphatidyl-serine (P.S.) and phosphatidyl-ethanolamine (P.E.).

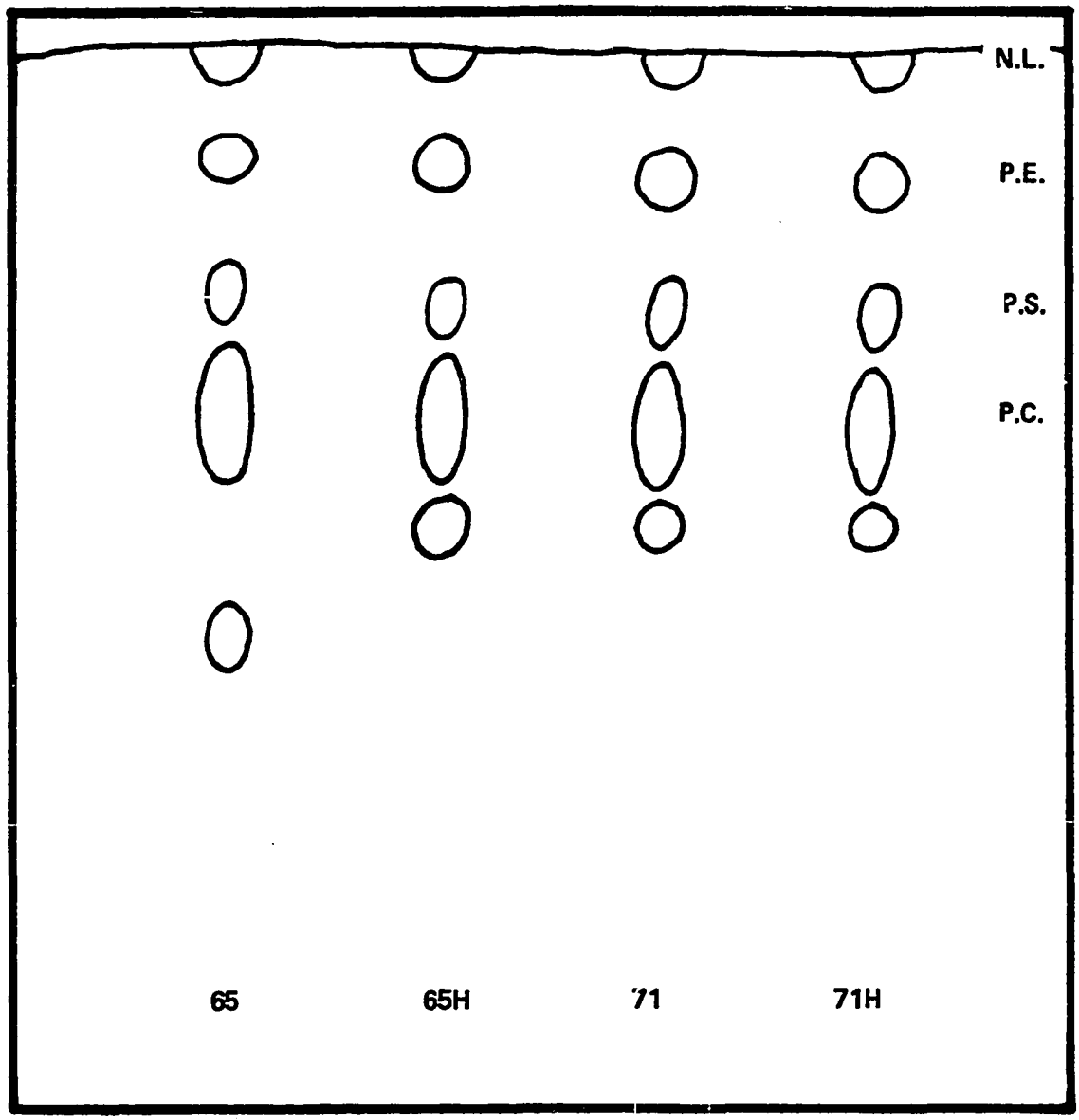


Figure 5--Schematic drawing of a preparative thin layer chromatogram of lipids from whole cell extracts of HeLa 65 and HeLa 71 cells in control and Hcr states. The plate was coated with Anacil H and the spots detected in an iodine chamber. The spots are identified as neutral lipid (N.L.), phosphatidyl-ethanolamine (P.E.), phosphatidyl-serine (P.S.) and phosphatidyl-choline (P.C.) which had two spots with the standard as well as the experimental material. The two spots of phosphatidyl-choline were combined for analysis.

TABLE 6

QUANTITATION OF PHOSPHOLIPIDS FROM HeLa 65 AND HeLa 71 CELLS IN CONTROL AND Hcr STATES

	65 Control nmoles/10^6 cells	65 Hcr nmoles/10^6 cells	71 Control nmoles/10^6 cells	71 Hcr nmoles/10^6 cells
Phosphatidyl-ethanolamine	16.0	32.0	8.6	19.0
Phosphatidyl-serine	5.4	17.0	8.5	27.0
Phosphatidyl-choline	31.0	35.0	31.0	65.0

TABLE 7

A COMPARISON OF RECOVERIES OF PHOSPHOLIPIDS FROM WHOLE CELL EXTRACTS AND PREPARATIVE THIN LAYER PLATES

	HeLa 65		HeLa 71	
	Control	Hcr	Control	Hcr
	μμmoles/cell		μμmoles/cell	
Whole cell	.090	.120	.080	.130
Σ Phospholipid Fractions	.051	.084	.048	.111

represent hydrolysis during chromatography, or if they are due to other factors involved in the biology of these cells, since quantitative recovery of standard phospholipids was obtained in this chromatographic system.

From one run, the neutral lipids for both clones in both states were eluted from the scraped spots and weighed on a microbalance. This determination gave the following amounts on a per cell basis: HeLa 65 control, 0.013 μμmoles; HeLa 65 Hcr, 0.033 μμmoles; HeLa 71 control, 0.048 μμmoles; and HeLa 71 Hcr, 0.109 μμmoles. These values are based on the molecular weight of cholesterol, since it was observed by analytical thin layer chromatography that this compound constitutes the majority of neutral lipids present in these cells.

The phospholipid to neutral lipid ratio (P.L./N.L.) for HeLa 71 in both states is near unity, while HeLa 65, which contains much less neutral lipid, exhibits P.L./N.L. ratios of four in the control state and 2.6 in the Hcr state. This data suggests that the neutral lipid composition of membranes in these two clones is different in their control states. Furthermore, HeLa 65 evidences an increase in neutral lipid which is disproportionate to the increase in phospholipid. However, this comparison must be viewed with caution due to the unequal yields of phospholipid from the thin layer plate compared to the yield for the whole cell. If one corrects for the estimated loss in yields, one obtains P.L./N.L. ratios as follows: HeLa 65 control, 6.5; HeLa 65 Hcr, 3.5; HeLa 71 control, 1.6; HeLa 71 Hcr, 1.1. This represents a 46 percent reduction in this ratio from HeLa 65 to HeLa 65 Hcr, and a 31 percent reduction in the ratio from HeLa 71 to HeLa 71 Hcr.

PART II

CHAPTER IV

DISCUSSION

Though the sialic acid results related here for HeLa 65 differ from those previously reported (5), both in magnitude and direction, the previous paper dealt with HeLa 65 cells in the induced state, i.e., after no more than 72 hours in growth medium containing hydrocortisone. This apparent reversal of response to hydrocortisone by a HeLa cell strain has been noted in our laboratory with regards to alkaline phosphatase activity. Both HeLa 65 and HeLa 71 exhibit increased alkaline phosphatase activity when first subjected to hydrocortisone-containing medium, but after two weeks of growth in the medium, only HeLa 65 retains its elevated enzyme level. The activity of alkaline phosphatase in HeLa 71 Hcr cells decreases to below the control level and remains stable for at least two years.

It has been reported (16) that glycoprotein synthesis is intimately associated with the smooth internal membranes of HeLa cells. This is in complete accord with the present findings for HeLa 65 Hcr in which elevated amounts of sialic acids occur in the smooth endoplasmic reticulum concomitant with a total cellular decrease. It has also been suggested (17) that the Golgi, which sediments with the smooth endoplasmic

reticulum, is in part responsible for the synthesis of glycoproteins and more specifically, the attachment of the more peripheral carbohydrate moiety to the protein backbone of glycoproteins. This fraction shows increased amounts of sialic acid in both clones in the hydrocortisone regulated state. It would seem, then, that both HeLa cell strains respond to hydrocortisone by accumulating sialic acids in the smooth endoplasmic reticulum, but one clone, HeLa 65 Hcr has an overall decrease in sialic acids and may catabolize or secrete these compounds more efficiently than does HeLa 71 Hcr. Another possible explanation for these observations is that trans-sialidation reactions from the smooth endoplasmic reticulum to cell membrane glycoproteins are inhibited in HeLa 65 Hcr. Cell membrane glycoprotein synthesis proceeds from soluble CMP-sialic acid through the smooth endoplasmic reticulum and from this membrane system to other cell membrane fractions. The above observations could be explained by an inhibition of trans-sialidation reactions from the smooth endoplasmic reticulum to developing cell membrane fractions in HeLa 65 Hcr. This might still allow an increase in sialic acids of the smooth endoplasmic reticulum itself. In HeLa 71 Hcr, an acceleration of all trans-sialidation reactions in the cell membranes would account for the increases observed. In this regard it is interesting to note that growth of a Ruber rat liver hepatoma line with hydrocortisone _in vitro_ reduces the level of fructose-6-phosphate:L-glutamine amino transferase (Dr. Albert Chandler, private communication). This enzyme is believed to be rate-limiting in the formation of UDP-N-acetyl-glucosamine which eventually is transferred to the peptide backbone of glycoprotein.

The necessity of dealing effectively with increased amounts of

membrane associated sialic acid could possibly underly the inhibition of movement of HeLa 71 cells through the G_1-S interface of the cell cycle, and this would suggest a membrane associated DNA mediated DNA polymerase initiation site on the nuclear membrane which is sensitive to the amount of sialic acid in its microenvironment. It is interesting to note that chromatin contains sialic acid, and this fraction also evidences an increase in this material in HeLa 71 Hcr.

Another characteristic of cells which is affected to a large extent by the presence or absence of sialic acid is the surface charge, which determines the adhesiveness of a cell. Decreases in the adhesiveness of a cell can occur in two ways; (a) a decrease in attractive forces between surfaces and (b) an increase in repulsive forces. It is this property of adhesiveness which Ambrose (18) declares is related to behavioral changes of cells after carcinogenic transformation. This change in tumor cells is manifested by easier separation from one another and from the substrate when compared to normal cells of the same type, and a higher net negative surface charge in transformed cells as shown by cell electrophoresis. It may be that a general increase in net surface charge occurs during carcinogenesis. Coman (19) suggests on the basis of his studies that the reduced adhesiveness found in cancer cells constitutes a physical basis of malignancy and indicates that it might explain both invasion and metastases.

Ambrose also suggests that the material responsible for the change in surface charge is a sialic-acid containing component, and cites as evidence his determination of increased electrophoretic mobility in C_{13} hamster fibroblasts transformed with polyma virus in which

neuraminidase treatment returns the electrophoretic mobility of these cells to non-transformed, or control cell values.

Sialic acids appear to be an integral part of membranes of both normal and transformed cells. Since the carbohydrate moiety of glycoproteins may be an important cell-surface localized immunological determinant for tissue rejection (cell mediated through lymphocyte associated antibody), corticosteroid therapy may alter the sialic acid composition of the transplanted cells, and so hide them from normal tissue rejection reactions. Conversely the antibody determinant located on the surface of the lymphocyte may be altered by lymphocyte membrane changes and thus affect this complex immunologic interaction.

Alkaline phosphatase activity of both cell lines studied in our laboratories is inducible in the transition state, i.e., after 72 hours of growth with hydrocortisone. This increase in enzyme activity is reflected in the steady-state condition in only one line, HeLa 65, which retains its high level of alkaline phosphatase activity for as long as hydrocortisone remains in the medium. Whether the activity reported for the mitochondria-lysosome fraction is due to the presence of this enzyme in the membranes of this fraction or whether it is due to contaminating membranes from the fractionation procedure cannot be determined from these results. In any event, the three fractions richest in total alkaline phosphatase activity are also the richest in sialic acids (rough endoplasmic reticulum, nuclear membrane and chromatin). This correlates with the previous report that HeLa alkaline phosphatase is a sialoprotein (20).

The membrane fractions obtained by this method appear to be

relatively discreet. The nuclear membrane fraction was free of any DNA, and therefore can be considered reasonable pure, although the chromatin fraction may contain some contaminating membrane pieces. For HeLa 65, the cytoplasmic membrane fractions total 8 μg protein/10^6 cells which is similar to the amount found by Bosmann, et al., (1), for this strain in suspension culture (about 9 μg protein/10^6 cells).

The lipid data presented in this portion of the paper may represent one possible explanation for the physiological changes observed in the presence of hydrocortisone in the previous section. Present data indicates that there is more lipid per mg of membrane protein in the Hcr state of both clones, but HeLa 71 contains a proportionately greater amount of neutral lipid in both states than does HeLa 65. This increase in membrane associated lipid, in a system where protein levels remain relatively constant, results in a transport boundary which is more hydrophobic. It was suggested in the previous section that transport properties in these two clones were different, especially in HeLa 71 Hcr. It is possible that the increased hydrophobicity (due to increased lipid in the membranes) coupled with the increase in net negative charge (due to increased sialic acid in the membranes) in the HeLa 71 Hcr cell could account for the differences seen in the measurements of physiological responses that are membrane associated, e.g., water content, ion content, increased glycogen, protein and RNA levels, response to lytic agents, and the secretory activity of the cell. The phospholipid results reported here are for whole cell extracts only, and the sub-cellular localization of these compounds must still be done before any definitive answer regarding their part in membrane constitution can be given.

PART II

CHAPTER V

SUMMARY

The membranes of two HeLa cell clones, HeLa 65 and HeLa 71, were analyzed for several biochemical parameters in two separate steady-state conditions, control and hydrocortisone regulated. These two HeLa cell clones appear to have differently constituted membranes, and each reacts differently to growth with the steroid. The most exaggerated response is seen in the membranes of the HeLa 71 cells in the hydrocortisone regulated state. The sialic acid content of the membranes of this clone, as well as membrane protein, are increased in the Hcr state. Total cell phospholipid and neutral lipid content is also increased. By contrast, HeLa 65 exhibits a mild decrease in membrane sialic acid content, and no increase in membrane protein. This clone shows whole cell increases in neutral lipids and two of the three major phospholipids. Alkaline phosphatase is increased in HeLa 65 but decreased in HeLa 71 in the hydrocortisone regulated state, and is associated with major sialic acid containing membrane fractions.

Histochemistry corroborates the biochemical findings with regard to the sialic acid changes observed in these two HeLa cell clones.

These studies should be extended to other membrane biochemical

constituents in order to clarify the alterations suggested by these results. However, these data strongly suggest a difference in the membrane mosaic of these two HeLa cell clones, and a difference in how these membrane systems respond to the presence of hydrocortisone.

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