SIALIC ACIDS ON THE PLASMA MEMBRANE OF CULTURED HUMAN LYMPHOID CELLS

Chemical Aspects and Biosynthesis

STEVEN A. ROSENBERG and ALBERT B. EINSTEIN, JR.

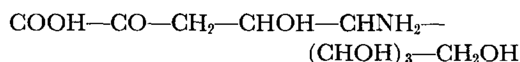
From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT

From 61 to 92% of the total sialic acid of a variety of human lymphoid cell lines maintained in tissue culture is present on the cell surface as measured by its susceptibility to cleavage by *Clostridium perfringens* neuraminidase. These cells contain from 1.22×10^8 to 6.99×10^8 molecules of surface sialic acid per cell. In synchronized cultures synthesis of surface sialic acid occurs only during a limited time in the late G_2 phase of the cell cycle. The amount and density of surface sialic acid vary considerably throughout the cell cycle.

INTRODUCTION

"Sialic acids" refer to a family of compounds derived from an unsubstituted nine-carbon chain called neuraminic acid:



$$COOH—CO—CH_2—CHOH—CHNH_2—(CHOH)_3—CH_2OH$$

They account for a major part of the negative charge on the surface of a variety of mammalian cells and are important in regulating intercellular contacts and the interaction of charged macromolecules with the cell surface (1). Recent studies have also indicated a role for sialic acids in the antigenic expression of tumor cells, trophoblastic tissue, and lymphoid cells (2–4). Evidence has been presented that removal of plasma membrane (cell-surface membrane) sialic acids by neuraminidase can increase the immunogenicity of the surface antigens of these cells (2–4).

Neuraminidase is an enzyme which cleaves terminal sialic acids bound by 2–3′ and 2–6′ glycosidic linkages (5). Because of its large size and its lack of effect on cell viability, this enzyme is thought not to enter viable cells and therefore can be used to remove sialic acids from the plasma membrane without destroying the cell permeability barrier. The enzyme is thus useful in identifying plasma membrane molecules that contain sialic acids and in separating them from intracellular macromolecules which also contain sialic acids.

The present report is a study of the amounts and distribution of sialic acids on the surface of human lymphoid cells maintained in long-term tissue culture. Kinetic and chemical aspects of the removal of sialic acids by neuraminidase were determined. Synchronized cultures were used to study the biosynthesis of sialic acids on the cell plasma membrane. Surface sialic acid synthesis during the cell cycle may serve as a marker for the biosynthesis of other plasma membrane components. Knowledge of the chemistry and biosynthesis of plasma sialic acids forms the basis for studies on the manipulation of these molecules to change the immunogenicity of the cell surface.

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

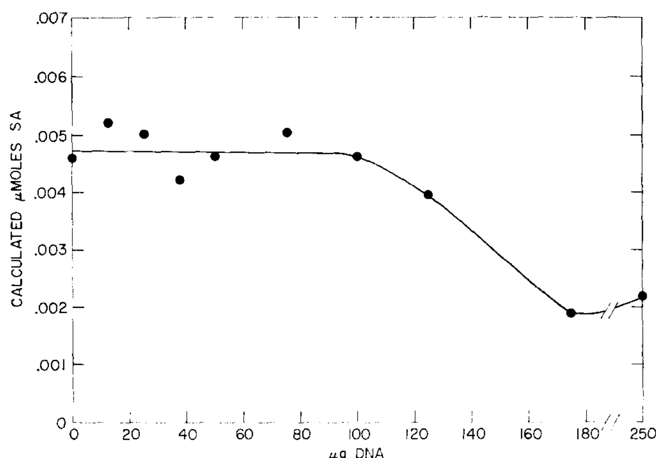


FIGURE 1 The effect of DNA on measurements of sialic acid (SA). Varying amounts of DNA were added to a constant amount of mucin, and the total sialic acid was determined after digestion in 0.1 N H_2SO_4 for 60 min. The correction factor described in the text is adequate to correct for the presence of less than 100 μg DNA, but it results in underestimation of the amount of sialic acid if the amount of DNA present is greater than 100 μg.

MATERIALS AND METHODS

Materials

Chemical reagents obtained from Fisher Scientific Co., Pittsburgh, Pa., were of analytical grade. *N*-acetylneuraminic acid and thymidine were obtained from Sigma Chemical Co., St. Louis, Mo. *Clostridium perfringens* neuraminidase (0.5 units per mg) and mucin were obtained from Worthington Biochemical Corp., Freehold, N. J.

Eagle's minimum essential medium with penicillin and streptomycin was obtained from the Media Unit of the National Institutes of Health. Fetal bovine serum was obtained from Grand Island Biological Co., Grand Island, N. Y. and Microbiological Associates, Inc., Bethesda, Md.

Sialic Acid Assay

The thiobarbituric assay of Warren was employed (6). The three naturally occurring sialic acids, *N*-acetyl-, *N*-glycolyl-, and *N*-diacetylneuraminic acid are all detected by this assay (6). With the use of *N*-acetylneuraminic acid as a standard, the production of the chromophore varied linearly between 0.004 and 0.090 moles. The molecular extinction coefficient we obtained was 56,800, which is similar to 57,000 described by Warren (6).

Release of total sialic acid from both mucin and tissue culture cells was found to be maximum after 60 min treatment with 0.1 N sulfuric acid at 80°C. These conditions were therefore used for all measurements of total cell sialic acid.

The presence of 2-deoxyribose produces color in this assay, and corrections for the presence of this moiety in whole cell digests must be applied. The correction used was (6):

$$\mu\text{moles sialic acid} = 0.09 \times A_{549} - 0.033 \times A_{532}$$

As is shown in Fig. 1, this equation was adequate for correcting for amounts of DNA less than 100 μg but resulted in overcorrection (i.e., underestimation of the amount of sialic acid present) if larger amounts of DNA were present.

In all measurements of the sialic acid of tissue culture cells, the cells were first washed twice with a 50-fold volume of isotonic buffer solution at pH 7.4. This was necessary to remove the sialic acid containing glycoproteins present in the serum of the culture medium. Increased washing of the cells removed no further sialic acid.

Tissue Culture Cells

All of the tissue culture cell lines used in these experiments were derived from human lymphoid tissue and have been maintained in continuous suspension culture utilizing Eagle's minimum essential medium with penicillin and streptomycin, supplemented with 10–20% fetal bovine serum.

IM-1 cells were derived from lymphoid cells in a gingival biopsy of a patient with lymphoblastic lymphoma. IM-9 and IM-10 cells were derived, respectively, from the bone marrow and peripheral blood of different patients with multiple myeloma.

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

Cell lines 8866 and 4265 were derived from the peripheral blood of patients with acute myelogenous leukemia and chronic myelogenous leukemia, respectively. Cell line 8226 was derived from a plasma cell tumor, and the Raji cell line was derived from a Burkitt lymphoma.

Cell Synchrony Procedure

The human lymphoid tissue culture cells were synchronized by the double thymidine block procedure originally described by Puck (7) and Bootsma et al. (8), and elaborated upon by Galavazi et al. (9). This procedure results in cells that are synchronized at the beginning of the S phase of the cell cycle.

The cells were grown in suspension culture, in 1000-ml spinner flasks, utilizing Eagle's minimal essential medium with penicillin, streptomycin, nonessential amino acids, and 10% fetal bovine serum. When the cells were at a concentration of approximately 0.8×10^6 cells/ml and in the log growth phase, thymidine was added to a final concentration of 3 mM and the culture was incubated for 16 hr. The cell suspension was then centrifuged in 400-ml sterile beakers at 250 *g* for 10 min at room temperature. The supernatant was decanted and the cells were washed by resuspension in fresh media and recentrifugation. The cells were resuspended in fresh medium at a concentration of 0.8×10^6 cells per ml in a sterile 1000 ml spinner flask, and the cell suspension was incubated for 12 hr. After this time thymidine was again added to 3 mM final concentration and the cell suspension was incubated for 16 hr. The cells were then centrifuged at 250 *g* for 10 min at room temperature, washed once with fresh media, and resuspended at a concentration of 0.8×10^6 cells per ml in a sterile 1000 ml spinner flask. Though the recovery of cells from this second thymidine block may not in fact be immediate, this time has been used as the zero time in our experiments.

Cell Counts and Cell Volume

Cell counts were performed in a hemocytometer. Cells were photographed on a Neubauer hemocytometer grid in culture medium immediately after removal from the culture flask. Photographs of the cells were projected and the average cell diameter of about 100 cells was measured. Cell volume and surface area were calculated on the assumption that the cell is spherical.

Mitotic Index

A sample of cells was centrifuged and the pellet resuspended in 5% bovine serum albumin in isotonic buffer. The cells were spread on microscope slides, air dried, fixed with glacial acetic acid:ethanol (1:2), and stained with modified Giemsa stain. 200 cells were examined for the presence of mitotic figures. The mitotic index is expressed as the percentage of cells in mitosis.

RESULTS

Removal of Cell-Surface Sialic Acid by Neuraminidase

Neuraminidase from *Clostridium perfringens* was used at final concentrations of 0.01–0.06 units per ml at 37°C in 0.01 M sodium phosphate with 0.85% sodium chloride. 1 unit of neuraminidase is defined as that amount of enzyme causing the release of 1 μmole of sialic acid per min from bovine submaxillary mucin at 37°C. These concentrations of neuraminidase resulted in maximum sialic acid release from lymphoid cells.

The effect of pH on the action of neuraminidase on lymphoid cells is presented in Fig. 2. Maximum release of sialic acid was obtained in the pH range 6.0–6.5. The experiments described in this report were performed at pH 6.4.

Release of sialic acid from lymphoid cells by neuraminidase was time dependent as is shown in Fig. 3. Release of sialic acid was maximum by 90 min, and this time was used to determine the levels of neuraminidase-susceptible sialic acid on the lymphoid cell surface. Under these conditions, cell viability as determined by trypan blue dye exclusion and by subsequent cell growth was not significantly affected.

Most of the total cell sialic acid was susceptible to neuraminidase cleavage (Table I). This varied

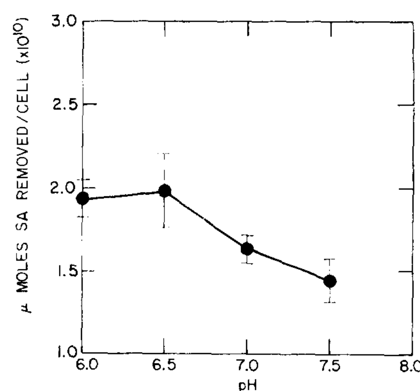


FIGURE 2 The effect of pH on neuraminidase action. pH 6.4 was chosen for the experiments described in this paper.

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

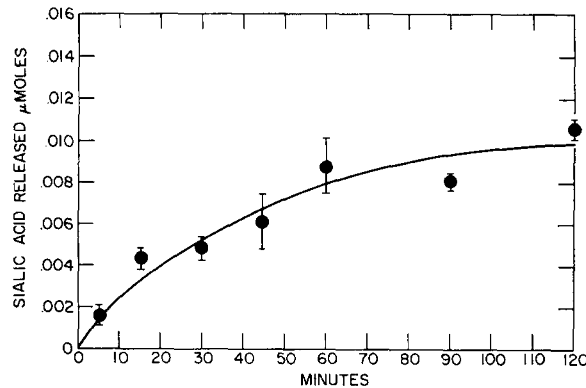


FIGURE 3 Raji lymphoid cells were digested with 0.03 unit/ml neuraminidase, and samples were assayed for the presence of free sialic acid. Release of sialic acid was time dependent and was complete after 60–90 min.

TABLE I

The Total Sialic Acid and the Neuraminidase-Susceptible Sialic Acid Present on Cells from Different Lymphoid Lines

	Total SA*/cell‡	NASE§-susceptible SA / Cell	NASE-susceptible SA / Total SA	Cell-surface area	NASE-susceptible SA / Cell-surface area
	× 10^{10} *μmoles*	× 10^{10} *μmoles*		μ^2	× 10^{12} *μmoles*/μ^2
IM-1	18.40 ± 6.51	11.28 ± 2.75	0.61	455.4	2.48
IM-9	9.48 ± 2.87	8.76 ± 4.51	0.92	418.4	2.09
IM-10	5.71 ± 0.74	4.58 ± 1.50	0.80	280.0	1.64
Raji	2.95 ± 0.84	2.02 ± 0.19	0.68	347.7	0.58
4265	6.16 ± 0.82	5.42 ± 1.00	0.88	357.0	1.52
8226	9.65 ± 4.15	6.66 ± 2.55	0.69	463.0	1.44
8866	6.30 ± 0.85	5.03 ± 0.90	0.80	289.5	1.74

* Sialic acid
‡ Mean ± SE of six experiments
† Neuraminidase

from 61% for IM-1 cells to 92% for IM-9 cells. Because neuraminidase is thought not to enter cells, this neuraminidase-susceptible sialic acid was presumed to be present on the external surface of the cell.

Sialic Acid of Human Lymphoid Cell Lines

The total sialic acid and the neuraminidase-susceptible sialic acid present on cells from different lymphoid lines are given in Table I. These results represent the mean of six separate experiments. The amount of surface sialic acid per cell varied considerably, from 2.02 × 10^{-10} moles for the Raji cell line to 11.28 × 10^{-10} moles for the IM-1 cells. This corresponds to 1.22 × 10^{8} to 6.99 × 10^{8} molecules of surface sialic acid for the respective cells. The surface density of sialic acid, computed by dividing the surface sialic acid by the cell-surface area, is also presented in Table I, and showed considerable differences among the cell types. It should be emphasized that these calculations were performed on the assumption that the cell is spherical. These measurements were all performed in cells in the stationary phase of cell growth.

Variations of Surface Sialic Acid in the Cell Cycle

The Raji cell line was used in studies on the plasma membrane sialic acid of synchronized cultures. By the use of the double thymidine block procedure, synchronous cultures were established

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

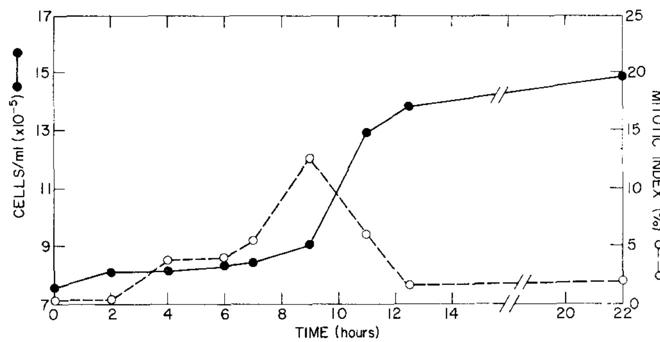


FIGURE 4 Raji lymphoid cells synchronized by the double thymidine block procedure. The mitotic phase begins from 8 to 10 hr after removal of the second thymidine block. The mitotic index reaches a peak of about 14% at 9 hr.

TABLE II

Presence of Cell-Surface Sialic Acid (Neuraminidase-Susceptible) through the Cell Cycle

Time	Mean cell volume	Mean cell-surface area	NASE-susceptible SA / Cell	NASE-susceptible SA / Surface area
hr	μ^3	μ^2	$\times 10^{10}$ *µmoles*	$\times 10^{13}$ *µmoles/µ²*
0	1027	492	1.64	3.33
2	1276	569	1.65	2.90
4	1949	754	1.58	2.09
6	1934	750	2.17	2.89
8	1934	750	2.33	3.10
11	1352	591	1.98	3.34
13	1271	567	1.42	2.52

at the start of the S phase of the cell cycle. The growth of a typical synchronized cell culture is presented in Fig. 4. In all experiments, the cell number remained constant for 7–9 hr after removal of the second thymidine block. During this time the cells progressed through the S and G_2 phases of the cell cycle. Between 8 and 12 hr from the start of the culture, the cells entered mitosis; after this phase of activity, the cell number remained constant for the next 12 hr when the experiment was terminated. The mitotic index rose to a maximum of 14% between 8 and 10 hr after the start of the culture.

The presence of cell-surface sialic acid (neuraminidase-susceptible) through the cell cycle is shown in Table II and Fig. 5 which represents the mean results of six experiments. The amount of cell-surface sialic acid remains constant for the first 4 hr of the culture. The amount of sialic acid per cell then shows a rapid increase, reaching a peak at about 8 hr, immediately before the onset of mitosis. As the cell divides, the amount of sialic acid per cell declines.

Because of changing cell size throughout the cell cycle, the cell-surface density of sialic acid shows a somewhat different pattern. This is shown in Fig. 5 A. The cell-surface area increases during the first 4 hr of the cell cycle and then remains constant until the onset of mitosis. As the cell enlarges, the surface density of sialic acid falls. After the cell has reached maximum size (about 4 hr), the synthesis of surface sialic acid continues and at the onset of mitosis the sialic acid has regained its earlier surface density.

In three experiments in which the total cell sialic acid in the cell cycle was measured, a similar pattern of sialic acid distribution was seen. Total sialic acid per cell also exhibited a constant level for 4 hr, and then increased to a peak at about 8 hr and declined as the cells divided.

DISCUSSION

We have found that from 61 to 92% of the total sialic acid of different human lymphoid cell lines is susceptible to neuraminidase cleavage and, thus, is present on the surface of these cells. This is sub-

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

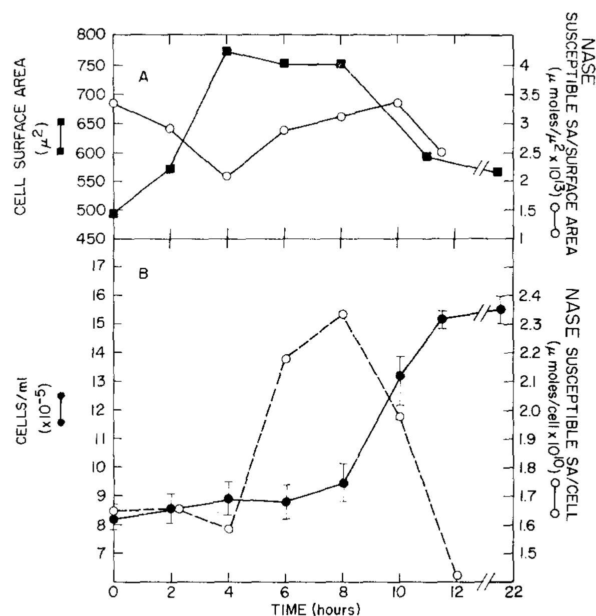


FIGURE 5 (A) Cell-surface area changes during the cell cycle. Because of a rapid increase in cell volume during the first 4 hr of the culture, the surface density of sialic acid falls during this time. The appearance of new sialic acid on the membrane after 4 hr returns the surface sialic density to its previous value before the onset of cell division. *NASE*, neuraminidase; *SA*, sialic acid. (B) The amount of sialic acid per cell in the synchronized Raji lymphoid cultures remains constant for the first 4 hr of the culture. It reaches a peak at about 8 hr and falls as the cell divides.

stantially in agreement with the results of others who used a variety of animal cell tissue culture lines. Studies on C13 and P183 cell lines from the Syrian hamster, L5178Y murine lymphoma cells, HeLa human cervical cancer cells, L mouse fibroblast cells, and the CHO cell line from the Chinese hamster ovary have shown that 59–68% of the total cell sialic acid is removed by treatment with neuraminidase (10). Wallach and Eylar showed that 73% of the total sialic acid of mouse Ehrlich ascites tumors is susceptible to neuraminidase cleavage and can be isolated in a subcellular fraction of the cell presumed to contain plasma membranes (11). Lichtman and Weed reported that 63% of the sialic acid of human leukemic lymphocytes was susceptible to neuraminidase cleavage (12).

In our studies, all of the neuraminidase-susceptible sialic acid is cleaved from the cell after 90 min without affecting cell viability. The growth characteristics of treated and untreated cell populations were identical. It is important to note, however, that an actively metabolizing cell can replenish its surface sialic acid (13). In preliminary experiments with the Raji cell line, we have determined that the regeneration of normal levels of surface sialic acid is complete within 12 hr after treatment with neuraminidase.

In the seven human lymphoid cell lines studied, the amount of surface sialic acid per cell varied over a fivefold range from 1.22×10^8 to 6.99×10^8 molecules per cell. This is somewhat lower than the values, ranging around 8×10^8 molecules per cell, that have generally been found for epitheliod or fibroblastic tissue culture cell lines (10). The difference may be related to the fact that lymphoid cells grow in suspension culture as opposed to most other cell lines which have glass-adherent properties. It is of interest that the L5178Y murine lymphoma cell line, which grows well in suspension culture, has a low surface sialic acid content (2.16×10^8 molecules per cell [10]) and that the Erlich ascites tumor, which in vivo grows free in suspension, also has a low level of surface sialic acid content (2.03×10^8 molecules per cell [11]). Circulating normal human lymphocytes contain only 0.84×10^8 surface sialic acid molecules per cell (12). It thus seems possible that lymphoid cells have a lower surface sialic acid content than other tissue cells, and that this is re-

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

lated to their ability to remain in suspension in the blood, in vivo, and to grow in suspension culture, in vitro.

Because an approaching cell or macromolecule interacts with only a small area of the surface of the cell, the local surface density of sialic acid is probably a more important biologic parameter than the total amount of sialic acid per cell. Information concerning the distribution pattern of sialic acid on the cell surface is not now available. By assuming that the surface sialic acid is distributed equally on the cell membrane and that a valid approximation of the cell-surface area is obtained by measuring the cell diameter and considering the cell spherical, the cell-surface density of sialic acid can be approximated. These data are presented in Table I. Despite adjustment for varying cell sizes, considerable differences exist among the different lymphoid cell lines.

Sialic acids are easily identifiable components of the cell plasma membrane and, as such, can serve as a marker for the biosynthesis or turnover of membrane components during the cell cycle. It is well established that specific biochemical events may occur only at certain phases of the cell cycle (14, 15). For example, human lymphoid cells produce immunoglobulin only during the late G_1 and early S phases of the cell cycle (16).

Mayhew, using synchronized RPMI No. 41 human osteogenic sarcoma cells in tissue culture, has shown that these cells have the highest electrophoretic mobility and, thus, the highest density of negative charge at the cell surface during the late G_2 and mitotic phases (17).[1] This characteristic electrophoretic mobility was eliminated by neuraminidase treatment. On the basis of these findings, Mayhew suggested that cell-surface sialic acid was synthesized during the late G_2 and mitotic phases of the cell cycle. However, in studies on parasynchronous Chinese hamster ovary cells in tissue culture, Kraemer was unable to find any sudden shifts in sialic acid synthesis or surface sialic acid density through the cell cycle (18).

Our measurements on synchronized Raji cells in tissue culture demonstrate that cell membrane sialic acid is being synthesized primarily in the late G_2 phase of the cell cycle (Fig. 4). An alternate interpretation is that the natural rate of destruction of surface sialic acid is decreased. These data provide a molecular basis for Mayhew's hypothesis (17). Further, in our experiments we found no sialic acid synthesis during the S and early G_2 phases of the cell cycle, followed by a rather sudden burst of synthesis in the 2–4 hr before the onset of cell mitosis. Over-all cell-surface charge density drops during the S and early G_2 phases due to increasing cell size, but rises when the synthesis of surface sialic acid begins (Fig. 5).

The demonstration that at least a portion of membrane sialoproteins are synthesized during the late G_2 phase of the cell cycle does not imply that all membrane components are also synthesized at this time. In fact, Gerner et al. have shown that incorporation of radioactive precursors into the membranes of synchronized KB cells occurs just after cell division, in the G_1 phase of the cell cycle (19).

The biological role of sialic acid on lymphoid cells is presently unknown. We are currently using the lymphoid cells, described in this report, to study the effect of neuraminidase treatment on the expression of complement and antibody binding sites and of histocompatibility antigens on the cell surface.

We wish to thank Mr. John Williams for excellent technical assistance, Dr. Donald Buell for his help in performing the synchronized culture experiments, and Dr. John Fahey and Dr. William Terry for many stimulating discussions.

Received for publication 22 November 1971, and in revised form 17 January 1972.

[1] After submission of this manuscript, Shank and Burki, 1971, *J. Cell. Physiol.*, **78**:243, have reported that cells synchronized with the use of thymidine and Colcemid show no electrophoretic differences throughout the cell cycle.

REFERENCES

1. Gottschalk, A. 1960. The Chemistry and Biology of Sialic Acids and Related Substances. Cambridge University Press, London.
2. Currie, G. A., W. van Doorninck, and K. D. Bagshawe. 1968. *Nature (London)*. **219**:191.
3. Bagshawe, K. D., and G. A. Currie. 1968. *Nature (London)*. **218**:1254.
4. Simmons, R. L., A. Rios, G. Lundgren, P. K. Ray, C. F. McKhann and G. R. Haywood. 1971. *Surgery*. **70**:38.
5. Drzeniek, R. 1967. *Biochem. Biophys. Res. Commun.* **26**:631.
6. Warren, L. 1959. *J. Biol. Chem.* **234**:1971.
7. Puck, T. T. 1964. *Science (Washington)*. **144**:565.
8. Bootsma, D., L. Budke, and O. Vos. 1964. *Exp. Cell Res.* **33**:301.
9. Galavazi, G., H. Schenk, and D. Bootsma. 1966. *Exp. Cell Res.* **41**:428.

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023

10. KRAEMER, P. M. 1966. *J. Cell. Physiol.* **67**:23.
11. WALLACH, D. F. H., and E. H. EYLAR. 1961. *Biochim. Biophys. Acta.* **52**:594.
12. LICHTMAN, M. A., and R. I. WEED. 1970. *Blood.* **35**:12.
13. KRAEMER, P. M. 1966. *J. Cell. Physiol.* **68**:85.
14. KLEVECZ, R. R., and F. H. RUDDLE. 1968. *Science (Washington).* **159**:634.
15. MITCHISON, J. M. 1969. *Science (Washington).* **165**:657.
16. BUELL, D. N., and J. L. FAHEY. 1969. *Science (Washington).* **164**:1524.
17. MAYHEW, E. 1966. *J. Gen. Physiol.* **49**:717.
18. KRAEMER, P. M. 1967. *J. Cell Biol.* **33**:197.
19. GERNER, E. W., M. C. GLICK, and L. WARREN. 1970. *J. Cell. Physiol.* **75**:275.

Downloaded from http://rupress.org/jcb/article-pdf/53/2/466/1626026/466.pdf by guest on 26 September 2023