MOLECULES AT THE EXTERNAL NUCLEAR SURFACE

Sialic Acid of Nuclear Membranes and Electrophoretic Mobility of Isolated Nuclei and Nucleoli

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ABSTRACT

The molecules occurring as terminal residues on the external surfaces of nuclei prepared from rat liver by either sucrose-$CaCl_2$ or citric acid methods and nucleoli derived from the sucrose-$CaCl_2$ nuclei were studied chemically and electrokinetically. In 0.0145 M NaCl, 4.5% sorbitol, and 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1 at 25°C, the sucrose-$CaCl_2$ nuclei had an electrophoretic mobility of −1.92 μm/s/V/cm, the citric acid nuclei, −1.63 μm/s/V/cm, and the nucleoli, −2.53 μm/s/V/cm. The citric acid nuclei and the nucleoli contained no measurable sialic acid. The sucrose-$CaCl_2$ nuclei contained 0.7 nmol of sialic acid/mg nuclear protein; this was essentially located in the nuclear envelope. Treatment of these nuclei with 50 μg neuraminidase/mg protein resulted in release of 0.63 nmol of sialic acid/mg nuclear protein; treatment with 1% trypsin caused release of 0.39 nmol of the sialic acid/mg nuclear protein. The pH-mobility curves for the particles indicated the sucrose-$CaCl_2$ nuclei surface had an acid-dissociable group of pK ~ 2.7 while the pK for the nucleoli was considerably lower. Nucleoli treated with 50 μg neuraminidase/mg particle protein had a mobility of −2.53 μm/s/V/cm while sucrose-$CaCl_2$ nuclei similarly treated had a mobility of −1.41 μm/s/V/cm. Hyaluronidase at 50 μg/mg protein had no effect on nucleoli mobility but decreased the sucrose-$CaCl_2$ nuclei mobility to −1.79 μm/s/V/cm. Trypsin at 1% elevated the electrophoretic mobility of the sucrose-$CaCl_2$ nuclei slightly but decreased the mobility of the nucleoli to −2.09 μm/s/V/cm. DNase at 50 μg/mg protein had no effect on the mobility of the isolated sucrose-$CaCl_2$ nuclei but decreased the electrophoretic mobility of the nucleoli to −1.21 μm/s/V/cm. RNase at 50 μg/mg protein also had no effect on the electrophoretic mobility of the sucrose-$CaCl_2$ nuclei but decreased the nucleoli mobility to −2.10 μm/s/V/cm. Concanavalin A at 50 μg/mg protein did not alter the nucleoli electrophoretic mobility but decreased the sucrose-$CaCl_2$ nuclei electrophoretic mobility to −1.64 μm/s/V/cm. The results are interpreted to mean that the sucrose-$CaCl_2$ nuclear external surface contains terminal sialic acid residues in trypsin-sensitive glycoproteins, contains small amounts of hyaluronic acid, is completely devoid of nucleic acids, and binds concanavalin A. The nucleolus surface is interpreted to contain a complex made up of protein, RNA, and primarily DNA, to be devoid of sialic acid and hyaluronic acid, and not to bind concanavalin A.

INTRODUCTION

A great deal of work has been centered of late on the plasma membrane surface oligosaccharides and in particular on terminal monosaccharide residues of the exposed cell surface. utilizing plant lectins as molecular probes for their identification (Kornfeld et al., 1971; Inbar and Sachs, 1969; Burger and Goldberg, 1967; Cline and Livingston, 1971) Furthermore, much earlier work utilizing whole cell particle electrophoresis as the tool for examining the nature of biological surfaces tended to be concerned with oligosaccharides and terminal monosaccharides of mammalian plasma membranes, in particular the plasma membrane of the erythrocyte (Ambrose, 1965; Heard and Seaman, 1960; Seaman and Heard, 1960). In short, comparatively little work has been concerned with the nature of the exposed surfaces of cellular organelles such as mitochondria, lysosomes, and nuclei, and essentially no work has been performed on terminal residues of macromolecules of the less convoluted membranes of the smooth and rough endoplasmic reticulum and the Golgi apparatus. The nature of the terminal residues of the macromolecules of these internal cellular membranes is of extreme importance from the standpoints of their biogenesis, evolution, and role in cellular processes and also because in neoplasia and other instances of uncontrolled growth, changes in the plasma membrane seem to occur (it would be of interest to determine whether similar changes occurred in the internal cellular membranes).

Kaneko et al. (1972) and Nicolson et al. (1972), using specific plant agglutinins, have demonstrated the presence of exposed oligosaccharides on the surface of rat and bovine liver nuclei; the latter group also reported the presence of similar terminally bound oligosaccharides on the surface of bovine liver mitochondria. Bosmann et al. (1972) have shown electrokinetically that isolated rat liver mitochondria have terminal sialic acid residues on their surfaces. Thus the mitochondrial surface, although unique, may be, in terms of constituents, exposed on the external surface, quite similar to the exposed surface of the cell plasma membrane.

Although the two papers on nuclei referred to above (Kaneko et al., 1972; Nicolson et al., 1972) did not deal with sialic acid residues in the exposed surface of the nucleus, several investigators have studied this point. Initially, Lillie (1903) showed that migration of isolated nuclei toward the anode was greater in those containing more "nucleic acid." Lieberman and his colleagues, in a series of papers reporting their work on regenerating liver, indicated the presence of nuclear surface sialic acid (Kishimoto and Lieberman, 1964; Chaudhuri and Lieberman, 1964; Lieberman and Short, 1965; Tsukada and Lieberman, 1965). Vassar et al. (1967) concluded that in a series of rat liver nuclear preparations no sialic acid was demonstrable on the nuclear surface, while Nordling and Mayhew (1966) indicated the presence of sialic acid on the surface of avian erythrocyte nuclei. Others reporting the presence of sialic acid in nuclear preparations include Marcus et al. (1965), Molnar (1967), and Wu et al. (1969). In addition, Keshgegian and Glick (1973) described differences in nuclear glycopeptides from normal and virus-transformed cells and reported the presence of sialic acid in their nuclear preparations.

The present report describes a detailed study of the electrophoretic mobilities and electrokinetic properties of isolated rat liver nuclei; the results indicate that depending on the method of nuclear isolation used, surface sialic acid can be demonstrated in these liver nuclei. Because nucleoli can be isolated as well-defined particles, their electrokinetic properties were also studied.

Nucleolar structure has been studied by Robbins and co-workers (Robbins and Borum, 1967; Robbins et al., 1972) and, in great detail by Busch and Smetana (1970), although little work has been done on properties of either nucleolar or nuclear surfaces. The rationale of the present work was to perform experiments on the electrokinetic properties of these surfaces and to determine the role of glycoconjugates, and in particular sialoprotein, in the structure of these surfaces.

MATERIALS AND METHODS

Solutions

All solutions were prepared in water which was distilled, passed through a deionizer, and then redistilled in an all-glass still. The solution utilized in most of the studies was termed either saline-sorbitol of low ionic strength or saline-sorbitol and contained 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1, and 4.5% sorbitol. This solution and iso-osmotic solutions of NaOH and HCl for adjusting solution

pH were prepared as described by Heard and Seaman (1960).

Materials

Bovine testicular hyaluronidase (EC 3.2.1.35), bovine pancreas DNase (EC 3.1.4.5), beef pancreas RNase (EC 2.7.7.16), neuraminidase (EC 3.2.1.18) (purified and proteolytic activity-free, with an activity of 0.7 units/mg where 1 U of activity equals 1 μmol of *N*-acetylneuraminic acid released per minute at 37°C at pH 5), and purified trypsin (EC 3.4.4.1) were purchased from Worthington Biochemical Corporation, Freehold, N.J. Concanavalin A and albumin (crystalline from bovine serum) were purchased from Calbiochem, San Diego, Calif. Density gradient grade sucrose was purchased from Mann Research Labs. Inc., New York. Other biochemicals were purchased fron Sigma Chemical Co., St. Louis, Mo.

Preparation of Nuclei

Several procedures for preparing rat liver nuclei were utilized and in general each procedure gave a preparation that varied in some parameter from the others. Two procedures were used for the experiments described herein, and one procedure was used to provide most of the data described. This procedure is referred to as the sucrose-$CaCl_2$ procedure, is the procedure used for preparation of the nuclei used in a generic sense in this paper and is the preparation from which all subnuclear fractions (i.e., nucleoli and nuclear "envelopes") were prepared.

SUCROSE-$CaCl_2$ NUCLEI: Since great differences (see below) in the surface properties of nuclei occur depending on the mode of preparation, the procedure for preparing sucrose-$CaCl_2$ nuclei is described in detail. The procedure is essentially that devised without use of lead by Dounce and coworkers (Magliozzi et al., 1971), the stabilization of the nuclei by calcium ions being the result of the work of Schneider and Petermann (Schneider and Petermann, 1950; Petermann and Schneider, 1951; Mizen and Petermann, 1952) and the ultracentrifugation flotation step being the result of the work of Chauveau et al. (1956). Sprague-Dawley male rats (180–200 g) were starved with water ad libitum before decapitation. Blood was drained from the animals, livers were removed, connective tissue was dissected away, and the livers were weighted. All subsequent steps were carried out at 2°–4°C. For every g of liver, 40 ml of 0.44 M sucrose-3.3 mM $CaCl_2$ were added (this is 1 vol in description below) and the livers were diced with scissors. The suspension was then homogenized for six strokes in a loose Dounce homogenizer. The homogenate was then filtered through coarse cheesecloth (no. 60, four layers) and through two layers of fine mesh (no. 120) cheesecloth. The filtrate was homogenized vigorously for 15 strokes in a tight Dounce homogenizer taking precautions to avoid air bubbles in the homogenate. An equal volume of the 0.44 M sucrose-3.3 mM $CaCl_2$ preparation was added and the suspension was centrifuged at 700 × *g* for 30 min. The supernatant fluid was discarded and the pellet was resuspended in the original 1 vol of 0.44 M sucrose-3.3 mM $CaCl_2$ which was then diluted fourfold with the same solution. This suspension was centrifuged at 500 × *g* for 20 min and the resultant pellet drained for 1 min. The pellet was then resuspended in 3 vol of 2.2 M sucrose-3.3 mM $CaCl_2$ and centrifuged at 78,410 × *g* for 10 min and the resulting pellet was considered the "sucrose-$CaCl_2$ nuclei." If the pellet was creamy white it was considered acceptable; if it was ringed with a brown outline, the above step was repeated with 6 vol of the above solution; this usually resulted in a clean, creamy white pellet, but if it didn't the preparation was rejected. This nuclear pellet was washed once with cold glass-distilled water (resuspended and resedimented) and then used as described below.

CITRIC ACID NUCLEI: Citric acid (pH 3.8) nuclei, which were used in some experiments, were prepared by the method of Dounce (1955) from rats similar to those described above.

Nuclear Envelopes

Nuclear envelopes or outer membranes were prepared from the sucrose-$CaCl_2$ nuclei by the method of Agutter (1972).

Nucleoli

Nucleoli were prepared from the sucrose-$CaCl_2$ nuclei by the sonication method of Busch and Smetana (1970).

Morphology

Purity of the above preparations was monitored by light microscopy of preparations in 0.06% azure C in 0.25 M sucrose. All nuclear and nucleolar preparations were monitored for the plasma membrane marker enzyme 5′-nucleotidase (Bosmann and Pike, 1971) which proved undetectable.

Electrophoretic Mobilities

Measurements were made at 25° ± 0.1°C in a horizontal cylindrical chamber of small volume (10 ml) equipped with reversible, blacked platinum electrodes (Bosmann, 1972 *a* and *b*; Bosmann and Carlson, 1972; Bosmann et al., 1972). The chamber was viewed by transillumination in the apparatus obtained from Rank Brothers, Bottisham, England.

The mobilities of the particles were calculated in μm/s/V/cm; each value was obtained by timing the movement of at least 20 particles with reversal of polarity after each measurement. The alignment of the apparatus was checked by the method of Heard and Seaman (1960). Determinations of the mobility of washed human erythrocytes were made in 0.0145 M NaCl and 4.5% sorbitol made at 0.6 mM with respect to $NaHCO_3$. Normal blood for this purpose was obtained from healthy donors of the phenotype A Rh^+, taken into EDTA, and immediately washed; the electrophoretic mobilities were determined. Heard and Seaman (1960) reported a value for the electrophoretic mobility of human erythrocytes of -2.78 ± 0.08 μm/s/V/cm while in the present experiments a value of -2.80 ± 0.06 μm/s/V/cm was found in saline-sorbitol. A minimum of three independent experiments were performed for each electrophoretic mobility determination, and all values are the means of at least 80 readings $\pm$ SD. In all cases homogeneous populations were encountered for the particles studied herein; no bimodal or trimodal distributions were found and only normal distributions were encountered.

Protein Determination

Protein was determined by the method of Lowry et al. (1951) utilizing bovine serum albumin as standard.

Enzyme Treatment of Nuclei

The procedure used was to treat the nuclei or nucleoli with neuraminidase, DNase, RNase, hyaluronidase, or trypsin and to measure the electrophoretic mobility of a portion of the treated nuclei or nucleoli. Nuclei or nucleoli corresponding to 0.1–1 mg (as protein) were treated with 1 ml of various concentrations of enzymes dissolved in physiological saline. The pH was adjusted to 6.5–7.0 and the tubes were incubated in a gently rocking water bath at 37°C for 30 min. The treated nuclei or nucleoli were centrifuged at 5,000 $\times$ g for 10 min, washed three times with physiological saline, centrifuged for 10 min at 2,500 $\times$ g, and finally washed (resuspended and resedimented) in saline-sorbitol of low ionic strength. The washed nuclei or nucleoli were resuspended in 1 ml of saline-sorbitol, and four drops of this solution were added to 30 ml of saline-sorbitol to produce a dilute mixture for observation of electrophoretic mobility. The particles were timed successively in both directions at 60 V for 2 grids (166 μm). Between runs the chamber was washed with dichromate, distilled water, and saline-sorbitol. The pH of the solution was kept at 7.2 $\pm$ 0.1.

Treatment with Trypsinized Concanavalin A

Monovalent conconavalin A was utilized so that binding but not agglutination would occur (Burger and Noonan, 1970); trypsination was used to prepare the monovalent concanavalin A. Concanavalin A was trypsinized by a modified method of Burger and Noonan (1970). 25 mg of concanavalin A were dissolved in 2.4 ml of 0.2 M phosphate, pH 7.0. 0.1 ml of 2.5% trypsin was added, and the solution was incubated at 37°C for 5 h. The trypsinization was stopped by adding 2.5 ml of 1% trypsin inhibitor. 0.5 ml of nuclei or nucleoli was incubated with 0.1 ml of trypsinized concanavalin A solution or control solution plus 0.4 ml of physiological saline for 30 min. The particles were centrifuged at 7,000 $\times$ g for 10 min and washed twice with physiological saline. Suspensions for the observation of electrophoretic mobility were made and measurements were taken as outlined above.

Sialic Acid

Released or free sialic acid was measured by the Warren (1959) procedure; total sialic acid was determined by the Svennerholm (1958) procedure.

Anthrone-Positive Material

Anthrone-positive material was analyzed as described (Bosmann and Kessel, 1970).

DNA

DNA was determined by the method of Burton (1956).

RNA

RNA was determined by the method of Schneider (1957).

Calculation of Parameters

Conversions of electrophoretic mobilities to other electrokinetic parameters were made using the Helmholtz-Smoluchowski equation and the generalized Gouy equation for a uni-divalent ionic system, as described by Abramson (1934) and Heard and Seaman (1960). The following diameters as approximations derived from electron micrographs were used in these calculations: rat liver nuclei, $d = 7 \times 10^{-6}$m; rat liver nucleoli, $d = 12 \times 10^{-6}$m. The equation for surface area of a sphere was used to calculate the following surface areas, although the particles in fact may not be spherical and invaginations, shrinkage, swelling, or folding of the surfaces (depending upon the suspending media) might cause the area calculation to be at best an approximation: rat liver nuclei, $SA = 154 \times 10^{-12}m^2$; rat liver nucleoli, $SA = 4.5 \times 10^{-12}m^2$.

Carbohydrate Determinations

Neutral sugars were isolated and analyzed by gas-liquid chromatography of alditol acetate derivatives

as described previously (Martin and Bosmann, 1971). Hexosamine concentration was determined as described previously (Bosmann and Jackson, 1968).

RESULTS

Electrophoretic Mobilities of Isolated Nuclei and Nucleoli

The data presented in Table I indicate that in 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2, and 4.5% sorbitol, the rat liver nuclei prepared by two different procedures and the nucleoli had vastly different electrophoretic mobilities. The nuclei prepared by the sucrose-$CaCl_2$ procedure had an electrophoretic mobility of -1.92 μm/s/V/cm while those prepared by the citric acid method had a mobility of -1.63 μm/s/V/cm, indicating fundamental differences in these two surfaces. The nucleoli prepared from the sucrose-$CaCl_2$ nuclei of rat liver had a very high electrophoretic mobility in the defined solution of -2.53 μm/s/V/cm, indicating a surface greatly different from that of the parent nuclei. The number of electrons per particle surface was 6.9 million for the sucrose-$CaCl_2$ nuclei, 5.8 million for the citric acid nuclei, and 0.26 million for the nucleoli (Table I). The data given in Fig. 1 for the pH vs. electrophoretic mobility relationships of the nuclei and nucleoli indicate that the surface of nuclei isolated in sucrose $CaCl_2$ exhibits a pK of about 2.7 while the pK of the nucleoli prepared from these nuclei is much lower. The electrophoretic mobility of the nuclei is much lower than that of the nucleoli at all pH values studied except pH 2 and below (Fig. 1). The pK of the rat liver nuclear surfaces prepared by the sucrose-$CaCl_2$ method is consistent with the presence on these surfaces of terminal sialic acid residues with a carboxyl group with pK_a of approximately pH 2.7.

The data of Fig. 2 give the electrophoretic mobility-ionic strength relationships for the nuclei prepared by two different procedures and for the nucleoli prepared from the sucrose-$CaCl_2$ nuclei. At all ionic strengths at which observations were made the three particles behaved with respect to electrophoretic mobility as unrelated particles; at ionic strengths above 0.145, the three particles had rather similar electrophoretic mobilities of about -1 μm/s/V/cm.

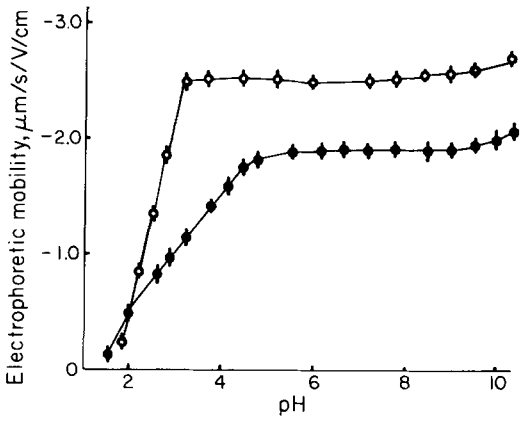

FIGURE 1 Electrophoretic mobility of isolated rat liver nuclei or nucleoli as a function of pH. Experiments were performed as given in Materials and Methods and points are means ± 1 SD. The solution for measurement was 0.0145 M NaCl and 4.5% sorbitol at 25°C; pH adjustments were made with iso-osmotic NaOH or HCl. ○, nucleoli; ●, nuclei.

TABLE I

Electrophoretic Mobility of Rat Liver Nuclei and Nucleoli and Other Electrokinetic Parameters

Particle	Mobility	Zeta potential	Surface charge	No. of electrons
	μm/s/V/cm	*mV*	*esu/cm²*	*millions/ particle surface*
Nuclei (sucrose-$CaCl_2$)	-1.92 ± 0.01	25.1	2.14×10^3	6.9
Nuclei (citric acid)	-1.63 ± 0.01	21.4	1.81×10^3	5.8
Nucleoli (sucrose-$CaCl_2$)	-2.53 ± 0.03	33.0	2.81×10^3	0.26

Data for electrophoretic mobilites are means ± 1 SD. The total number of observations was in each instance greater than 800; that is, mobilities of over 800 particles were measured independently. The data are for saline-sorbitol of ionic strength 0.0145 at 25°C at 0.6 mM $NaHCO_3$ and 4.5% sorbitol, pH 7.2 ± 0.1. Experiments were performed as given in the text. Calculations assumed particle conductance was negligible.

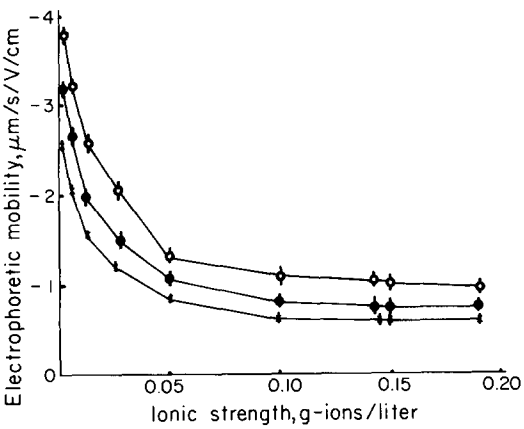

FIGURE 2 Electrophoretic mobility of isolated rat liver nuclei or nucleoli as a function of ionic strength. Experiments were performed as given in Materials and Methods, and points are means ± 1 SD. The solution for measurement was 4.5% sorbitol and 0.6 mM $NaHCO_3$, with pH 7.2 ± 0.1 at 25°C; the 0.6 mM $NaHCO_3$ was omitted at low ionic strengths. Ionic strength was varied by changing concentrations of NaCl. ×, nuclei (citric acid); ○, nucleoli (sucrose-$CaCl_2$); ●, nuclei (sucrose-$CaCl_2$).

Electrophoretic Mobilities of Isolated Rat Liver Nuclei and Nucleoli Treated with Neuraminidase

Neuraminidase treatment of either nuclei prepared by the citric acid method or the nucleoli did not affect the electrophoretic mobilities of these particles at any of the concentrations studied, as shown in Fig 3. However, neuraminidase treatment of the rat liver nuclei prepared by the sucrose-$CaCl_2$ method resulted in a dramatic decrease in the electrophoretic mobility of these particles. At 50 μg of neuramindase/mg nuclear-protein the electrophoretic mobility was reduced from a control value of −1.92 μm/s/V/cm to −1.41 μm/s/V/cm while boiled neuraminidase at 50 μg/mg nuclear protein resulted in a mobility of −1.93 μm/s/V/cm (Fig. 3). The decreases in electrophoretic mobility of the sucrose-$CaCl_2$ nuclei followed a dose-response relationship upon treatment with the neuraminidase; even at 1 μg of neuraminidase/mg nuclear protein the electrophoretic mobility was decreased to −1.81 μm/s/V/cm. The numbers in Table II further substantiate these data and show that no *N*-acetylneuraminic acid was detectable in the "citric acid" nuclei or the sucrose-$CaCl_2$ nucleoli. However, as shown in Table II, 0.7 nmol of sialic acid/mg nuclear protein in the sucrose-$CaCl_2$ nuclei was found. This sialic acid was preferentially located in the nuclear envelope, as shown by the 13-fold purification on a milligram protein basis of the amount of sialic acid present in the various fractions (nuclear membranes, 9 nmol of sialic acid/mg protein). Furthermore almost all of the sialic acid of the sucrose-$CaCl_2$ nuclei was susceptible to release by neuraminidase (of the 0.7 nmol/mg protein present in the nuclei, the 50 μg of neuraminidase released 0.63 nmol/mg protein; Table II), indicating that it was most likely located on the external nuclear envelope surface and occurred there as terminal residues susceptible to the endoglycosidase neuraminidase. The data of Fig. 4 indicate that the release of sialic acid from the sucrose $CaCl_2$ rat liver nuclei by neuraminidase was related to the amount of neuraminidase incubated with the nuclei between 1 and 50 μg of neuraminidase; at 50 μg of neuraminidase essentially all of the nuclear sialic acid was released. Higher levels of neuraminidase did not substantially lower the electrophoretic mobility of the rat liver nuclei (sucrose $CaCl_2$) below that of −1.41 μm/s/V/cm or cause additional release of sialic acid above 0.65 nmol/mg nuclear protein.

Electrophoretic Mobilities of Isolated Rat Liver Nuclei or Nucleoli Treated with Hyaluronidase

The data given in Fig. 5 indicate that hyaluronidase treatment has no effect on the electrophoretic mobilities of isolated nucleoli but did slightly but significantly decrease the electrophoretic mobilities of the isolated sucrose-$CaCl_2$ nuclei. The electrophoretic mobility of the nuclei treated with 50 μg of hyaluronidase/mg of nuclear protein was −1.79 μm/s/V/cm compared to a control value of −1.92 μm/s/V/cm. These data indicate that hyaluronic acid may be a minor constituent of the external nuclear envelope.

Electrophoretic Mobilities of Isolated Rat Liver Nuclei or Nucleoli Treated with Trypsin

The electrophoretic mobility of nuclei treated with trypsin actually increases after treatment (Fig. 6) in spite of the fact that the trypsin treatment caused release of considerable amounts of sialic acid from the nuclear surface (Table II; 1% trypsin treatment released 0.39 nmol of sialic acid/mg nuclear protein of the 0.7 nmol total

present in the nuclei). This indicates that much of the external sialic acid of the nuclear membrane is located in trypsin-susceptible linkages and probably occurs as glycoprotein constituents as opposed to glycolipid constituents of the membrane. The reason the electrophoretic mobility of the nuclei actually increases slightly after trypsin treatment despite liberation of the carboxyl group of sialic acid is probably that the action of trypsin on the surface glycoproteins generates terminal amino acid carboxyl groups in the cleaved glycoproteins. Thus an amino acid carboxyl group is substituted on the surface for the sialic acid carboxyl group, the net result being a similar or slightly elevated electrophoretic mobility; a similar explanation has been used by Seaman and Uhlenbruck (1963) to explain analogous behavior of certain erythrocytes.

The nucleoli decrease significantly in electrophoretic mobility when treated with trypsin (Fig. 6). Treatment with 1% trypsin decreased the mobilities from −2.53 to −2.09 μm/s/V/cm. This decrease probably means that the nucleic acid primarily responsible for the electrophoretic

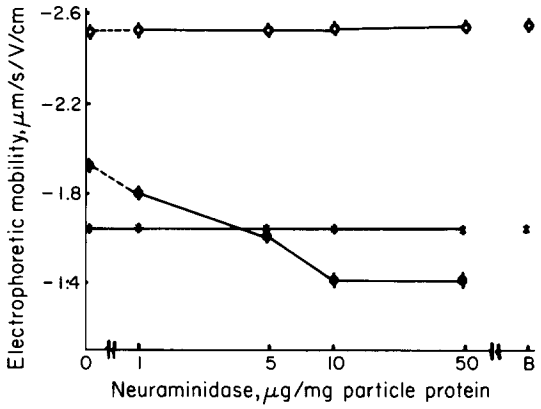

FIGURE 3 Electrophoretic mobilities of neuraminidase-treated rat liver nuclei or nucleoli. Data are the mean value ± 1 SD. *B* refers to neuraminidase (50 μg/mg particle protein) which was boiled for a 5-min period prior to use in the experiment. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1, and 4.5% sorbitol at 25°C. ×, nuclei (citric acid); ○, nucleoli (sucrose-$CaCl_2$); ●, nuclei (sucrose-$CaCl_2$).

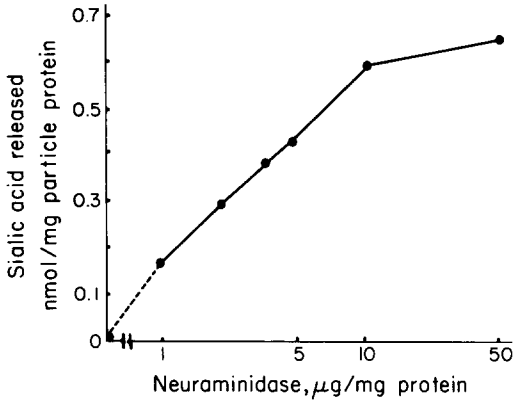

FIGURE 4 Amount of sialic acid released from sucrose-$CaCl_2$ nuclei as a function of amount of neuraminidase present in the incubation. Incubations were carried out as given in Materials and Methods and free sialic acid released into the supernate was measured. Data are means ± 1 SD.

TABLE II

Sialic Acid Content of Rat Liver Nuclei and Subnuclear Fractions

Particle or fraction	Total	Sialic acid released upon treatment with: Neuraminidase: Boiled (50 μg/mg)	Neuraminidase: 10 μg/mg	Neuraminidase: 50 μg/mg	Trypsin: Boiled (1%)	Trypsin: 0.01%	Trypsin: 1%
	nmol/mg particle protein	*nmol/mg particle protein*					
Nuclei (citric acid)	0*	0	0	0	—‡	—	—
Nuclei (sucrose-$CaCl_2$)	0.7	0	0.58	0.63	0	0.17	0.39
Nucleoli (sucrose-$CaCl_2$)	0	0	0	0	—	—	—
Nuclear membranes	9	—	—	—	—	—	—

Experiments were performed as given in Materials and Methods.

* 0 denotes not detectable.

‡ — denotes an experiment not performed.

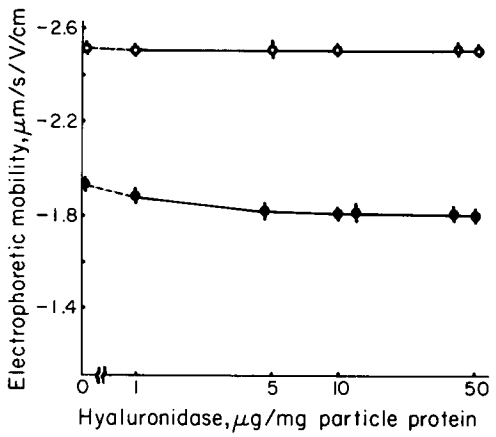

FIGURE 5 Electrophoretic mobilities of hyaluronidase-treated rat liver nuclei or nucleoli. Data are mean values ± 1 SD. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1, and 4.5% sorbitol at 25°C. ●, nuclei; ○, nucleoli.

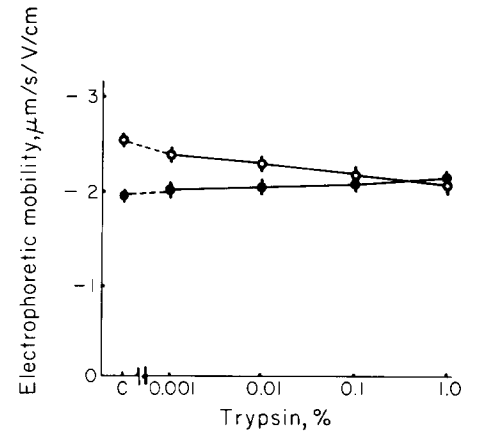

FIGURE 6 Electrophoretic mobilities of trypsin-treated rat liver nuclei or nucleoli. *C* refers to control or non-treated nuclei or nucleoli. Experiments were performed as given in Materials and Methods, and the data are means ± 1 SD. The solution for measurement was 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1 at 25°C, and 4.5% sorbitol. ○, nucleoli; ●, nuclei.

mobility of the nucleoli (see below) is held somewhat in trypsin-susceptible linkages; i.e., the "surface" of the nucleolus may be composed of nucleic acid-protein macromolecules. The data also clearly demonstrate further fundamental differences between the rat liver and nucleoli.

Electrophoretic Mobilities of Isolated Rat Liver Nuclei or Nucleoli Treated with DNase

The data in Fig. 7 are of extreme interest because they show that (*a*) the nuclei had no change in electrophoretic mobility upon treatment with DNase and (*b*) the nucleoli had a most dramatic decrease upon DNase treatment. The electrophoretic mobility of nucleoli treated with 50 μg DNase/mg nucleoli protein was −1.21 μm/s/V/cm compared to a control value of −2.53 μm/s/V/cm; at this level of DNase the nucleoli began to lose their structural integrity. These results clearly indicate the presence on the external surface of the nucleoli of DNA and the complete lack of DNA on the nuclear surface in rat liver. Analysis of the nuclear envelopes indicated that they contained 6.7% (by weight) DNA; this DNA must be located within the membrane or on the internal surface of the nuclei but not on the nuclear external surface.

Electrophoretic Mobilities of Isolated Rat Liver Nuclei or Nucleoli Treated with RNase

Fig. 8 shows results which demonstrate that RNase had no effect on nuclear electrophoretic mobility and at high levels decreased the electrophoretic mobility of nucleoli substantially but not to the same degree as DNase. At 50 μg of

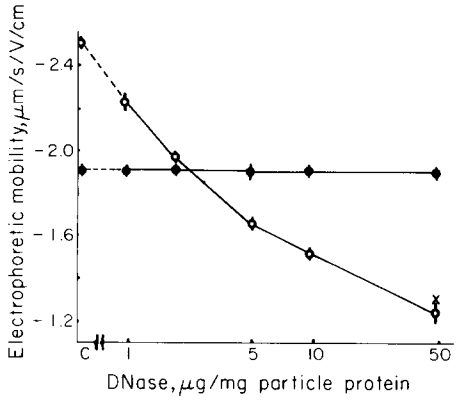

FIGURE 7 Electrophoretic mobilities of DNase-treated rat liver nuclei or nucleoli. *C* refers to control or non-treated nuclei or nucleoli. *X* indicates that at this level and higher levels of DNase lysis or breakdown of the nucleoli occurred. Experiments were performed as given in Materials and Methods, and the data are means ± 1 SD. The solution for measurement was 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1 at 25°C, and 4.5% sorbitol. ○, nucleoli; ●, nuclei.

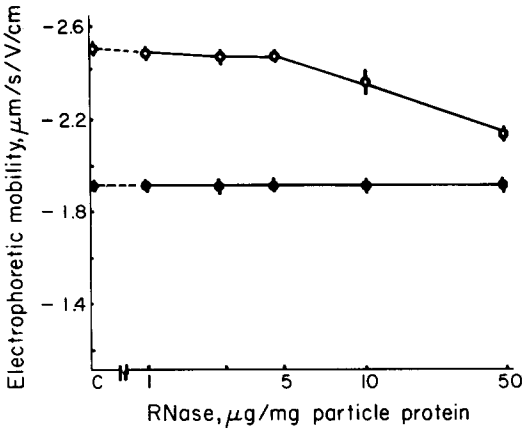

FIGURE 8 Electrophoretic mobilities of RNase-treated rat liver nuclei or nucleoli. *C* refers to control or non-treated nuclei or nucleoli. Experiments were performed as given in Materials and Methods, and the data are means ± 1 SD. The solution for measurement was 0.0145 M NaCl, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1 at 25°C, and 4.5% sorbitol. ○, nucleoli; ●, nuclei.

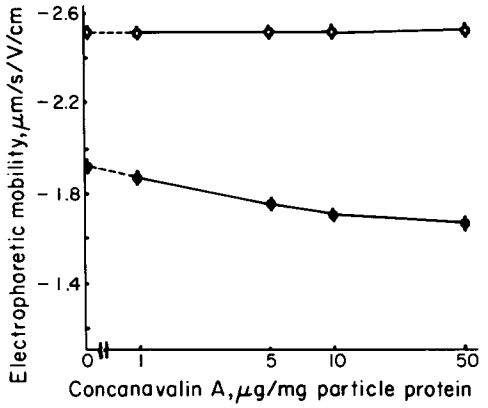

FIGURE 9 Electrophoretic mobilities of isolated rat liver or nucleoli treated with trypsinized concanavalin A. Experiments were performed as given in Materials and Methods and each point is the mean ± 1 SD. The solution for measurement was 4.5% sorbitol, 0.6 mM $NaHCO_3$ with pH 7.2 ± 0.1 at 25°C, and 0.0145 M NaCl. ○, nucleoli; ●, nuclei.

RNase the electrophoretic mobility of the nucleoli was −2.11 compared to a control electrophoretic mobility of −2.53 μm/s/V/cm. Higher levels of RNase did not decrease the mobility of the nucleoli below −2.10 μm/s/V/cm. The data suggest that on the nucleolus periphery an RNA component is also present along with the DNA and protein (see above).

Electrophoretic Mobilities of Isolated Rat Liver Nuclei or Nucleoli Treated with Concanavalin A

Concanavalin A, which binds to certain carbohydrate residues of cell surface glycoconjugates may affect surface sialyl residues by masking these residues electrokinetically. Treatment of the nucleoli with trypsinized concanavalin A (Fig. 9) resulted in no change in their electrophoretic mobility. However, treatment of the rat liver nuclei prepared by the sucrose-$CaCl_2$ method with trypsinized concanavalin A resulted in a reduction in their electrophoretic mobility. Nuclei treated with 50 μg of concanavalin A/mg nuclear protein had an electrophoretic mobility of −1.66 compared to a control mobility of −1.92 μm/s/V/cm. Incubation of the nuclei with greater concentrations of trypsinized concanavalin A did not decrease the electrophoretic mobility below −1.64 μm/s/V/cm.

Carbohydrate Composition of Sucrose-$CaCl_2$ Rat Liver Nuclei and their Subfractions

The foregoing data indicated the presence of glycoconjugates on nuclear surfaces and in nuclear subfractions. The data of Table III demonstrate the actual total carbohydrate composition of sucrose-$CaCl_2$ rat liver nuclei, nuclear envelopes, and nucleoli. Nuclei were characterized by high levels of mannose, glucose, and glucosamine; relatively low levels of galactose, fucose, xylose, and galactosamine were present. Up to a 10-fold enrichment of the monosaccharides in the membrane fraction occurred (Table III); little carbohydrate was found in the nucleoli fraction.

Integrity of Sucrose-$CaCl_2$ Rat Liver Nuclei after Enzyme Treatment and Electrophoresis

It was considered important to determine whether the nuclei were intact and structurally unaltered (except for the molecules removed by the enzyme) after enzyme treatment and electrophoresis. The data in Table IV indicate that after electrophoresis or after enzyme treatment and electrophoresis no change in nuclear RNA, DNA, protein, sialic acid, or anthrone-positive material occurred except for cases in which a particular molecule was removed by enzymatic action. No rupturing of the nuclei occurred and no non-specific solubilization of the membranes occurred.

TABLE III

Carbohydrate Composition of Sucrose-$CaCl_2$ Rat Liver Nuclei and their Subfractions

Monosaccharide	Nuclei	Nuclear membrane	Nucleoli
Glucose	4.1 ± 0.3	51.3 ± 8.2	1.3 ± 0.3
Galactose	1.0 ± 0.1	4.3 ± 0.9	0.2 ± 0.1
Mannose	5.7 ± 0.4	42.8 ± 4.7	0.4 ± 0.1
Fucose	0.8 ± 0.1	3.1 ± 0.4	0*
Xylose	1.7 ± 0.4	1.6 ± 0.4	0*
Glucosamine	3.6 ± 0.2	11.7 ± 0.6	0.4 ± 0.1
Galactosamine	0.4 ± 0.1	0.8 ± 0.2	0*

* 0 denotes not detected.

Data are expressed as nmoles per milligram of fraction protein. Experiments were performed as given in Materials and Methods. Means ± 1 SD.

TABLE IV

Integrity of Rat Liver Nuclei after Enzyme Treatment and Electrophoresis

Treatment	RNA	DNA	Protein	Sialic acid	Anthrone positive*
	μg/mg protein	*μg/mg protein*	*pg/nucleus*	*nmol/mg protein*	*nmol/mg protein*
Control	80 (0)‡	795 (0)	21 (0)	0.7 (0)	22 (0)
Electrophoresis (no enzyme)	81 (0)	797 (0)	21 (0)	0.7 (0)	21 (0)
50 μg/mg neuraminidase	82 (0)	792 (0)	22 (0)	0.1 (0.63)	21 (1.1)
50 μg/mg hyaluronidase	82 (0)	780 (0)	21 (0)	0.7 (0)	22 (0.4)
50 μg/mg DNase	80 (0)	788 (0)	22 (0)	0.7 (0)	22 (0)
50 μg/mg RNase	81 (0)	794 (0)	21 (0)	0.7 (0)	22 (0)
1% trypsin	80 (0)	796 (0)	20 (0.1)	0.38 (0.39)	9 (11.6)

Data are for sucrose-$CaCl_2$ nuclei treated with enzyme, subjected to electrophoresis, centrifuged out of solution, and analyzed chemically, as given in Materials and Methods. Numbers in parentheses refer to amount of given material in the supernate after centrifugation of the electrophoresis sample and lyophilization.

* Given as glucose equivalents.

‡ 0 denotes "not detectable."

TABLE V

Chemical Composition of Rat Liver Nuclear Envelopes after Enzyme Treatment and Electrophoresis

Treatment	DNA	RNA	Protein	Anthrone positive*
		% by weight‡		
Control	6.7 ± 0.7	4.2 ± 0.6	60 ± 3	0.7 ± 0.02
Electrophoresis (no enzyme treatment)	6.7 ± 0.7	4.3 ± 0.7	61 ± 2	0.7 ± 0.06
50 μg/mg neuraminidase	6.8 ± 0.4	4.2 ± 0.1	60 ± 4	0.6 ± 0.06
50 μg/mg hyaluronidase	6.9 ± 0.4	4.2 ± 0.3	61 ± 3	0.6 ± 0.06
50 μg/ml DNase	6.7 ± 0.9	4.2 ± 0.3	60 ± 4	0.7 ± 0.04
50 μg/mg RNase	6.8 ± 0.7	4.3 ± 0.3	61 ± 6	0.6 ± 0.04
50 μg/mg trypsin	6.7 ± 0.6	4.2 ± 0.4	58 ± 4	0.3 ± 0.01

Experiments were carried out as given in Table IV except that nuclear envelopes were isolated and the chemical composition was determined as given in Materials and Methods. Data are means ± 1 SD.

* Given as glucose equivalents.

‡ Dry weight.

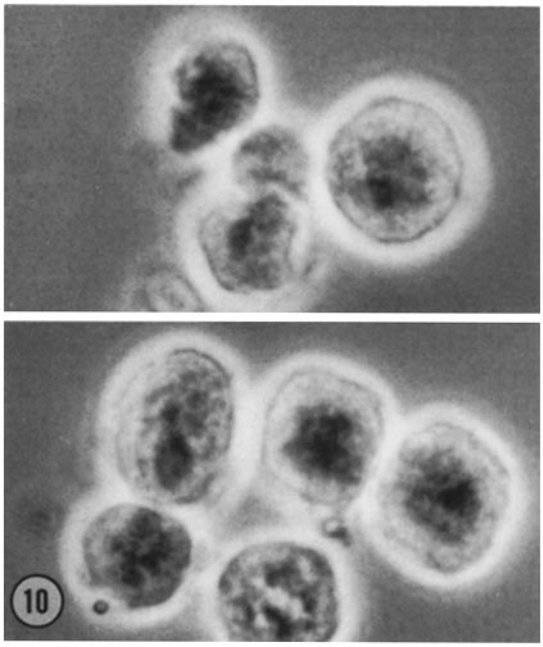

FIGURE 10 Phase micrographs of isolated sucrose $CaCl_2$ rat liver nuclei before and after enzyme treatment and electrophoresis. Top is control nuclei. Bottom is nuclei after treatment with 50 μg/mg neuraminidase and 1% trypsin and electrophoresis.

The data on the supernatant content also clearly show that there was no membrane solubilization (the anthrone data are especially appropriate here) or nuclear rupture during the procedures employed in these experiments.

Preparation of nuclear envelopes from nuclei that had been subjected to enzyme treatment and electrophoresis also showed no chemical deviation from the normal unmanipulated nuclei (Table V). Finally, Fig. 10 shows light micrographs of untreated nuclei and those subjected to neuraminidase *and* trypsin treatment followed by electrophoresis; no visible difference is found. Thus, no lysis or solubilization of the nuclei occurred after the experimental procedures and true surface phenomena were being studied.

DISCUSSION

The results presented here allow several points to be made: (*a*) Nuclei prepared by the sucrose-$CaCl_2$ method have nuclear envelopes whose membranes have terminal sialic acid residues and some hyaluronic acid but are devoid of nucleic acid on their surfaces. (*b*) Nuclei prepared by the citric acid technique present a completely different surface, one devoid of sialic acid residues. (*c*) Nucleoli have a defined structure, the surface or periphery of which is devoid of sialic acid but contains external DNA, RNA, and protein, possibly together in a macromolecular complex; indeed the entire nucleolus may be made of this material, the nature of which is responsible for the integrity of the nucleolus. (*d*) The external membrane of the cell nucleus may indeed be in continuum with the rough endoplasmic reticulum, and this membrane may have a glycoprotein structure similar to that of other cell membranes.

It should be noted that the present data are consistent with those of Vassar et al. (1967), who found nuclei to be free of surface nucleic acid and citric acid nuclei of rat liver to be free of sialic acid. The data differ, however, from those of Vassar et al. (1967) in that the sucrose-$CaCl_2$ nuclei have been demonstrated to have surface *N*-acetylneuraminic acid in agreement with the data of Nordling and Mayhew (1966), who prepared chicken erythrocyte nuclei in sucrose-$CaCl_2$; Vassar et al. (1967) did not study sucrose-$CaCl_2$ nuclei. Several criticisms of utilizing the present rat liver nuclei are (*a*) that the cells in the liver are in different stages of the cell mitotic cycle and this might result in different nuclei (McBride and Peterson, 1970); (*b*) that liver cells contain diploid, tetraploid, and higher ploidy nuclei; (*c*) that liver contains several cell types, each of which has a slightly different nucleus (Johnston et al., 1968); and (*d*) that the nuclei could be enveloped in plasma membrane fragments, as found for avian erythrocyte nuclei (Zentgrab et al., 1969). In all instances in the present experiments, the nuclear (and nucleolar) preparations behaved as homogeneous, not heterogeneous, populations with respect to their electrophoretic mobilities, and the analysis of the nuclear fractions for the plasma membrane marker enzyme 5′-nucleotidase was negative. Another problem with the interpretation of the data is that proteins and other macromolecules readily enter the nucleus. The neuraminidase, hyaluronidase, DNase, RNase, and trypsin might indeed be entering the isolated nuclei, but this should not adversely affect the surface phenomena described here. Finally, it is not clear whether the changes observed here occur in vivo since in breaking the cell, the liberated degradative enzymes (proteases, glycosidases, and nucleases) could conceivably alter the nuclear surface properties in the present in vitro experiments.

Some workers who have isolated nuclear enve-

lopes (Monneron et al., 1972; Franke et al., 1970; Berezney et al., 1970) did not determine whether or not these envelopes contained sialic acid, but Zbarsky et al. (1969) reported that nuclear envelopes from a variety of sources contained 0.08–0.1% sialic acid. Furthermore, Kashing and Kaspar (1969) found that rat liver nuclear envelopes contained 2.9–3.9% carbohydrate and 0.06–0.09% sialic acid. These data, the studies discussed in the introduction, and the results of the experiments presented here indicate that sialic acid is a surface constituent of nuclear membranes. Since it has been shown that various plant lectins agglutinate nuclei (Kaneko et al., 1972; Nicolson et al., 1972) and since mitochondria have recently been shown to contain surface *N*-acetylneuraminic acid (Bosmann et al., 1972), it seems probable that, at least in terms of sialic acid residues and glycoprotein structures, cell plasma membranes and cell organelle membranes are similar. It was originally reported that HeLa cell endoplasmic reticulum contained sialic acid (Bosmann et al., 1968), but it was not known whether it was present in precursors for glycoprotein synthesis (Bernacki and Bosmann, 1973) or whether the sialic acid was indeed an integral part of the endoplasmic reticulum membrane. Work by Larsen et al. (1972) has established that sialic acid is indeed an integral part of rat liver microsomal membrane structures. It thus appears likely that not only do the plasma membranes and organelle outer membranes of the cell contain sialic acid but also the less convoluted internal membranes of the cell contain sialyl residues in their structural components.

It is interesting that the treatment of the sucrose-$CaCl_2$ nuclei with neuraminidase results in a mobility of -1.41 μm/s/V/cm while the mobility of the citric acid nuclei is -1.63 μm/s/V/cm. Thus the citric acid nuclei have a completely different surface from the sucrose $CaCl_2$ nuclei. This difference is not simply a matter of the presence of sialic acid on the sucrose-$CaCl_2$ nuclear surface; if it were, then one would expect the electrophoretic mobility of the neuraminidase-treated sucrose-$CaCl_2$ nuclei to be -1.63 instead of -1.41. Dounce and co-workers (Magliozzi et al., 1971) believe that high levels of citric acid used in preparation of liver nuclei actually "strip off" the outer nuclear membrane.

It is tempting to speculate about the function of the sialic acid at the terminus of the macromolecules at the periphery of membranes. Because of its dissociable carboxyl group the sialic acid undoubtedly is important for imparting charge properties to the membranes. However, it is likely that the sialic acid exerts a more important specific function as a control molecule or receptor site on the various membranes to which it is bound. On the whole cell external plasma membrane surface, sialic acid terminal molecules are thought to be important in cell recognition, antigenicity, adhesiveness, permeability, communication, contact inhibition, catabolism, etc. In the nucleus it is possible that the terminal sialic acid residues might act in an analogous fashion for similar functions within the cell nucleus.

It should be noted that the measurement of electrophoretic mobility gives a *net* mobility for the surface under consideration and that after treatment of organelles of cells with enzymes the ionogenic groups which remain are as important in the interpretation of the data as the molecule which is removed by the enzymatic digestion. A particular example of this is the lowered electrophoretic mobility of the nucleoli after digestion with DNase. Evidently the surface exposed in the nucleoli after removal of the DNA has a lower electrophoretic mobility than when the DNA is present. The fact that the trypsin treatment only lowers the mobility of the nucleoli slightly is evidence that a portion of the nucleic acid may be linked via a trypsin-sensitive protein linkage. RNase treatment of the nucleoli does not lower the electrophoretic mobility except at high RNase concentrations, presumably because the DNA is still present and the RNA gives only a minor contribution to the nucleoli electrophoretic mobility. These data are consistent then with the nucleolus being a particle containing protein, RNA but predominantly DNA on its periphery.

Cell or cell organelle electrokinetic measurements made in relatively simple electrolytes as utilized herein should be viewed with caution with respect to the in vivo condition since the cells or organelles *in situ* are suspended in a much more complex and variable medium. The facts, however, that homogeneous mobilities obtained for all of the particles studied and the biochemical data complemented the electrokinetic data indicate that the results were probably not complicated by adsorption of cellular debris to the particles and the true surface phenomena were being measured.

The data indicating that concanavalin A binds to nuclei are of interest and complement the data

of Kaneko et al. (1972). In their study, they indicated that rat ascites hepatoma (AH 108A) nuclei bound ten times as much *Ricimus communis* agglutinin as did rat liver nuclei. On the basis of this fact and the present data, it would be of extreme interest to study electrophoretic mobilities of nuclei (and nucleoli) of "normal cells" and their "neoplastic counterparts." Just as each cell type and perhaps each cell type mitochondrion (Bosman et al., 1972) has a characteristic electrophoretic mobility, perhaps each cell type nucleus has a characteristic electrophoretic mobility and response to binding of plant agglutinins.

It is clear that definition of the preparation of nuclei utilized in biochemical or electrokinetic studies is of prime importance since two readily accepted methods of preparation give exactly opposite results in the present work. In any event, it is clear that the nucleoli have a completely dissimilar surface from the nuclei. The present work provides a basis for investigating further structural differences in various nuclei and nucleoli surfaces. The results showing that the majority of the sucrose-$CaCl_2$ nuclear sialic acid was released by trypsin indicates the glycoprotein nature of these sialyl-macromolecules. Thus the nucleus is characterized by external sialoproteins and hyalutonic acid but no external nucleic acid.

I thank Dr. G. V. F. Seaman for kind suggestions and Dr. A. Dounce for help with the nuclear preparations. I thank Ms. A. Spataro for helpful suggestions and Mr. K. R. Case, Mr. R. Ball, and Ms. D. DeHond for excellent technical assistance.

This work was supported in part by grant CA 13220 from the National Institutes of Health.

Dr. Bosmann is a Research Career Development Awardee of the National Institute of General Medical Sciences.

Received for publication 3 May 1973, and in revised form 20 July 1973

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