THE LYSOSOME PERIPHERY:

BIOCHEMICAL AND ELECTROKINETIC PROPERTIES

OF THE TRITOSOME SURFACE

DOUGLAS M. GERSTEN, THOMAS W. KIMMERER, and
H. BRUCE BOSMANN

From the Department of Pharmacology and Toxicology, University of Rochester School of Medicine
and Dentistry, Rochester, New York 14642

ABSTRACT

Normal rat liver lysosomes were isolated by the technique of loading with Triton WR-1339. Purity of the preparation was monitored with marker enzymes; a high enrichment in acid hydrolases was obtained in the tritosome fraction. In 0.0145 M NaCl, 4.5% sorbitol, 0.6 mM $NaHCO_3$, pH 7.2 at 25°C the tritosomes had an electrophoretic mobility of -1.77 ± 0.02 μm/s/V/cm, a zeta potential of 23.2 mV, a surface charge of 1970 esu/cm^2, and 33,000 electrons per particle surface assuming a tritosome diameter of 5×10^{-7} m. Treatment of the tritosomes with 50 μg neuraminidase/mg tritosome protein lowered the electrophoretic mobility of the tritosome to -1.23 ± 0.02 μm/s/V/cm under the same conditions and caused the release of 2.01 μg sialic acid/mg tritosome protein. Treatment of the tritosomes with hyaluronidase did not affect their electrophoretic mobility, while trypsin treatment elevated the net negative electrophoretic mobility of the tritosomes. Tritosome electrophoretic mobilities indicated a homogeneous tritosome population and varied greatly with ionic strength of the suspending media. pH vs. electrophoretic mobility curves indicated the tritosome periphery to contain an acid-dissociable group which likely represents the carboxyl group of N-acetylneuraminic acid; this was not conclusively proven, however, since the tritosomes lysed below a pH of 4 in the present system. Total tritosome carbohydrate (anthrone-positive material as glucose equivalents) was 0.19 mg/ mg tritosome protein while total sialic acid was 3.8 μg (11.4 nmol)/mg tritosome protein.

A tritosome "membrane" fraction was prepared by osmotic shock, homogenization, and sedimentation. Approximately 25% of the total tritosome protein was present in this fraction. Analysis by gas-liquid chromatography and amino acid analyzer showed the following carbohydrate composition of the tritosome membrane fraction (in microgram per milligram tritosome membrane protein): N-acetylneuraminic acid, 14.8 $\pm$ 3; glucosamine, 24 $\pm$ 3; galactosamine, 10 $\pm$ 2; glucose, 21 $\pm$ 2; galactose, 26 $\pm$ 2; mannose, 31 $\pm$ 5; fucose, 7 $\pm$ 1; xylose, 0; and arabinose, 0. The results indicate that the tritosome periphery is characterized by external terminal sialic acid residues and an extensive complement of glycoconjugates. Essentially all the tritosome N-acetylneuraminic acid is located in the membrane and about 53% of it is neuraminidase susceptible.

INTRODUCTION

In 1955, de Duve made the crucial observation that different acid hydrolases are localized within one compartment of the cell (1). Since then, several more acid hydrolases have been identified as being present in a subcellular particle, and the entire concept of lysosomes has evolved (2). That the lysosome could hold potent degradative enzymes without being degraded itself led to the speculation that the composition and properties of the lysosomal membrane might be vastly different from those of other membranous structures (3). In support of this premise were the observations of Goldstone et al. (4) that large amounts of lipoproteins and phospholipid-rich residues are present in the lysosomal membrane and recent reports that lysosomes possess remarkable osmotic stability (5, 6) and extremely low internal pH (7). By contrast, Tappel (8) found that the sialic acid content of lysosomes is similar to that of mitochondria and microsomes, although 60% greater than that of plasma membranes (9). Physical experiments such as electron microscopy (10) also have shown a similarity between lysosomal and mitochondrial membranes.

Except for the report of Henning et al. (9) on the gross chemical composition of rat liver tritosomes, comparatively little work has been concerned with the surfaces of intact lysosomes. The cell plasma membrane, the mitochondrion (11), and the nucleus (12) have all been extensively characterized electrokinetically. Our investigation was undertaken to measure the same surface electrokinetic parameters in the lysosome and to compare the characteristics of the lysosome periphery with those of other organelle membranes and cell plasma membranes.

Even though the properties of the lysosome and the enzymes it purportedly contains have been extensively documented (13–15), many questions remain concerning formation of the lysosome and its membrane, synthesis and correct integration of the lysosomal enzymes within the particle, and the mode of uptake by the particle and release from the lysosome of molecules which it is its function to catabolize. Much of the integrity of subcellular particles depends on the membranes—to separate subcellular compartments, to act as attachment sites for enzymes, or to perform specialized functions such as transport, binding, or release of macromolecules. Hence for the lysosome, which performs a very specialized

function within the cell, definition of its membrane surface properties should be of extreme importance.

This paper concerns itself with the biochemical composition and electrokinetic properties of tritosomes from isolated rat liver. Tritosomes are secondary lysosomes filled with Triton WR-1339, a nonionic, nonhemolytic detergent. By virtue of their large complement of Triton WR-1339, they are of a density which permits separation of the fraction from liver mitochondria and peroxisomes by flotation. This clean preparation of lysosomes has greatly aided the study of pinocytosis and phagocytosis, secondary lysosomes, and the general area of the intracellular digestive catabolic system. It was used in the experiments reported herein to give a clean preparation of secondary lysosomes for electrokinetic and membrane biochemical study.

MATERIALS AND METHODS

Preparation of Lysosomal Fraction

Male Sprague-Dawley rats were injected intraperitoneally with Triton WR-1339 (Ruger Chemicals, Irvington, N. J.) at 85 mg/100 g body weight by the method of Leighton et al. (16) and sacrificed 3½ days after injection. Tritosomes were isolated by the sucrose-gradient flotation method of Trouet (17). The centrifugation, however, was performed at 25,000 rpm in an SW 25.2 rotor of a Beckman model L3-50 centrifuge for 2.5 h. The tritosome fraction was removed from the upper interface with a syringe and bent 18-gauge needle and centrifuged at 27,000 g for 10 min in a refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The resultant pellet was washed and resuspended in 0.145 M NaCl. This fraction is referred to herein as rat liver tritosomes.

Tritosome Membrane Fraction

A tritosome membrane preparation was made by adding 40 vol. of distilled water to the tritosomes. The suspension was homogenized for 10 strokes with a loose Dounce homogenizer and centrifuged at 120,000 g for 1 h. The resulting pellet, dialyzed for 10 h against distilled water to remove sucrose, was considered the tritosome membrane fraction.

Solutions

All solutions were prepared in water that had been distilled, passed through a deionizer, and redistilled in an all-glass still. The solution used in most of the studies was termed saline:sorbitol of low ionic

strength or saline:sorbitol and contained 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 ± 0.1, 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 (18).

Materials

Bovine testicular hyaluronidase (EC 3.2.1.35), neuraminidase (EC 3.2.1.18; purified, proteolytic activity free, with an activity of 0.7 U/mg where 1 U of activity equals 1 μmol of N-acetylneuraminic acid released per min at 37°C at pH 5), and purified trypsin (EC 3.4.4.4) were purchased from Worthington Biochemical Corp., Freehold, N. J. Density gradient grade sucrose was purchased from Schwarz/Mann. Other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. All enzymes were analyzed for purity and in the case of the neuraminidase and hyaluronidase were found to be free of proteolytic activity between pH 5 and 9 utilizing [acetyl-³H]hemoglobin as substrate; similarly the trypsin and hyaluronidase were found to be free of neuraminidase activity between pH 5 and 9 using tritium-labeled AcNeu[7], 5-acetamido-3,5-dideoxy-L-arabino-2 heptulosonic acid fetuin, as substrate.

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 (11). 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 (18). Determinations of the mobility of washed human erythrocytes were made in 0.0145 M NaCl, 4.5% sorbitol made 0.6 mM with respect to NaHCO₃ . Normal blood for this purpose was obtained from healthy donors of the phenotype A Rh⁺, taken into EDTA and immediately washed, and electrophoretic mobilities were determined. Heard and Seaman (18) 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 −2.81 ± 0.02 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 60 readings ± standard error of the mean. 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 Determinations

Protein was determined by the method of Lowry et al. (19) utilizing bovine serum albumin as standard.

Enzyme Treatment of Tritosomes

The procedure used was to treat the tritosomes with neuraminidase, hyaluronidase, or trypsin and to measure the electrophoretic mobility of a portion of the treated tritosomes. Tritosomes 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 tritosomes were centrifuged at 15,000 g for 10 min, washed twice with physiological saline, centrifuged for 10 min at 15,000 g, and finally resuspended in 7 ml of saline:sorbitol for observation of electrophoretic mobility. The particles were timed successively in both directions at 60 V for two 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 ± 0.1.

Sialic Acid

Released or free sialic acid was measured by the Warren procedure (20), total sialic acid by the Svennerholm procedure (21).

Anthrone-Positive Material

Anthrone-positive material, as a measure of total carbohydrate, was analyzed as previously described (22).

Calculation of Parameters

Conversions of electrophoretic mobilities to other electrokinetic parameters were made by the Helmholtz-Smoluchowski equation and the generalized Gouy equation for a unidivalent ionic system, as described by Abramson (23) and Heard and Seaman (18). The diameter used in the calculations is an approximation derived from electron micrographs: $d = 0.5 \times 10^{-6}$ m. The equation for surface area of a sphere was used to calculate the surface areas.

Carbohydrate Determinations

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

as described previously (24). Hexosamines were determined as described previously (25, 26).

Enzyme Determinations

The starch-iodide determination for hydrogen peroxide of Maehley and Chance (27) was used as an indicator for peroxisomes, while the cytochrome oxidase (EC 1.9.3.1) method of Smith (28) was used as a mitochondrial indicator. Glycosidase activities were measured at pH 4 using p-nitrophenyl glycosides (Pierce Chemical Co., Rockford, Ill.) after the method of Bosmann and Bernacki (29). 5'-Nucleotidase (EC 3.1.3.5) was measured as described previously (30).

RESULTS

Properties of Tritosome Preparation

The data given in Table I indicate that the tritosome preparation utilized herein was essentially free of peroxisomes and mitochondria, as evidenced by the lack of the marker enzymes catalase and cytochrome oxidase, respectively. 5'-Nucleotidase, a plasma membrane marker enzyme (30), was slightly enriched in the tritosome preparation, consistent with the allegation that secondary lysosomal membranes are at least in part plasma membrane derived. The acid hydrolases, acid phosphatase, and glycosidases were enriched approximately 10-fold in each instance in the tritosome fraction; furthermore, homogeni-

zation of the tritosomes in the nonionic detergent Triton X-100 enhanced the activity of the acid hydrolases. For example, the acid phosphatase activity level of the total liver Triton X-100 homogenate was 0.8 μmol/h/mg protein, that of the tritosome fraction was 7.79 μmol/h/mg protein (a 9.7-fold purification), and that of the 0.05% Triton X-100 tritosome homogenate was 18.05 μmol/h/mg protein (a 22-fold purification). The glycosidase activity of the Triton X-100 tritosome homogenate was also elevated 20-fold or more over the crude homogenate.

Electrokinetic Parameters of Isolated Rat Liver Tritosomes

As shown in Table II, the tritosomes in 0.0145 M NaCl, 0.6 mM NaHCO$_3$, 4.5% sorbitol, pH 7.2, at 25 $\pm$ 0.1°C had an electrophoretic mobility of -1.77 ± 0.02. As shown in Fig. 1, these particles behaved as a homogeneous population with respect to their electrophoretic mobility, a non-bimodal or trimodal distribution of the tritosome electrophoretic mobilities obtained. Using this electrophoretic mobility, a zeta potential of 23.2 mV, surface charge of 1,970 esu/cm^2, and electron opacity of 33,000 electrons per particle surface can be calculated (ignoring any particle conductance) for the tritosome.

The data of Fig. 2 illustrate the pH-vs.-mobility relationships for the tritosomes. Above a pH of

TABLE I

Enzyme Activities in Whole-Liver Homogenate Compared with Tritosome Preparation

Enzyme	Liver (0.1% Triton X-100 homogenate)	Tritosomes (suspended in 0.145 M NaCl)	Tritosomes (0.05% Triton X-100 homogenate)
	μmol/h/mg protein		
Catalase (EC 1.11.1.6)	81.4	0*	0
5'-Nucleotidase (EC 3.1.3.5)	3.1	3.9	4.7
Cytochrome oxidase (EC 1.9.3.1)	14.2	0	0
β-Galactosidase (EC 3.2.1.23)	0.144	2.11	2.94
β-Fucosidase (EC 3.2.1.38)	0.002	0.013	0.040
β-N-acetylgalactosaminidase (EC 3.2.1.—)	0.127	1.78	2.97
α-Mannosidase (EC 3.2.1.14)	0.02	0.24	0.54
Acid phosphatase (EC 3.1.3.2)	0.8	7.79	18.05
β-N-acetylglucosaminidase (EC 3.2.1.29)	0.7	7.89	16.40

* Not detectable.

TABLE II

Electrokinetic Parameters for Isolated Rat Liver Tritosomes

Parameter	Unit	Tritosome value
Electrophoretic mobility	μm/s/V/cm	-1.77 ± 0.02
Zeta potential	mV	23.2
Surface charge	esu/cm^2	1,970
Electrons	no. per tritosome surface	33,000

Data for electrophoretic mobility are means ± 1 SD. The total number of observations was greater than 600; that is, mobilities of over 600 particles were measured independently. The data are for saline:sorbitol of ionic strength 0.0145 at 25°C in 0.6 mM NaHCO₃, 4.5% sorbitol, pH 7.2 ± 0.1. Experiments were performed as given in the text. Calculations are based on the assumption that particle conductance was negligible.
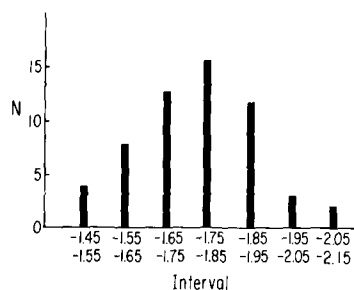
FIGURE 1 Distribution of electrophoretic mobilities of isolated rat liver tritosomes. 58 observations from three independent experiments are plotted where *N* equals the number of observations having the indicated electrophoretic mobility. All electrophoretic mobilities were plotted in the given interval. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO₃, pH 7.2 ± 0.1, 4.5% sorbitol, 25°C.

6.7 the mobility was rather constant between −1.75 and −1.80 μm/s/V/cm. Below pH 6.7 the mobilities fell off rather rapidly as they approached pH 4 (e.g., at pH 4.5 the electrophoretic mobility was −0.66 μm/s/V/cm). Below pH 4.5, however, the tritosomes appeared to lyse in the suspending medium, making measurement of electrophoretic mobility impossible; from the shape of the curve (Fig. 2), however, it is possible to characterize the surface as having a pKa of somewhere below pH 4—possibly around pH 2.7, which would be consistent with the pKa of
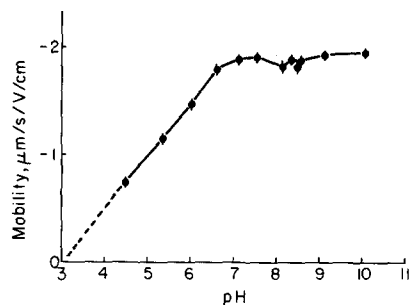

FIGURE 2 Electrophoretic mobility of isolated rat liver tritosomes as a function of pH. Experiments were performed as given in Materials and Methods and points are means ± 1 SD. The solution for measurement was 0.0145 M NaCl, 4.5% sorbitol, 25°C; pH adjustments were made with iso-osmotic NaOH or HCl. Below pH 4 the tritosomes lysed and measurement of mobility was impossible.
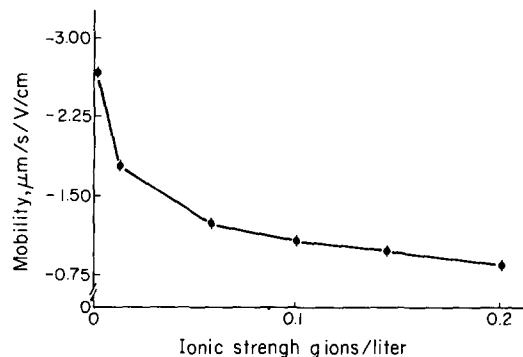

FIGURE 3 Electrophoretic mobility of isolated rat liver tritosomes as a function of ionic strength. Experiments were performed as given in Materials and Methods, and points are means ± 1 SD. The solution for measurement was 4.5% sorbitol, 0.6 mM NaHCO₃, pH 7.2 ± 0.1, 25°C; the 0.6 mM NaHCO₃ was omitted at low ionic strengths. Ionic strength was varied by changing concentrations of NaCl.

the carboxyl group of terminal sialic acid residues (see below). The reason for the fragility of the tritosome membrane in the present system is not known.

In Fig. 3 is given the ionic strength-vs.-electrophoretic mobility curve for the isolated rat liver tritosomes. Very high electrophoretic mobilities, approaching −3 μm/s/V/cm, were found as the ionic strength approached 0 g-ions/liter. With suspending media ionic strengths of 0.1 g-ions/liter and above, the electrophoretic mobility of the particles was essentially constant around −1 μm/s/V/cm.

At the conclusion of electrophoretic measurements performed at ionic strengths approaching 0 g-ions/liter, the lysosomes were visually intact by light microscopy, and no acid hydrolase activity was found in the 2000 g supernatant of the actual measuring media (see Table I for list of enzymes measured). Thus, in these experiments no rupture of lysosomes or reabsorption of molecules to lysosomal surfaces occurred at low ionic strengths.

Neuraminidase Treatment of Isolated Rat Liver Tritosomes

The curve in Fig. 4 indicates that treatment of the tritosome with neuraminidase greatly reduced the electrophoretic mobility of the particles. At 50 µg neuraminidase/mg tritosome protein the mobility was reduced from a control value of -1.75 µm/s/V/cm to -1.25 ± 0.02 µm/s/V/cm. That this decrease was probably not due to absorption of the neuraminidase onto the tritosome is shown by the value for the boiled control (B in Fig. 4). The electrophoretic mobility of the tritosome was reduced significantly with as little as 10 µg neuraminidase/mg tritosome protein. With treatments above 33 µg neuraminidase/mg tritosome protein the electrophoretic mobility of the particle remained constant at -1.25 µm/s/V/cm; levels of neuraminidase higher than 50 µg/mg protein did not result in a further lowering of the electrophoretic mobility below -1.25 µm/s/V/cm.

The data of Fig. 5 clearly indicate that the reduction in electrophoretic mobility with neuraminidase was indeed probably due to release of sialic acid from the tritosome periphery. The amount of sialic acid released from the tritosome was essentially linear up to about 33 µg neuraminidase/mg tritosome protein; above this level and at levels higher than 50 µg neuraminidase/mg tritosome protein 2.01 ± 0.14 µg sialic acid were released per mg tritosome protein. Since the tritosome contains 3.8 µg sialic acid/mg protein (Table III), this means that 53% of the total tritosome sialic acid is released by the neuraminidase. Also since 97% of the tritosome sialic acid is located in the "tritosome membrane" (Table III), the remaining 47% of the sialic acid, although membrane located, must not be neuraminidase susceptible. The remaining N-acetylneuraminic acid may be what determines the remaining -1.25 µm/s/V/cm of net negative electrophoretic mobility after neuraminidase treatment. Alternatively, the 47% of the remaining N-acetylneuraminic acid may be masked by other macromolecules embedded in the tritosome membrane or may reside as terminal residues on the internal face of the tritosome membrane.

Trypsin and Hyaluronidase Treatment of Isolated Rat Liver Tritosomes

Trypsin treatment of the tritosomes at a level of 50 µg/mg tritosome protein (Table IV) significantly elevated the mobility of the tritosomes, while hyaluronidase elevated the electrophoretic
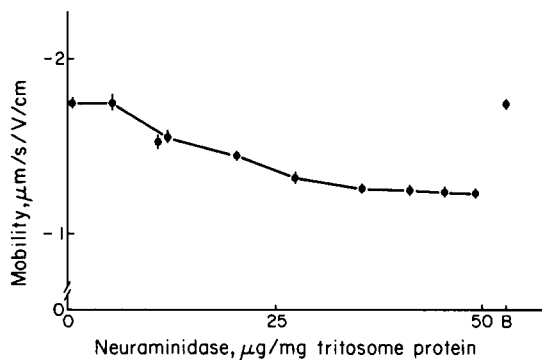

FIGURE 4 Electrophoretic mobilities of neuraminidase-treated rat tritosomes. Data are the mean value ± 1 SD. B refers to neuraminidase (50 µg/mg particle protein) which was boiled for 5 min before use in the experiment. Experiments were performed as given in Materials and Methods. The solution for measurement was 0.0145 M NaCl, 0.6 mM NaHCO₃ , pH 7.2 ± 0.1, 4.5% sorbitol, 25°C.
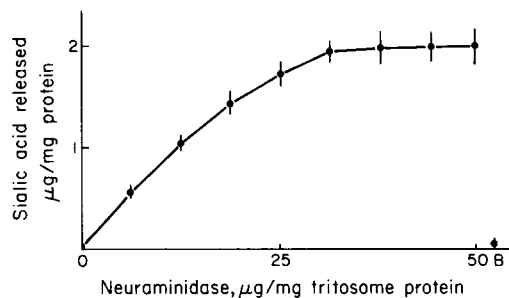
FIGURE 5 Amount of sialic acid released from rat liver tritosomes as a function of amount of neuraminidase present in the incubation. Incubations were carried out as given in Materials and Methods, and free sialic acid released into the supernatant was measured. Data are means ± 1 SD. B refers to neuraminidase (50 µg/mg particle protein) which was boiled for 5 min before use in the experiment.

TABLE III

Carbohydrate Present in Tritosomes and Tritosome Membrane Fraction

Constituents	Tritosome		Tritosome membrane		
			μg/mg fraction protein		
Protein (arbitrary units)	100	± 1	25	± 1	(25)*
Anthrone-positive material	190	± 23	230	± 60	(30)
N-Acetylneuraminic acid	3.8	± 0.2	14.8	± 1.2	(97)
Glucosamine	11.8	± 0.9	24	± 3	(51)
Galactosamine	4.3	± 0.3	10	± 2	(58)
(Total amino sugars)	[16]		[34]		(53)
Glucose	52	± 6	21	± 2	(10)
Galactose	11	± 2	26	± 2	(60)
Mannose	13	± 1	31	± 5	(57)
Fucose	2.5	± 0.3	7	± 1	(70)
Xylose	0‡		0		
Arabinose	0		0		
(Total neutral sugars)	[79]		[86]		(27)

Protein was determined as given in text. Anthrone-positive material is given as microgram per milligram fraction protein, glucose equivalents. Sialic acid was determined as given in the text, neutral and amino sugars by gas-liquid chromatography as described in Materials and Methods. Values are means ± 1 SD.

* Parentheses indicate percentage of total tritosome material contained in tritosome membrane.

‡ Not detectable.

TABLE IV

Electrophoretic Mobilities of Isolated Rat Liver Tritosomes Treated with Various Enzymes

Enzyme treatment (50 μg enzyme/mg tritosome protein)	Tritosome electrophoretic (μm/s/V/cm)
None (control)	−1.77 ± 0.02
Neuraminidase (EC 3.2.1.18)	−1.35 ± 0.02
Trypsin (EC 3.4.4.4)	−2.18 ± 0.06
Hyaluronidase (EC 3.2.1.35)	−1.88 ± 0.08

Enzyme treatments were as given in the text. Measurement of mobilities was in 0.0145 M NaCl, 0.6 mM NaHCO₃, 4.5% sorbitol, pH 7.2, 25°C. Experiments were carried out as given in Materials and Methods. Means ± 1 SD.

mobility slightly but not significantly. These data are interpreted to mean that although the trypsin treatment of the tritosomes undoubtedly releases tritosome sialopeptides and hence the terminal sialic acid residues, the amino acid terminal carboxy group generated by the trypsin action effectively replaces this group electrokinetically, and the amino acid instead of the sialic acid becomes the determinant of the electrokinetic properties of the tritosome periphery. Another

alternative is that even though trypsin treatment removes sialic acid-containing glycopeptides, it is possible that it also uncovers other N-acetyl-neuraminic acid-containing glycolipids or glyco-proteins. Analysis of the sialic acid-containing components remaining after trypsin treatment indicated that 46% of the tritosome membrane N-acetylneuraminic acid was removed; thus 54% of the tritosome membrane N-acetylneuraminic acid was not removed under the conditions of trypsin treatment (Table 4). From the results of this study it does not seem that hyaluronic acid is a constituent of the tritosome periphery.

Carbohydrate of Isolated Rat Liver Tritosomes and the Tritosome Membrane

The membrane carbohydrate present primarily in glycoconjugates is of extreme interest for any membrane and of especial interest for the tritosome membrane for comparison with the plasma membrane and other cellular membranes. The data of Fig. 3 indicate that the tritosomes contained 190 μg of anthrone-positive material per mg of tritosome protein, a rather high complement of carbohydrate; 30% of this anthrone-positive material was located in the tritosome membrane.

The data on the gas-liquid chromatography and hexosamine analysis, however, are much more accurate and reliable than the anthrone data since this reagent reacts differently with each monosaccharide and nonspecifically (22). Thus the fact that more anthrone-positive material is present than can be accounted for by summing of the amounts of the neutral amino sugars plus N-acetylneuraminic acid is not surprising. Essentially all of the N-acetylneuraminic acid, 70% of the fucose, and more than 50% of the amino sugars, galactose, and mannose are found in the membrane fraction. The large amount of glucose (not usually associated with glycoproteins) found in the tritosome and in the tritosome membrane fraction may represent (a) sucrose bound to or included in the fractions during the isolation procedure, which upon hydrolysis releases glucose, (b) glycolipid glucose, or (c) glycoprotein glucose in a molecule with a collagen-like or other structure. The high amount of carbohydrate not in the tritosome membrane (70%) probably derives from three sources: (a) glycoprotein acid hydrolases, since many lysosomal enzymes are known to be glycoproteins; (b) glycoconjugates and glycosaminoglycans in the tritosome ready for digestion, (c) intratritosome membrane matrix glycoconjugates, and (d) sucrose trapped within the lysosomes.

The amino and neutral sugar content of the tritosome membrane fraction was accurately measured by amino acid analyzer and gas-liquid chromatography of the alditol acetates, respectively. The tritosome membrane contained 34 μg/mg protein of amino sugar, probably present in the membrane as N-acetylhexosamine; 24 μg of this was glucosamine and 10 μg was galactosamine. 86 μg/mg protein of neutral sugar was found in the tritosome membranes by these techniques; the distribution was mannose 31, galactose 26, glucose 21, and fucose 7 μg/mg tritosome membrane protein, while xylose and arabinose were not detected.

DISCUSSION

The experimental data presented in this paper indicate that the lysosomal periphery can be studied by electrokinetic and biochemical means if the "tritosome" method is employed for their isolation in essentially pure form. However, it should be noted that although the technique allows a class of secondary lysosomes to be isolated relatively purely and although the technique has been

successfully utilized in a number of studies (16), the preparation may not be equivalent to native in vivo secondary lysosomes not obtained from animals under the trauma of the extensive Triton WR-1339 overload. The effect the detergent WR-1339 has on tritosome membrane properties (i.e., solubilization) is unknown and will not be resolved until secondary lysosomes can be isolated in pure form by a less potentially disruptive method. Furthermore, the effect of cell disruption in general, i.e. breakage of primary and secondary lysosomes and release of hydrolases, on the tritosome periphery is not known.

The results indicate that the tritosome and in particular the tritosome membrane are rich in N-acetylneuraminic acid. It is of interest that in the present study the tritosomes were found to contain 11 nmol sialic acid/mg protein while Glick et al. (31) found L cell lysosomes also to contain 11 nmol sialic acid/mg protein. Henning et al. (9) reported "tritosome membranes" to contain 45.6 μg/mg protein of neutral carbohydrate (as measured by the α-naphthol reaction), while in the present study neutral sugars were found to be present at 86 μg/mg protein (as measured by gas-liquid chromatography). Henning et al. (9) also reported 16.1 μg sialic acid, 25.4 μg glucosamine, and 5.5 μg galactosamine per mg protein for their tritosome membranes; the present results were 14.8, 24, and 10 μg/mg protein, respectively. Thus our results compare favorably with those of Henning et al. (9) except for the galactosamine value. It should be noted, however, that the tritosomes used in our studies may not reflect particles actively engaged in pinocytosis since they were taken from animals sacrificed 3½ days after injection. According to the present results, the neutral sugars usually associated with glycoconjugates are present in the tritosome membranes in the order mannose > galactose > glucose > fucose, with xylose and arabinose absent.

Although, as noted in the Introduction, the lysosomal membrane seems to be called upon to perform special duties involving pH, cation, and macromolecule control, chemically and electrokinetically the tritosome membrane seems rather similar to the plasma membrane (8) and other subcellular membranes (10). To date the only "subcellular organelles" without appreciable terminal sialic acid residues at their periphery which have been studied by this laboratory,

using techniques similar to those described herein, are the nucleolus (12) and cerebral cortex synaptic vesicles (32). The nucleus (12), mitochondria (11), and various plasma membranes (31) have all been shown to have similar sialic acid-containing glycoconjugates on their surfaces. The tritosome membrane in particular appears similar to the cell plasma membrane, which may be an indication that the plasma membrane is the origin of portions of the tritosome membrane.

It should be emphasized that in experiments such as those described above the net negative electrophoretic mobility is being measured, and this is the sum of very complex macromolecules at the particle surface. Also the medium in which measurements are made is much simpler than that encountered by the particle in vivo. In any event, the results indicate the presence of terminal sialic acid residues on the tritosome surface. Other constituents of the tritosome surface and other macromolecules contributing to the electrophoretic mobility are unknown.

In the light of the postulated phenomenon of "sublethal autolysis" (9) and the fact that lysosomes contain proteases and glycosidases, it remains a question whether these hydrolases can modify the lysosome surface glycoconjugates by digestion of the particle periphery. Finally, the presence of these glycoconjugates on the surface of the lysosome means that the particle should be amenable to study with lectins.

Note Added in Proof: While the present paper was in press, Hening and co-workers (33) have demonstrated the ionic binding of certain enzymes (β-glucuronidase and acid phosphatase) to tritosomal outer membrane at pH 4. Enzyme binding to the membrane was significantly reduced by treatment with neuraminidase, but not by proteases or phospholipase C, suggesting that sialic acid is the main anionic group at that pH. It was also reported that the sialic acid content of rat liver tritosome membranes was 14.9 μg/mg protein, of which 35 $\pm$ 18% was neuraminidase susceptible. Electron micrographs were reported (33) to show a preferential localization of sialic acid on the inside of the lysosomal membrane, perhaps accounting for the membrane sialic acid not neuraminidase susceptible as reported herein.

REFERENCES

1. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**:605–617.
2. DE DUVE, C. 1963. The lysosome concept. Ciba Foundation Symposium on Lysosomes. Little, Brown and Company, Boston. 1–35.
3. KOENIG, H. 1962. Histological distribution of brain gangliosides. Lysosomes as glycoprotein granules. *Nature (Lond.).* **195**:782–784.
4. GOLDSTONE, A., E. SZABO, and H. KOENIG. 1970. Isolation and characterization of acidic lipoprotein in renal and hepatic lysosomes. *Life Sci.* **9**(Pt. 2):607–616.
5. LLOYD, J. B. 1971. A study of permeability of lysosomes to amino acids and small peptides. *Biochem. J.* **121**:245–248.
6. GOLDMAN, R., and H. ROTTENBERG. 1973. Ion distribution in lysosomal suspensions. *FEBS Lett.* **33**:233–238.
7. REIJNGOUD, D. J., and J. M. TAGER. 1973. Measurement of intralysosomal pH. *Biochim. Biophys. Acta.* **297**:174–178.
8. TAPPEL, A. L. 1963. Discussion in Ciba Foundation Symposium on Lysosomes. Little, Brown and Company, Boston. 31.
9. HENNING, R., H. D. KAULEN, and W. STOFFEL. 1970. Biochemical analysis of the pinocytotic process. I. Isolation and chemical composition of the lysosomal and plasma membrane of the rat liver cell. *Hoppe-Seyler's Z. Physiol. Chem.* **351**:1191–1199.
10. PREZBINDOWSKI, K. S., F. F. SUN, and F. L. CRANE. 1968. Characterization of microsomal membranes by negative staining techniques. *Exp. Cell Res.* **50**:241–256.
11. BOSMANN, H. B., M. W. MYERS, D. DEHOND, R. BALL, and K. R. CASE. 1972. Mitochondrial autonomy: sialic acid residues on the surface of isolated rat cerebral cortex and liver mitochondria. *J. Cell Biol.* **55**:147–160.
12. BOSMANN, H. B. 1973. Molecules at the external nuclear surface: sialic acid of nuclear membranes and electrophoretic mobility of isolated nuclei and nucleoli. *J. Cell Biol.* **59**:601–614.
13. DINGLE, J. T., editor. 1969. Lysosomes in Biology and Pathology. Vol. 1. Elsevier Publishing Company, New York.
14. DINGLE, J. T., editor. 1969. Lysosomes in

Biology and Pathology. Vol. 2. Elsevier Publishing Company, New York.

15. DINGLE, J. T., editor. 1973. Lysosomes in Biology and Pathology. Vol. 3. Elsevier Publishing Company, New York.

16. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHVIN, J. W. COFFEY, S. FOWLER, and C. DE DUVE. 1968. The large scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. J. Cell Biol. 37:482-513.

17. TROUET, A. 1964. Immunisation de lapins par des lysosomes hépatique de rats traités du Triton WR-1339. Arch. Int. Physiol. Biochem. 72:698-699.

18. HEARD, D. H., and G. V. F. SEAMAN. 1960. The influence of pH and ionic strength on the electrokinetic stability of the human erythrocyte membrane. J. Gen. Physiol. 43:635-654.

19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

20. WARREN, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:1971-1975.

21. SVENNERHOLM, L. 1958. Quantitative estimation of sialic acids. III. An anion exchange resin method. Acta Chem. Scand. 12:547-554.

22. DISCHE, A. 1962. Color reactions of carbohydrates. In Methods in Carbohydrate Chemistry. 1:477-514.

23. ABRAMSON, H. A. 1934. Electrokinetic Phenomena. Chemical Catalog Co., New York.

24. MARTIN, S. S., and H. B. BOSMANN. 1971. Glycoprotein nature of mitochondrial structural protein and neutral sugar content of mitochondrial protein and structural protein. Exp. Cell Res. 66:59-64.

25. BOSMANN, H. B., and J. J. JACKSON. 1968. Glycoprotein structure: the carbohydrate of bovine corneal collagen. Biochim. Biophys. Acta. 170:6-14.

26. BOSMANN, H. B., and D. KESSEL. 1970. Inhibition of glycoprotein synthesis in L5178Y mouse leukaemic cells by L-asparaginase in vitro. Nature (Lond.). 226:850-851.

27. MAEHLEY, A. C., and B. CHANCE. 1954. The assay of catalases and peroxidases. Methods Biochem. Anal. 1:357-424.

28. SMITH, L. 1965. Spectrophotometric assay of cytochrome c oxidase. Methods Biochem. Anal. 2:427-434.

29. BOSMANN, H. B., and R. J. BERNACKI. 1970. Glycosidase activity. Exp. Cell Res. 61:379-386.

30. BOSMANN, H. B., and G. Z. PIKE. 1971. Membrane marker enzymes: Isolation, purification and properties of 5'-nucleotidase from rat cerebellum. Biochim. Biophys. Acta. 227:402-408.

31. GLICK, M. C., C. A. COMSTOCK, M. A. COHEN, and L. WARREN. 1971. Membranes of animals cells. VIII. Distribution of sialic acid, hexosamines and sialidase in the L cell. Biochim. Biophys. Acta. 233:247-257.

32. MCLAUGHLIN, J., K. R. CASE, and H. B. BOSMANN. 1973. Electrokinetic properties of isolated cerebral cortex synaptic vesicles. Biochem. J. 136:919-926.

33. HENNING, R., H. PLATTNER, and W. STOFFEL. 1973. Nature and localization of acidic groups on lysosomal membranes. Biochim. Biophys. Acta. 330:61-75.