Martinez, A. M. (1967), *J. Biol. Chem. 242*, 590.
Sternberger, N., and Stevens, A. (1966), *Biochem. Biophys. Res. Commun. 24*, 937.
Stevens, A., Emery, A. J., Jr., and Sternberger, N. (1966), *Biochem. Biophys. Res. Commun. 24*, 929.
Zillig, W., Fuchs, E., and Millette, R. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 323.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Glycoproteins of Ehrlich Ascites Carcinoma Cells. Incorporation of [^{14}C]Glucosamine and [^{14}C]Sialic Acid into Membrane Proteins*

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ABSTRACT: Washed Ehrlich ascites carcinoma cells were incubated with labeled glucosamine which resulted in the appearance of radioactive glucosamine and sialic acid in trichloroacetic acid insoluble cell constituents. The subcellular localization of the products of this reaction have been investigated by separating the labeled cell homogenates to nuclei, mitochondrial, rough and smooth microsomal, and supernatant fractions by using differential and density centrifugation methods. The fractions were characterized in terms of protein, hexosamine, sialic acid, radioactivity, ribonucleic acid, deoxyribonucleic acid, and also by electron microscopy. The smooth and rough microsomal fractions were analyzed further by sucrose density gradient centrifugation and a few enzymatic activities were also determined. Three methods were tested for separating subcellular particles. By the best method more than 40% of the cellular hexosamine, sialic acid, and radioactivity could be isolated with the smooth microsomal fraction.

The data supported the view that most of the sialic acid and hexosamine of Ehrlich cells is localized in the plasma membrane and smooth endoplasmic reticular membrane structures. Incorporation of glucosamine occurred mainly into the macromolecules containing these constituents.

In a previous paper it was shown that glucosamine-^{14}C was readily incorporated into proteins by Ehrlich ascites carcinoma cells (Molnar *et al.*, 1965a). Two major types of products were observed. One appeared in the incubation media (*in vitro*), or in the ascites plasma (*in vivo*), as a heterogeneous mixture of glycoproteins (Molnar *et al.*, 1965b) and the other remained associated with cellular elements. The "secreted macromolecules" contained about equal amounts of radioactivity in their galactosamine and glucosamine moieties and in a hitherto unidentified compound. The cellular elements, on the other hand, had radioactivity mainly in glucosamine and about 10% of the total activity was in sialic acid.

The present paper describes our attempts to find the subcellular localization of the cellular products of glucosamine-^{14}C incorporation. We were guided in these experiments by the observations of Wallach and his co-workers (1961, 1966) who reported that 73% of sialic acid of Ehrlich cells could be released by treatment of intact cells with neuraminidase with a concomitant decrease of electrophoretic mobility. These experiments and some others suggested that about 70% of the cellular sialic acid is located on the outer surface of plasma membranes. A similar conclusion was reached by Langley and Ambrose (1964, 1967) who subjected Ehrlich cells to limited digestion by trypsin and found that about 70% of sialic acid was released with mucopeptides which also contained galactosamine. These experiments suggested that the plasma membrane fraction, if separable from the other membranes, should contain about 70% of cellular sialic acid and hexosamine. Since both of these sugar moieties became labeled in our experiments we were expecting that about 70% of the incorporated radioactivity should be present in the plasma membrane fraction too, unless some of the products localized in other types of structural elements have higher, or lower, turnover rates.

It will be shown that by our best method 40–50%

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of the incorporated radioactivity, hexosamine, and sialic acid could be isolated with the smooth microsomal fraction rich in plasma membrane fragments.

Experimental Procedure

Preparation of Cell Suspensions. The Ehrlich ascites tumor, originally obtained in 1964 from Dr. H. Monsen, was maintained by weekly intraperitoneal transfer of 0.2 ml of diluted ascites fluid into 30–40-g Swiss female albino mice (five- to tenfold dilution was made with 0.85% NaCl containing 1000 units of penicillin G and 10 units of heparin per ml). Cells were harvested by puncturing the abdomens of sacrificed mice 7–8 days after inoculation and collecting the ascites fluid in a beaker containing ice-cold Krebs–Ringer buffer (Molnar *et al.*, 1965a). The cancer cells were washed and freed of contaminating red cells by accelerating the rotor no. SS34 of a Sorval RC-2B refrigerated centrifuge, at 0°, containing the diluted ascites fluid, till 2000 rpm was reached and then stopping the centrifugation rapidly. This caused sedimentation to most of the cancer cells, while the red cells remained mainly in suspension. The supernatant was carefully decanted and the sediment was suspended in ten volumes of Krebs–Ringer solution and centrifuged as above. This washing step was repeated until the sediment was practically free of red cell contamination (usually six to ten washing steps were required). The last sediment was suspended in three volumes of Krebs–Ringer solution and centrifuged at 1000*g* for 10 min. The sediment was suspended in a known volume of Krebs–Ringer and the packed cell volume was calculated.

Reaction with Glucosamine-^{14}C. Incubations were carried out at 37° in a 500-ml erlenmeyer flask, in air, by using a metabolic shaker. Each 100 ml of reaction mixture contained about 10 ml of packed cells, 2.5 μc of glucosamine-1-^{14}C (11 μc/μmole, New England Nuclear Corp. or Tracerlab), 2 mmoles of sodium pyruvate, and 80 ml of Krebs–Ringer buffer. After 1 or 2 hr of reaction the suspension was centrifuged at 0° for 10 min at 1000*g*. The sediment was suspended in ten volumes of homogenizing medium and the centrifugation was repeated.

Preparation of Homogenates (Wallach and Ullrey, 1962). METHOD A. The homogenizing medium contained 0.25 M sucrose, 5 mM Tris-HCl buffer (pH 7.6), 0.2 mM $MgSO_4$, and 10% of packed cells (v/v). The cells were ruptured by applying 800 psi to a stainless-steel homogenizer, made according to Hunter and Commerford (1961), and after stirring with a magnet at 0° for 20 min, the pressure was released and the homogenate was adjusted to 1 mM EDTA by using a 0.1 M EDTA stock solution. The suspension was centrifuged at 16,000*g* (average) for 15 min. In our experiments one homogenization was not adequate to release all the microsomes to the 16,000*g* supernatant and therefore the homogenization was repeated two more times by using the 16,000*g* sediment each time with a fresh sucrose medium.

METHOD B. Washed labeled cells were suspended to obtain 15% concentration of packed cells (v/v) in a medium containing 0.25 M sucrose, 0.04 M NaCl, 0.1 M KCl, 0.02 M Tris (pH 7.6), and 5 mM $MgSO_4$. (This is essentially the sucrose–medium M solution of Munro *et al.* (1964) used for the isolation of polysomes from rat liver.) The packed cell volume is related to that obtained in Krebs–Ringer buffer, since in 0.25 M sucrose–medium M about one-half of this value was observed. Homogenization was carried out as in method A except EDTA was omitted this time. The 16,000*g* sediment was rehomogenized twice by using one-half of the original volume of sucrose–medium M. Usually the second homogenization was the most effective in terms of microsomal yield.

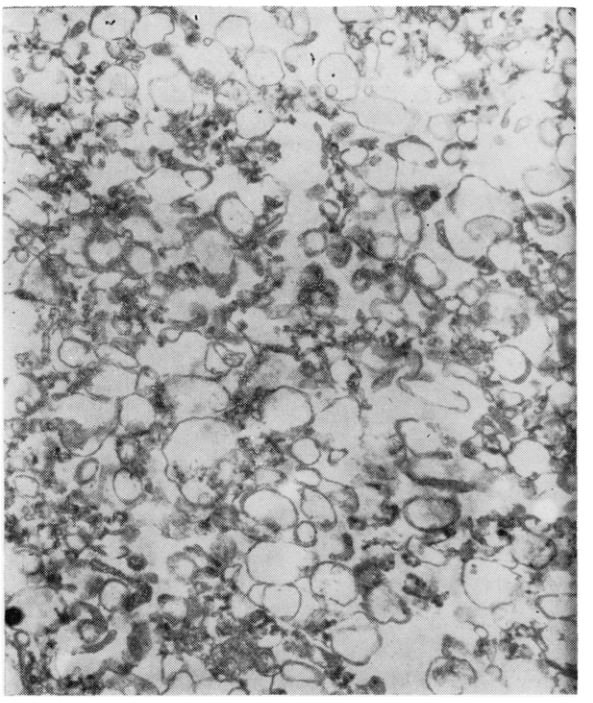

FIGURE 1: Electron micrograph of a plasma membrane fraction prepared by method I. Details are given in text. Magnification is about 23,000.

Isolation of Plasma Membranes and Fragments of Endoplasmic Reticulum. METHOD I. The procedure described by Kamat and Wallach (1965) was applied. In this method the 16,000*g* supernatant prepared by method A was used.

Isolation of Smooth and Rough Microsomal Fractions. METHOD II. The 16,000*g* supernatant obtained by method B was centrifuged for 60 min at 78,000*g*. The sediment was suspended by gentle homogenization with a Potter–Elvehjem-type homogenizer in a 10 mM Tris-HCl buffer (pH 8.4) containing 1.0 mM $MgSO_4$ to obtain 0.1–0.15% protein concentration and centrifuged as above. This washing step was repeated by using a 1 mM Tris buffer (pH 8.4) containing 0.2 mM $MgSO_4$. The magnesium salt was used in the washing solutions to increase the yield of microsomes and prevent dissociation of ribosomes from the rough microsomes.

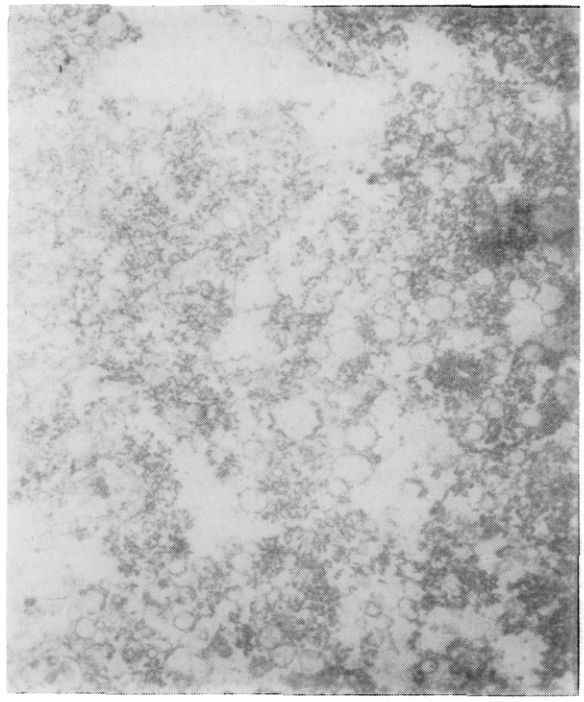

FIGURE 2: Electron micrograph of an endoplasmic reticulum membrane fraction prepared according to method I. Details are given in text. Magnification is about 23,500.

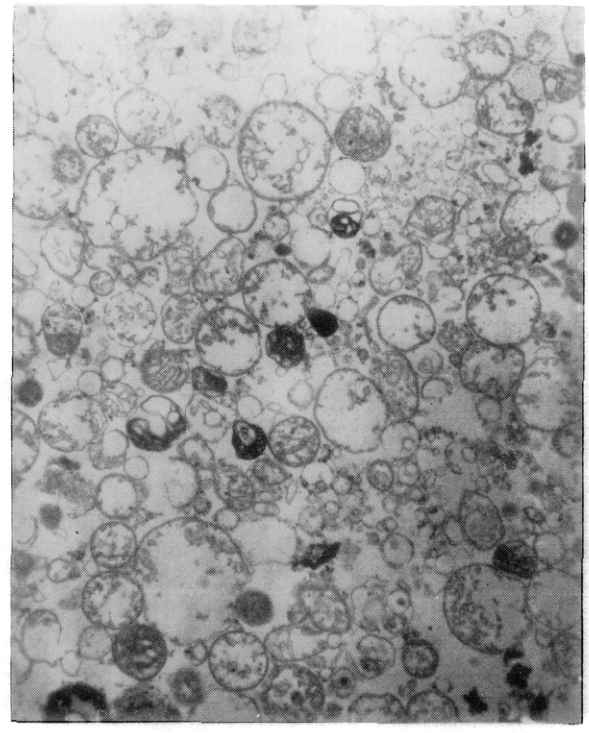

FIGURE 3: Electron micrograph of a mitochondrial fraction prepared by method II. Details are given in the text. Magnification is about 11,000.

In most experiments very little loss of radioactivity occurred during these washing steps. The final sediment was suspended in 5 mM Tris-HCl (pH 7.6) to obtain 2–3 mg of protein/ml and each 15 ml of suspension was layered over 20 ml of 1.3 M sucrose containing 5 mM Tris and centrifuged at 78,000*g* for 16 hr. (Ordinarily 3–4 hr of centrifugation was adequate and the longer time was used only for convenience.) The smooth microsomal fraction formed dispersed whitish flocculated particles at the interphase region and the rough microsomal fraction appeared as a loose sediment. The smooth microsomal fraction was separated from the pellet by careful aspiration, diluted threefold with 5 mM Tris-HCl buffer (pH 7.6), and centrifuged for 90 min at 78,000*g*. The rough microsomal fraction was suspended in 5 mM Tris buffer containing 1 mM $MgSO_4$ (pH 7.6, 18 ml/pellet) and centrifuged as the smooth microsomal suspension. At the end of this centrifugation the smooth microsomes formed a tight white pellet, while the rough microsomal sediment was slightly brownish. Both of the sediments were suspended in 5 mM Tris-HCl (pH 7.6) to get 5–10 mg of protein/ml and used either directly for analysis, or after storage at −15°.

Isolation of Smooth and Rough Microsomes. METHOD III. The procedure of Dallner (1963) described for the isolation of smooth- and rough-surfaced microsomes of rat liver was adopted. The 16,000*g* supernatant, prepared by method A, was adjusted to 10 mM CsCl by adding solid CsCl and each 15 ml of this was layered over 20 ml of a 1.3 M sucrose containing 10 mM CsCl and 5 mM Tris-HCl (pH 7.6). The mixtures were centrifuged for 16 hr at 78,000*g*. The smooth and rough microsomes were separated and washed with Tris-HCl buffer as described in method II.

Isolation of Nuclei, Mitochondria, and Residual Microsomal Fractions. The 16,000*g* sediment, obtained after the third homogenization by method B, was suspended in 0.25 M sucrose–medium M to get 7 mg of protein/ml, and to each milliliter of the suspension 2 ml of 2.3 M sucrose–medium M and on top of this 5 ml of 0.25 M sucrose–medium M was applied. The mixtures were centrifuged for 60 min at 78,000*g* (Blobel and Potter, 1966). Slightly brownish particles were floating at the two interphase regions and a grayish pellet was at the bottom and also on the side of the centrifuge tube. The suspended particles were separated by aspiration and diluted fivefold with medium M. The sediments, both from the bottom and the side, were combined by suspending in medium M (20 ml/pellet). The three suspensions, so obtained, were centrifuged for 20 min at 1000*g*. The 1000*g* sediments were combined by suspending them in medium M to obtain 5–10 mg of protein/ml and this was used as the nuclear fraction. The 1000*g* supernatants were centrifuged further at 16,000*g* for 20 min. The brownish sediments were suspended in medium M and this was termed as the mitochondrial fraction. The 16,000*g* supernatants were centrifuged at 78,000*g* for 1 hr. This caused the sedimentation of the residual

microsomal fraction, which was occasionally analyzed for smooth and rough microsomal contents by method II.

Isolation of Postmicrosomal Particles. The 78,000*g* supernatant obtained by method II was centrifuged at 78,000*g* for 16 hr. The very fine transparent pellet (adhering strongly to the centrifuge tubes) was suspended in 5–10 ml of medium M and was named as the "postmicrosomal particles."

Miscellaneous Fractions. When the 16,000*g* sediment was not fractionated further it was suspended in the 0.25 M sucrose–Tris buffer for analysis. The supernatant fraction in method III included the supernatant aspirated from above the smooth microsomal layer; the supernatant of the smooth microsomes obtained after the 90-min centrifugation; and the supernatant of the rough microsomes and the 1.3 M sucrose layer remaining between the smooth microsomal layer and rough microsomal pellet. Occasionally these supernatants were analyzed individually.

Analysis of Membranes by Sucrose Gradient Centrifugation. Linear sucrose gradients were prepared in centrifuge tubes for the SW 25.1 rotor of the Spinco Model L centrifuge with a mixing device similar to that described by Britten and Roberts (1960). Centrifugation was carried out for 16 hr at 63,600*g*. Tubes were punctured, and 1–1.2-ml fractions were collected and analyzed for optical density at 280 mμ, radioactivity, and density. The densities were measured by using a 500-μl calibrated micropipet. In all experiments samples were analyzed directly after the completion of the isolation steps.

Electron Microscopy. Pellets of given fractions were sampled with a spatula and fixed by a 1% osmium tetroxide solution containing Veronal buffer (pH 7.4; Palade, 1952). Sections were double "stained" with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958). These procedures, the viewing, and photography were carried out by personnel of the Electron Microscopical Laboratory of the Medical College, using a RCA Model EMU 3H or EMU 4D electron microscope.

Enzymatic Assays. Adenosinetriphosphatase activity was determined as described by Wallach and Ullrey (1964). *DPNH-diaphorase activity*, with potassium ferricyanide as a substrate, was estimated according to Kamat and Wallach (1965) and the results are expressed in terms of initial rates determined from the slope of the rate curves and also by double-reciprocal plots made according to Foster and Niemann (1953). These plots indicated that the initial rates were about 70% of maximal rates and the K_M was 3.7×10^{-5} M^{-1} for DPNH[1] by using the mitochondrial, smooth, or rough microsomal fractions as enzymes. *Acid phosphatase activity*, using β-glycerophosphate as a substrate, was estimated according to Appelmans *et al.* (1955). *Phosphatase activity*, with *p*-nitrophenyl phosphate as a substrate, was determined as described by Linhardt and Walter (1965), with modifications. Reactions were carried out in a 1-ml volume containing 0.8 ml of Veronal–acetate buffer (prepared according to Michaelis (1931) with the modification that it contained 0.03 M KCl and 0.06 M NaCl), 10 mM *p*-nitrophenyl phosphate, 5 mM $MgSO_4$, and the enzyme. The reaction was conducted at 37° for 30 min and stopped by adding 4 ml of 0.1 M NaOH. The color was read at 400 mμ. Under these conditions two pH optima were observed for the liberation of *p*-nitrophenol; one was between 5.2 and 6.2 and the other between 7.2 and 7.8. Magnesium enhanced the reaction rate at pH 7.4 and reduced it somewhat at acidic pH values.

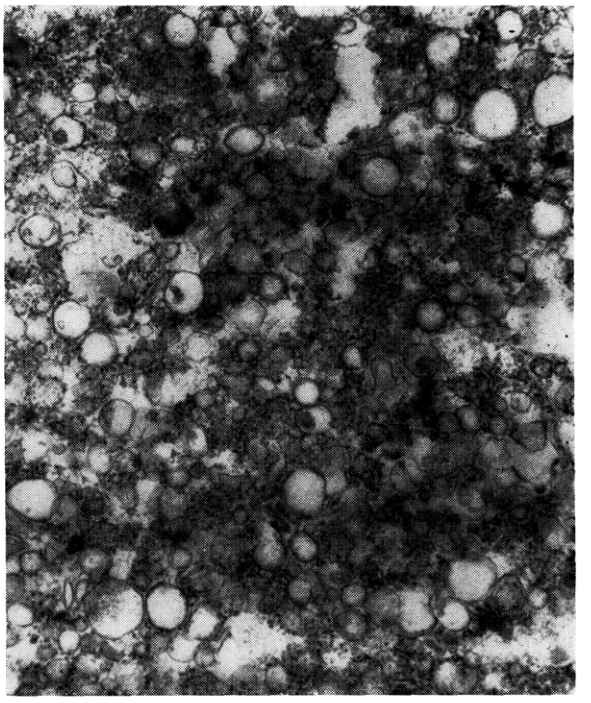

FIGURE 4: Electron micrograph of a rough microsomal fraction prepared by method II. Details are given in the text. Magnification is about 23,500.

All the enzymatic determinations were carried out at three different enzyme concentrations and the reported values are averages of these measurements.

Chemical Analysis. Protein was determined by either the biuret reaction (Gornall *et al.*, 1949) or according to Lowry *et al.* (1951). *Hexosamines* were purified and determined as described by Boas (1953). These carbohydrates were released by hydrolysis with 3 N HCl at 100° for 4 hr. The hexosamines were analyzed occasionally on the short, or long, column of a Technicon automatic amino acid analyzer (Piez and Morris, 1960). To carry out the Morgan–Elson (1934) reaction for *N-acetylhexosamine* determination, hexosamine-containing samples (after purification by Dowex 50 resin) were *N*-acetylated as described by Levvy and McAllan (1959) and estimated according to Reissig *et al.* (1955). *Sialic acid* was released from

[1] Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; ATPase, adenosine triphosphatase.

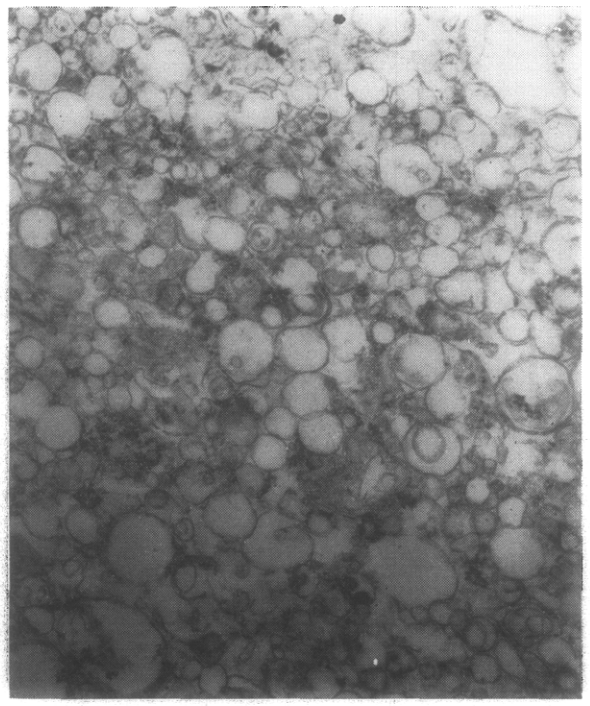

FIGURE 5: Electron micrograph of a smooth microsomal fraction prepared by method II. Details are given in the text. Magnification is about 23,500.

given samples by hydrolysis with 2.5% trichloroacetic acid at 80° for 1 hr. The hydrolysates were purified further by chromatography on a Dowex 1-Cl column (Molnar *et al.*, 1965c), and sialic acid was estimated according to Warren (1959). *Inorganic phosphate* was determined according to Fiske and Subbarow (1925). *RNA* was measured by the orcinol reaction and *DNA* by the diphenylamine reaction (Volkin and Cohn, 1954). For standardization yeast RNA (NBC) and thymus DNA (Sigma) were used; the weights of these nucleic acids were corrected for moisture and cations. These standards were compared with either DNA isolated from the nuclear fraction of Ehrlich cells by using saturated NaCl for extraction (Volkin and Cohn, 1954) or with RNA isolated from the rough microsomal fraction of Ehrlich cells according to Kirby (1956). The following analytical values were obtained and used in calculating the purified nucleic acids of Ehrlich cells: $E_{260\ m\mu}$(P) for DNA is 7800 and for RNA $E_{260\ m\mu}$(P) is 9100; μg of DNA/ml per $OD_{260\ m\mu}$ is 34 and μg of RNA/ml per $OD_{260\ m\mu}$ is 32.

Measurement of Radioactivity. Samples of subcellular fractions were precipitated with trichloroacetic acid and dissolved in 0.2 N sodium hydroxide. Aliquots of these solutions were used for protein determination and counting in a liquid scintillation system (Molnar *et al.*, 1965c). Radioactivity of sialic acid, hexosamine, and fractions from sucrose density centrifugation were assayed by using 0.2–0.5-ml aliquots of given samples in the counting system.

Results

Incorporation of Glucosamine-^{14}C into Cell Components Separated According to Kamat and Wallach. METHOD I. In this procedure glucosamine-^{14}C-labeled microsomes were isolated first and then washed with hypotonic buffers. The plasma membrane fraction was separated from the vesicles of endoplasmic reticulum by density centrifugation using a dense Ficoll solution. The centrifugation was carried out in the presence of a magnesium salt, which rendered the endoplasmic reticulum fragments to aggregate and sediment while the material suspended at the interphase region was derived mainly from the plasma membranes. Electron microscopic observations indicated that the suspended fraction was composed mainly of membranes with diameters between 200 and 500 mμ and of some vesicles of about 80 mμ in diameter (Figure 1). The sediment on the other hand (Figure 2) contained mainly smaller vesicles and lots of electron-dense small particles (some of which may have been derived from the fragmentation of nuclei which occurred during homogenization). The specific radioactivity of the "plasma membrane" fraction (related to protein) was about three times larger than that of the sediment in all six experiments carried out by this procedure. The specific activity of the 16,000*g* sediments were considerably smaller than that of the microsomal subfractions (Table I). The total amount of radioactivity, sialic acid, and hexosamine of the plasma membrane rich fraction was always less than that of the sediment. Some of the radioactivity (and also sialic acid and hexosamine) could be extracted from the sediment by recycling it in the separation procedure. In none of the experiments could we get a better yield in the plasma membrane rich fraction than 22% of the total incorporated radioactivity. This was partially due to coprecipitation with the endoplasmic reticulum fragments; however the major loss was present in the 78,000*g* supernatant and in the washing solutions of microsomes.

Properties of Smooth and Rough Microsomal Fractions Isolated by Method II. In the previous method EDTA was added to the cell suspension after homogenization to prevent aggregation of microsomes caused by bivalent cations. In method II the homogenization of cells and isolation of microsomes were carried out in the presence of sodium, potassium, magnesium, and Tris salts and EDTA was not used. The microsomes were washed subsequently with hypotonic buffers, as in method I, except magnesium sulfate was also included to minimize dissociation of ribosomes from membranes. The washed microsomal fractions contained regularly 50–60% of the incorporated radioactivity. The separation of smooth and rough microsomes was achieved by sucrose density centrifugation. Addition of a magnesium salt was omitted in this step since this would have caused sedimentation for most of the smooth microsomal membranes. After separation and isolation the smooth microsomal fraction contained reproducibly about 40% of the

TABLE I: Distribution of Radioactivity among Subcellular Fractions Using Ficoll Density Centrifugation for Separation.

Fraction	mg of Protein/ml of Cells[a]	dpm/mg of Protein[b]	dpm/ml of Cells
16,000*g* sediment	12.5 (6.7–15.8)	1,120	14,000
Endoplasmic reticulum fragments	16.1 (12.6–18.1)	5,200	83,700
Plasma membranes	3.0 (1.3–4.1)	15,000	45,000
Supernatant	50.0	2,600	130,000

[a] Average values and ranges obtained in six experiments. [b] Data obtained in a representative experiment. Cells were incubated and subcellular fractions were isolated as described in Experimental Procedures according to method I.

TABLE II: Distribution of Protein, Hexosamine, Sialic Acid, and Radioactivity among Subcellular Fractions Obtained by Method II.[a]

Fraction	mg of Protein	mμmoles of HN	mμmoles of SA	% of Whole Cells			
				Protein	HN	SA	Counts
Cells	110	1540	440				
16,000*g* sediment	19 (13–25)	400	152	17.2	26.0	34.5	14.5
"Rough" microsomes	7.8 (7.1–9.1)	168	43	7.1	10.9	9.8	9.4
"Smooth" microsomes	5.6 (4–6.5)	560	168	5.1	36.3	38.1	40.0
Supernatant	54.5 (46–60)	134	54	49.5	8.7	12.3	16.0
Postmicrosomal sediment	16.0	200		14.5	13.0		5.0
Total recovered				93.4	94.9	94.7	84.9

[a] Related to 1 ml of packed cells. The results are average values of eight experiments. Cells were incubated for 1 hr with glucosamine-^{14}C and homogenized, and subcellular fractions were isolated as described in the Experimental Procedures. [b] HN, hexosamine; SA, sialic acid.

cellular hexosamine, sialic acid, and radioactivity and 5.1% of protein (Table II). About 10% of radioactivity, hexosamine, and sialic acid and 7.1% of protein were present in the rough microsomal fractions. The smooth microsomal fraction and the 16,000*g* sediment had two to three times more protein and radioactivity than the corresponding fractions obtained by method I. The hexosamine, sialic acid, and radioactivity contents of the rough microsomal fraction and supernatant, however, decreased considerably.

In three experiments the 16,000*g* sediment, remaining after the third homogenization, was fractionated further into nuclear, mitochondrial, and "additional microsomal" fractions. The radioactivity of the 16,000*g* sediment was about equally distributed among these fractions (Table III). This table also shows the protein and hexosamine specific activities of all the subcellular fractions obtained in a representative experiment. The smooth microsomal fraction contained more than four times larger protein specific activity than the rough microsomal fraction, and the supernatant fraction had the lowest activity. The high specific activity of the "additional microsomal" fraction indicated that this may have contained a large percentage of the smooth microsomal variety. When this fraction was subjected to sucrose density centrifugation about 70% of its radioactivity appeared in the smooth microsomal region. Thus by such manipulations the yields in radioactivity, hexosamine, and sialic acid of the smooth microsomal fraction could be increased close to 50%.

The hexosamine specific activities of the smooth and rough microsomal fractions were about the same. The lowest hexosamine specific activity was found for the mitochondrial fraction and the largest for the supernatant, while the nuclear fraction had intermediate values. The supernatant fraction in this experiment was composed of the 78,000*g* supernatant and of the combined microsomal washing solutions.

When viewed by electron microscope, the nuclear fraction contained mainly electron-dense nuclear fragments, a few mitochondria, and some microsomal membranes. Most of the cellular DNA was retained within these fragments, probably due to the presence

TABLE III: Protein and Hexosamine Specific Activities of Subcellular Fractions Obtained by Method II.[a]

Fractions	mg of Protein	dpm Total	dpm/mg of Protein	dpm/μmole of HN
Cells	100	170,000	1,700	103,000
Nuclei	15	12,000	780	74,000
Mitochondria	4.8	10,000	2,100	47,000
Additional microsomes	1.8	11,000	6,100	
Rough microsomes	7.6	19,000	2,500	95,000
Smooth microsomes	6.5	71,000	11,000	95,000
Supernatant	66.0	49,000	750	165,000

[a] Washed Ehrlich cells were incubated with glucosamine-^{14}C for 1 hr and homogenized, and subcellular fractions were isolated. Data are related to 1 ml of packed cells. The additional microsomes were mainly smooth microsomes as judged by sucrose density centrifugation.

of magnesium sulfate, and no difficulty was encountered in suspending this fraction. However, if such nuclear suspensions were frozen and thawed, the DNA formed a gel and suspending was impossible.

The mitochondrial fraction appeared fairly homogeneous (Figure 3). A few smaller and larger membranous elements representative of the microsomal fractions were visible.

The rough microsomal fraction (Figure 4) contained numerous electron-dense particles. Some of these were free and many were attached to the vesicles. The size of the vesicles varied appreciably and a few very large "smooth" membrane fragments could be identified.

The smooth microsomal fraction was also very heterogeneous in size distribution. Membrane fragments 60–500 mμ in diameter were present and a few rough microsomes and free ribosomal particles were also visible (Figure 5).

Sucrose density gradient centrifugation of smooth microsomes revealed one radioactive peak which was located at d = 1.15 g/ml (Figure 6) and coincided with the protein peak (measured by ultraviolet absorption at 280 mμ). The specific activity (counts per minute per $OD_{280\ m\mu}$) was, however, higher at lighter densities than at heavier ones, thus indicating heterogeneity.

The rough microsomal fractions under similar conditions had three radioactive peaks. About two-thirds of the radioactivity was present in the region of the first peak (d = 1.206 g/ml). The second radioactive peak area, containing about 10% of activity, was at the same region as the smooth microsomal peak (d = 1.16 g/ml), and the third radioactive area was in the top layer (Figure 7).

The nucleic acid contents of the various subcellular particles are shown in Table IV. In this experiment the nuclear fraction contained 61% of the cellular DNA and 11% of RNA. Some contaminating DNA was present in all the other fractions in the range of 4.7–9.5%. The largest amount of RNA (54%) was present in the supernatant fraction (most of which could be sedimented if this fraction was centrifuged overnight at 78,000g). The rough microsomal fraction contained 35% of the cellular RNA while the smooth microsomal fraction had only 5.5% of it.

The sodium–potassium–magnesium-activated ATPase was suggested by Kamat and Wallach (1965) as a characteristic enzymatic activity of plasma membranes. For this reason this activity was determined under the conditions used by the above authors. Table V shows the results obtained in a number of experiments for the smooth and rough microsomal fractions. The average value of specific ATPase activity of smooth microsomes was 6.4 (micromoles of P_i per hour per milligram of protein) which could be compared with 7.95 specific activity of "pure" plasma membranes of Kamat and Wallach. The specific activity of rough microsomal fraction was 2.8, considerably larger than the value of 0.74 reported by Kamat and Wallach for their endoplasmic reticulum fragments (free of ribosomes). Since the rough microsomal fractions in our

TABLE IV: DNA and RNA Content of Subcellular Fractions Obtained by Method II.[a]

Fraction	mg of DNA/ml of Cells	%	mg of RNA/ml of Cells	%
Cells	3.2		5.4	
Nuclei	1.96	61	0.6	11.0
Mitochondria	0.2	6.2	0.2	3.7
Rough microsomes	0.3	9.3	1.9	35.1
Smooth microsomes	0.15	4.7	0.3	5.5
Supernatant	0.3	9.3	2.9	53.6

[a] Cells were incubated for 1 hr with glucosamine-^{14}C and homogenized, subcellular fractions were isolated, and the nucleic acid contents were determined as described in the Experimental Procedures.

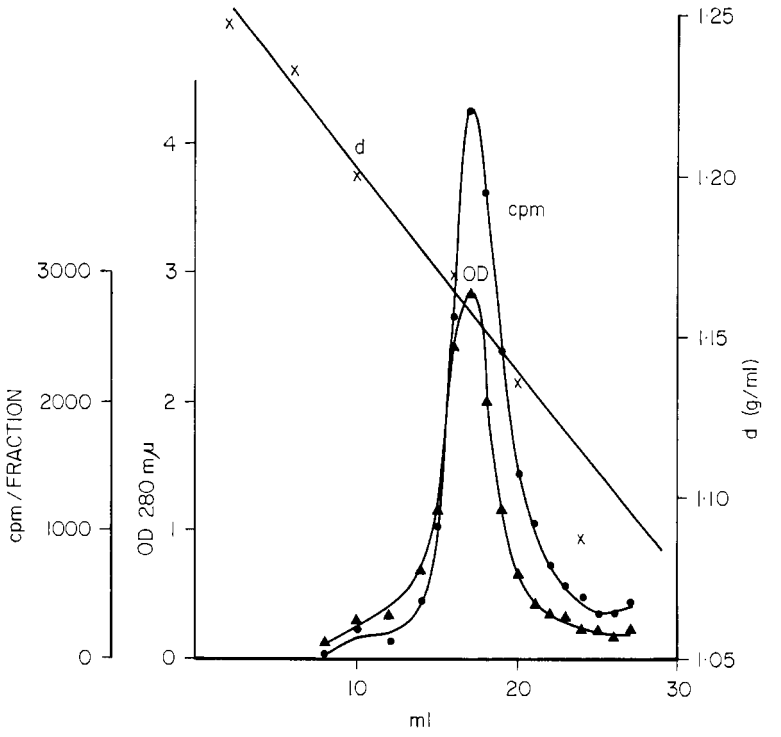

FIGURE 6: Sucrose density gradient centrifugation of a smooth microsomal fraction prepared by method II. Linear sucrose density gradient was prepared by mixing 12 ml each of 1 and 2 M sucrose solutions, both containing 1 mM Tris-HCl (pH 7.9). On top of the gradient 5 ml of a smooth microsomal fraction (3 mg of protein) was layered. The mixture was centrifuged for 16 hr at 63,600*g* at 0°.

TABLE V: Enzymatic Activities of Subcellular Fractions Prepared by Method II.[a]

Fraction	ATPase (μmole/P_i hr mg of Protein)	Prepn	DPNH-diaphorase (μmole/hr mg of Protein)	Prepn	Phosphatase (μmole/P_i hr mg of Protein)[b] pH 5.2	pH 7.5	Prepn
Rough microsomes	2.8 (2.4–3.3)	6	114	3	1.9	1.1	2
Smooth microsomes	6.4 (7.2–5.6)	6	95	3	3.2	1.4	2

[a] Cells were incubated with glucosamine-^{14}C and homogenized, and subcellular fractions were isolated. The enzymatic activities were determined as described in the Experimental Procedures. [b] *p*-Nitrophenyl phosphate was used as a substrate.

experiments contained about 25% of ribosomal protein the specific ATPase activity of ribosome-free membranes could be as high as 3.7 (assuming that the ribosomes do not contain such an activity).

The DPNH-diaphorase activities of smooth and rough microsomes were very similar in our experiments (Table V) while the plasma membrane fractions of Kamat and Wallach had about eight times less specific activity than the endoplasmic reticulum fractions.

These experiments supported the electron microscopic observations that the smooth microsomal fractions prepared by method II were more heterogeneous than those obtained by method I.

In a few experiments the phosphatase activities of smooth and rough microsomal fractions were determined. In the presence of sodium, potassium, and magnesium salts both of the fractions catalyzed the hydrolysis of *p*-nitrophenyl phosphate. Two pH optima were found for this reaction (about 5.2 for one and 7.5 for the other). The reaction at the acidic pH was faster than at the slightly basic pH. Reactions with β-glycerophosphate as a substrate were not measurable at pH 5.

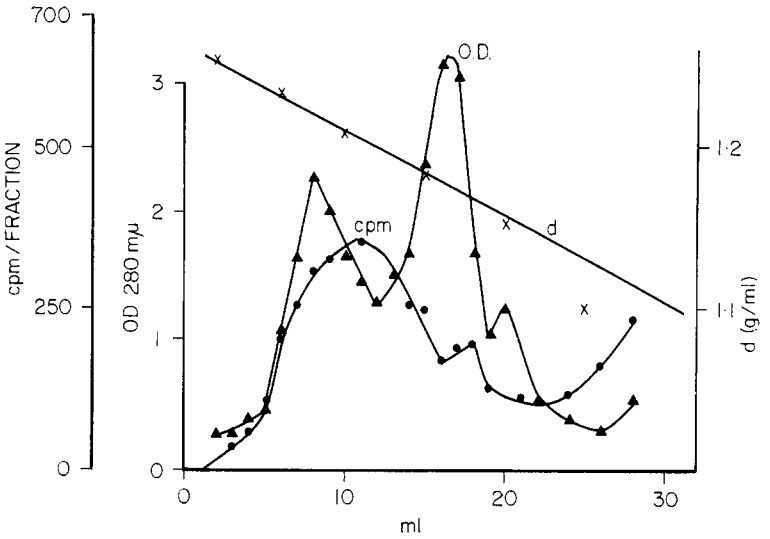

FIGURE 7: Sucrose density gradient centrifugation of a rough microsomal fraction prepared by method II corresponding to the smooth microsomal preparation of Figure 6. On top of linear sucrose density gradient 5 mg of protein of the rough microsomal fraction was applied.

Properties of Smooth and Rough Microsomal Fractions Isolated by Method III (Dallner's (1963) Procedure). The protein, hexosamine, sialic acid, and radioactivity contents of subcellular fractions isolated by this method are shown in Table VI. The smooth and rough microsomal fractions contained about equal amounts of hexosamine, sialic acid, and radioactivity falling in the range of 17–25.6%. The protein content of the rough microsomal fraction was about three times larger than that of the smooth microsomal fraction. The least amounts of radioactivity and also hexosamine and sialic acid were present in the 16,000*g* sediments. The combined supernatants had high amounts of radioactivity (45%). This could be due to incomplete sedimentation of microsomes during the isolation and washing steps, or disintegration of membranes through physical or enzymatic processes.[2]

When viewed by the electron microscope the smooth and rough microsomal fractions resembled the respective fractions isolated by method I.

The Identity of Hexosamine of Smooth and Rough Microsomal Fractions. Using either the long or short column of the automatic amino acid analyzer, the only amino sugar found in the rough and smooth microsomal fractions was glucosamine. Cook *et al.* (1965) and Langley and Ambrose (1967) have reported the presence of galactosamine in the smooth microsomes or in the cell surface mucopeptides of Ehrlich cells; our results therefore were in disagreement with these authors'. To ascertain the identity of hexosamine in our preparations a smooth microsomal fraction was hydrolyzed to release hexosamine and analyzed, after purification by Dowex 50-H^+, for hexosamine by the Elson–Morgan reaction and for *N*-acetylhexosamine, after acetylation with acetic anhydride, by the Morgan–Elson reaction. Glucosamine and galactosamine gives equal color values in the Elson–Morgan reaction, but the color value for *N*-acetylglucosamine is about two times higher than that of the *N*-acetylgalactosamine in the Morgan–Elson reaction and thus it is possible to distinguish between these two hexosamines (Levvy and McAllan, 1959). Table VII shows that equal color values were obtained in both of the reactions for the hexosamine of smooth microsomes. This supported the results of the amino acid analyzer, namely the presence of glucosamine and absence of galactosamine in the microsomal fractions.

Discussion

The ubiquitous presence of carbohydrate-containing macromolecules in the membranes of mammalian tissues have been shown recently by Rambourg and Leblond (1967). The carbohydrates may be bound to lipids (*e.g.*, gangliosides and certain blood group substances) or to proteins (glycoproteins or muco-

[2] When the centrifugation time of the sucrose density step was reduced from 16 to 4 hr the yields in smooth microsomes increased substantially while much less radioactivity occurred in the supernatants. Increasing the centrifugation time from one to several hours for sedimenting the smooth and rough microsomes after their separation resulted in the decrease of radioactivity in the supernatants. In many experiments, in which 1-hr centrifugation was used for sedimentation, the supernatant of smooth microsomes had two to three times higher protein-specific activity than the supernatant of the rough microsomes, or the supernatant aspirated from above the smooth microsomal layer after the sucrose density centrifugation step. In one experiment the separation was carried out at 7° instead of 0°. This caused very low yields of radioactivity in the particulate fractions. These experiments suggested that all the possibilities mentioned above (inadequate sedimentation time as well as physical and chemical disintegration) may explain the high radioactivity content of the supernatant fractions.

TABLE VI: Distribution of Protein, Hexosamine, Sialic Acid, and Radioactivity among Subcellular Fractions Isolated by Sucrose CsCl Density Centrifugation.[a]

Fraction	mg of Protein	mμmoles of HN	mμmoles of SA	% of Whole Cells			
				Protein	HN	SA	dpm
Cells	110 (90–126)	1560	440				
16,000*g* sediment	10.6 (8.5–13.7)	110	23	9.5	7.1	5.2	4.5
"Rough" microsomes	12.3 (10–15)	320	75	11.2	20.5	17.0	18.0
"Smooth" microsomes	3.8 (2.04–5.7)	390	113	3.4	25.0	25.5	24.7
Supernatant	53.0 (50–55)	530	110	48.0	34.0	25.0	45.0
Total recovered					86.6	72.7	92.2

[a] Washed Ehrlich cells were incubated with glucosamine-^{14}C for 1 hr and homogenized, and subcellular fractions were isolated by method III as described in the Experimental Procedures. The results are average values of seven experiments. Data are related to 1 ml of packed cells.

TABLE VII: Identification of the Hexosamine Constituent of Smooth Microsomes by the Elson–Morgan (E–M) and Morgan–Elson (M–E) Reactions.

	Hexosamine Std	μmoles of Hexosamine/mg of Protein		OD_{530}/ml	OD_{544}/ml
		E–M	M–E	E–M	M–E
Sample	Glucosamine[a]	0.094	0.095	0.64	1.14
Sample	Galactosamine[a]	0.105	0.300		
				OD_{530}/μmole	OD_{544}/μmole
	Glucosamine			2.25	3.82
	Galactosamine			2.33	1.28

[a] Standards used for calculation of micromoles from optical density.

proteins). The glycoproteins of plasma membranes have been suggested to participate in the mutual recognition and adhesiveness of like cells to form tissues (Margoliash *et al.*, 1965; Rambourg and Leblond, 1967). The red cell stroma glycoproteins are known to be specific receptor sites of various viruses and may have antigenic activities (Uhlenbruck, 1964). Sialic acid bound to macromolecules of liver plasma membranes may play a role in a certain phosphatase activity (Emmelot and Bos, 1966). Increased sialic acid content of malignant cells, compared to related normal tissues, may be responsible for the decreased affinity of these cells to each other and for the metastasis of many malignant cell types (Ambrose, 1966). These observations suggest that carbohydrate-containing macromolecules have important roles in the structure and function of mammalian membranes.

With the exception of the glycoproteins of red cell stroma, not much is known about the nature and composition of membrane-bound glycoproteins of mammalian tissues. Owing to the scarce knowledge of the nature, structure, quantity, distribution, and function of membrane-bound glycoproteins of mammalian tissues it seems desirable to investigate more thoroughly such constituents. Ehrlich ascites carcinoma cells were selected for such a purpose in the present studies because such glycoproteins could be labeled specifically with glucosamine-^{14}C and sialic acid-^{14}C (derived intracellularly from glucosamine-^{14}C) and the radioactivity could be used as a convenient tool to purify subcellular fractions.

Relying on current views, presented in the introductory section, we were expecting about 70% of the cellular sialic acid, hexosamine, and radioactivity to be associated with the plasma membranes of Ehrlich cells. We have investigated three methods to separate quantitatively the various membranous elements and in none of them could we obtain a fraction, rich in plasma membranes, which reached the desired 70% value. The best yields were about 40%, which were obtained by method II. Considering that losses due to incomplete sedimentation during isolation and washing steps should have occurred, that additional microsomes could be extracted from the 16,000*g* sediments by sucrose density centrifugation, and that cross-contaminations with other fractions were still possible, the total

TABLE VIII: Comparison of Some of the Properties of "Smooth" and "Rough" Microsomal Fractions Obtained by the Three Methods Applied in This Work.

	dpm/mg of Protein	mg of Protein/ml of Cells	% of Total Counts[a]	mμmole/mg of Protein HN	mμmole/mg of Protein SA	ATPase (units/mg of Protein)
		Method I				
Endoplasmic reticulum fragments	5,200	16.1	31		14	0.74[b]
Plasma membranes	15,000	3.0	16		27	7.9[b]
		Method II				
"Rough"	2,500	7.8	9	22	5.5	2.8
"Smooth"	11,000	5.6	40	100	30	6.4
		Method III				
"Rough"	2,200	12.3	18	27	6.1	
"Smooth"	8,000	3.8	25	100	30	

[a] Related to cells. [b] Kamat and Wallach's data; see details in text.

quantities actually present in the smooth microsomal structures could be more than 60%. The major losses in the other two methods occurred toward the supernatant fractions probably due to physical disintegration of membranes and also to some enzymatic degradations.

For comparative purposes some properties of "smooth" and "rough" microsomal fractions obtained by the three methods are summarized in Table VIII. In all three of these methods the protein-specific activities of the "smooth" microsomal fractions were three to four times larger than the "rough" microsomal fractions, regardless whether isolated from cells incubated for 1 or 2 hr with glucosamine-^{14}C (see Tables I and III). This latter point is emphasized because Cook *et al.* (1965) have reported that the specific activities of smooth and rough microsomes approached each other after 2-hr incubation of intact cells with glucosamine-^{14}C. This discrepancy may be explained by the poor separation of smooth and rough microsomes observed under certain conditions. The hexosamine and sialic acid contents of "smooth" microsomes, related to protein, were similar for all three preparations (about 30 μmoles/mg of protein of sialic acid and 100 μmoles/mg of protein of hexosamine). The sialic acid content of the endoplasmic reticulum membrane fraction obtained by method I was about one-half as much as that of the corresponding plasma membrane fractions, in agreement with the values of Wallach and Kamat (1966). The sialic acid content of the rough microsomal fractions isolated by methods II and III, on the other hand, were about five times less than those of the corresponding smooth microsomal fractions. Assuming that sialic acid is associated with the plasma membranes mainly (Wallach and Eylar, 1961), the data would indicate that the endoplasmic reticulum membrane fractions obtained by method I were more contaminated with plasma membrane fragments than those isolated by the other procedures.

Electron microscopic pictures of smooth microsomes isolated by method II suggested more heterogeneity in size and quality of membranous fragments than those isolated by the other two methods. Assuming that the larger pieces of membrane (200–500-mμ diameter) represented plasma membrane fragments (Wallach *et al.*, 1966) and the smaller ones (about 80-mμ diameter) were derived mainly from the smooth endoplasmic reticulum, the pictures would suggest that comparable quantities of these two membranous structures were present in these smooth microsomal fractions. Since the sialic acid content of pure plasma membranes was 28 mμmoles/mg of protein (Wallach and Kamat, 1966) and that of smooth microsomes (method II) were 30 mμmoles/mg of protein we had to assume that both the plasma membranes and the smooth endoplasmic reticulum membranes contained about equal quantities of sialic acid (per milligram of protein).

The Na–K–Mg-activated ATPase activity of Ehrlich cells was suggested by Kamat and Wallach (1965) to be associated mainly with plasma membranes. In their preparations the plasma membrane fragments had 7.9 units/mg of protein and the endoplasmic reticulum membrane fractions (including the rough and smooth endoplasmic reticulum membranes) had 0.74 unit/mg of protein of ATPase activity. The specific ATPase activity of smooth microsomes (method II) was 6.4 units/mg of protein. Assuming that the values of Kamat and Wallach represent correct activities of the two different membranous fractions, the 6.4 value would suggest about 80% purity in respect to plasma membranes. However, in our experiments the rough microsomal fractions (method II) had 3.7 units

of ATPase activity/mg of protein (after correction for 25% ribosomal content); therefore the above assumption could not be held. We assumed therefore that the fragments of smooth endoplasmic reticulum membranes had about the same ATPase activity as the rough ones. Taking 3.7 units/mg of protein for the smooth endoplasmic reticulum membrane fragments and 7.9 for that of the plasma membranes the 6.4 units/mg of protein of ATPase ndicat d that about 65% of the smooth microsomal fraction (method II) was derived from the plasma membranes. This value was closer to the electron microscopic observations than the 80% above. The small ATPase activity of the endoplasmic reticulum membrane fractions reported by Kamat and Wallach might have been due to partial inactivation of the enzyme(s) during the isolation procedure. We have found such inactivation for the smooth microsomal fraction prepared by method III. These preparations had no measurable ATPase activity although in many respects they behaved as the plasma membrane fraction made by method I.

Hexosamine, sialic acid, and radioactivity were present in the nuclei and mitochondrial fractions in all experiments. Electron microscopic observations suggested the presence of some microsomal membranes in both. However, owing to the lower hexosamine and sialic acid specific activities of these fractions than those of the microsomal subfractions we concluded that these carbohydrates were genuine constituents of the nuclear and mitochondrial membranes.

The hexosamine constituent of smooth and rough microsomal fractions was identified as glucosamine by both the automatic amino acid analyzer and by the Morgan–Elson and Elson–Morgan reactions. This observation was in disagreement with the reports of Langley and Ambrose (1964, 1967) and Cook *et al.* (1965); both of these authors reported the presence of galactosamine in their preparations. The reason for this discrepancy will be investigated.

In conclusion we have found that glucosamine-^{14}C when incubated with Ehrlich cells *in vitro* appeared in their natural glycoprotein constituents; these were mainly localized in the plasma and smooth endoplasmic reticular membranes. The solubilization of smooth microsomal fractions and purification of glycoproteins from these solutions will be described in a following paper.

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References

Ambrose, E. J. (1966), *in* The Biology of Cancer, Ambrose, E. J., and Roe, F. J. C., Ed., Princeton, N. J., Van Nostrand, p 65.

Appelmans, F., Wattiaux, R., and DeDuve, C. (1955), *Biochem. J. 59*, 438.

Blobel, G., and Potter, V. R. (1966), *Science 154*, 1662.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Britten, R. J., and Roberts, R. B. (1960), *Science 138*, 32.

Cook, G. M. W., Laico, M. T., and Eylar, E. H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 247.

Dallner, G. (1963), *Acta Pathol. Microbiol. Scand., Suppl. 166*, 1.

Emmelot, P., and Bos, C. J. (1966), *Biochim. Biophys. Acta 120*, 369.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Foster, R. J., and Niemann, C. (1953), *Proc. Natl. Acad. Sci. U. S. 39*, 999.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hunter, M. J., and Commerford, S. L. (1961), *Biochim. Biophys. Acta 47*, 580.

Kamat, V. B., and Wallach, D. F. H. (1965), *Science 148*, 1343.

Kirby, K. S. (1956), *Biochem. J. 64*, 405.

Langley, O. K., and Ambrose, E. J. (1964), *Nature 204*, 53.

Langley, O. K., and Ambrose, E. J. (1967), *Biochem. J. 102*, 367.

Levvy, G. A., and McAllan, A. (1959), *Biochem. J. 73*, 127.

Linhardt, K., and Walter, K. (1965), Methods in Enzymatic Analysis, Bergmayer, H. U., Ed., New York, N. Y., Academic, p 779.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Margoliash, E., Shenk, J. R., Hargie, M. P., Burshas, S., Richter, W. R., Barlow, G. H., and Moscona, A. A. (1965), *Biochem. Biophys. Res. Commun. 20*, 383.

Michaelis, L. (1931), *Biochem. Z. 234*, 139.

Molnar, J., Lutes, R. A., and Winzler, R. J. (1965a), *Cancer Res. 25*, 1438.

Molnar, J., Robinson, G. B., and Winzler, R. J. (1965c), *J. Biol. Chem. 240*, 1882.

Molnar, J., Teegarden, D. W., and Winzler, R. J. (1965b), *Cancer Res. 25*, 1860.

Morgan, W. T. J., and Elson, L. A. (1934), *Biochem. J. 28*, 988.

Munro, A. J., Jackson, R. J., and Korner, A. (1964), *Biochem. J. 92*, 289.

Palade, G. E. (1952), *J. Exptl. Med. 95*, 285.

Piez, K. R., and Morris, L. (1960), *Anal. Biochem. 1*, 187.

Rambourg, A., and Leblond, C. P. (1967), *J. Cell Biol. 32*, 27.

Reynolds, E. S. (1963), *J. Cell Biol. 17*, 208.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.

Uhlenbruck, G. (1964), *Vox Sanguinis 9*, 377.

Volkin, E., and Cohn, W. E. (1954), *Methods Biochem. Anal. 1*, 287.

Wallach, D. F. H., and Eylar, E. H. (1961), *Biochim.*

Biophys. Acta 52, 594.
Wallach, D. F. H., and Kamat, V. B. (1966), *J. Cell Biol. 30*, 660.
Wallach, D. F. H., Kamat, V. B., and Gaile, M. H. (1966), *J. Cell Biol. 30*, 601.
Wallach, D. F. H., and Ullrey, D. (1962), *Biochim. Biophys. Acta 64*, 526.
Wallach, D. F. H., and Ullrey, D. (1964), *Biochim. Biophys. Acta 88*, 620.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Watson, M. L. (1958), *J. Biophys. Biochem. Cytol. 4*, 475, 727.

Acid–Base Titrations of Tobacco Mosaic Virus and Tobacco Mosaic Virus Protein*

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ABSTRACT: Tobacco mosaic virus (TMV) is reversibly titrated between pH 2.5 and 9.5, and nucleic acid free TMV protein is reversibly titrated between pH 2.7 and 11. Neither an acid nor an alkaline end point is reached within the ranges of reversible titration of either the virus or the protein. The number of groups titrated within the reversible portion of the protein titration indicates that some functional groups of the protein are not exposed to the solvent. Comparison of the protein titration curves at 4 and 20° in the pH range where the protein is known to undergo endothermic polymerization indicates that about one hydrogen ion is bound per protein monomer as a result of polymerization.

Comparison of the protein titrations and the virus titration shows that under conditions permitting polymerization the ionization behavior of the protein resembles that of the virus.

Acid–base titration curves of tobacco mosaic virus (TMV) and TMV protein over a limited range of pH have been published previously (Ansevin *et al.*, 1964). The present study is an extension of the range of pH for the titration curves of both TMV and TMV protein and an extension of the range of temperature for the protein titration.

A unique feature of TMV is that it buffers more strongly in the range pH 7–9 than would be expected on the basis of amino acid content alone, histidine and free α-amino groups being absent (Anderer *et al.*, 1965). Furthermore, TMV protein at 4° exhibits a very strong buffering at about pH 6, caused by the binding of protons when the molecules polymerize into virus-like rods (Ansevin *et al.*, 1964). Estimates of the number of protons bound per monomer of the polymerizing protein have been made by several investigators. In this work a new estimate of this number is made from specific features of the protein titration curves.

Materials and Methods

Virus Preparation. Stock solutions of TMV were prepared by a method essentially the same as that of Boedtker and Simons (1958), involving centrifugation and treatment with EDTA of juice squeezed from Turkish tobacco plants systemically infected with the common strain of TMV.

Protein Preparation. Individual samples of TMV protein were prepared for titration from 2% TMV by a variation of the acetic acid method of Fraenkel-Conrat (1957). RNA was separated from the protein–67% acetic acid mixture by centrifugation at 4–6° for 10 min at 10,000 rpm. The protein was then separated from the concentrated acetic acid on a column of G-25 Sephadex in 0.1% HAc (pH 3.2) (C. L. Stevens, private communication). Effluent samples of about 15 ml were collected in 1 ml of 0.1 M phosphate buffer at pH 7.5, in which the protein precipitates. The tubes containing protein are then easily located and the protein is sedimented at 3000 rpm for 30 min. This step permits gentle packing of the protein and allows the solvent to be poured away. The protein is resuspended in 0.1 M KCl with dropwise addition of 0.1 M KOH to about pH 9. The preparation is then dialyzed against 0.1 M KCl, centrifuged at 40,000 rpm for 1.5 hr to remove aggregated protein, and dialyzed again.

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