ISOLATION AND CHARACTERIZATION OF

GOLGI MEMBRANES FROM BOVINE LIVER

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ABSTRACT

Zonal centrifugation has been used to isolate a fraction from bovine liver which appears
to be derived from the Golgi apparatus. Morphologically, the fraction consists mainly of
sacs and tubular elements. Spherical inclusions, probably lipoproteins, are occasionally
seen in negative stains of this material. The preparation is biochemically unique. UDP-
galactose:N-acetyl glucosamine, galactosyl transferase activity is concentrated about 40-
fold in this fraction compared to the homogenate. Rotenone- or antimycin-insensitive
DPNH– or TPNH– cytochrome c reductase activities are 60–80% of the level of activities
found in microsomes. Purified organelles from bovine liver such as plasma membranes,
rough microsomes, mitochondria and nuclei have negligible levels of galactosyl transferase.
Some activity is present in smooth microsomes but at a level compatible with the possible
presence of Golgi membranes in this fraction. The Golgi fraction does not contain ap-
preciable amounts of enzymes such as ATPase, 5'-nucleotidase, glycosidase, glucose-6-phos-
phatase, acid phosphatase, or succinate-cytochrome c reductase. Similar fractions isolated
from bovine epididymis also have very high levels of galactosyl transferase. The fraction
is heavily osmicated when incubated for long periods of time at elevated temperatures, a
characteristic property of Golgi membranes.

INTRODUCTION

The Golgi system of intracellular membranes has
been described primarily in morphological and
histochemical terms. Attempts at isolation have
been hampered by the lack of any criteria of
purity since no marker enzymes are known which
are localized exclusively in Golgi membranes.

In spite of the inherent difficulty in attempting
such an isolation, several investigators have
partially purified fractions from various sources
which, by morphological criteria at least, appear
to be derived from the Golgi apparatus. Thus,
Kuff and Dalton have described the isolation of
Golgi apparatus from rat epididymis, an organ
in whose epithelial cells Golgi membranes are in

great abundance (1). The fraction contained
many clusters of membranes which appeared to
originate from the Golgi complex but the purity
could not be assessed due to lack of any marker
enzyme activity. Morré and Mollenhauer (2)
have isolated morphologically identifiable Golgi
complexes from stem tissue of onion. Later studies
on the isolation of similar fractions from animal
tissues utilized glutaraldehyde in the homoge-
nizing medium as a stabilizing agent (3, 4). No
biochemical data on these preparations were
presented.

Several functions have, however, been ascribed
to the Golgi complex in situ by histochemical,

biochemical, and morphological techniques which indicate the kind of properties which might be used to identify the organelle after rupture of the cell, when morphological criteria are no longer critical enough to establish identity or purity.

In pancreas, Golgi vesicles mediate the intracellular transport of newly formed zymogens between the rough endoplasmic reticulum and the plasma membrane (5). In parenchymal cells of rat liver, Golgi vesicles have been shown to participate in the formation and secretion of very low density lipoproteins (6). By autoradiography, H^3-galactose has been found to be incorporated preferentially into the Golgi region of cells secreting glycoproteins or mucopolysaccharides (7). Thus, some enzymic functions which may be localized in Golgi membranes would be those involved in lipoprotein or glycoprotein formation and secretion.

The classical means of identifying the Golgi apparatus in situ for light microscopy has been its ability to reduce heavy metal salts. This organelle has been specifically stained for electron microscopy as well. Thus, incubation of tissues for long periods of time with OsO_4 at elevated temperature leads to the heavy deposition of osmium within the cisternae on one side of the Golgi complex (8). The reaction is probably not enzymatic but reflects some unique reductive component present in the Golgi complex, particularly in or on the cisternae of the outer or "immature" face.

In the present study we have attempted to isolate membranes of the Golgi apparatus from bovine liver homogenates and to develop chemical and biochemical criteria of purity which could be applied to these fractions. Recent advances in the elucidation of the steps involved in adding sugar moieties to proteins to form glycoproteins (9) were utilized to determine whether these activities were present in cell fractions known to be rich in Golgi membranes. In addition, impregnation with osmium was carried out in the isolated cell fractions, to see if this property of Golgi apparatus was retained on disruption of the cell. Contamination was estimated by the use of marker enzymes for organelles such as plasma membrane, endoplasmic reticulum, mitochondria and lysosomes.

MATERIALS AND METHODS

Preparation of Golgi

Bovine tissues were obtained from freshly killed animals at the slaughterhouse. They were placed in polyethylene bags and packed immediately in ice. The fractionation was begun within 1 hr of the death of the animal.

The liver was trimmed of connective tissue and ground in a small meat grinder. The mince (330 gms) was suspended in 900 ml of 0.5 M sucrose containing 0.1 M sodium phosphate buffer, pH 7.2, and 1% Dextran 500 (Pharmacia Fine Chemicals, New Market, N.J.). All sucrose solutions were prepared from "Ultra-Pure" sucrose from Mann Research Labs Inc., New York. The mixture was homogenized briefly (3 full strokes) with a Potter-Elvehjem homogenizer with a Teflon pestle with a known clearance of 0.026 inches at 1000 rpm. The final pH was 7.1. All centrifugations were made in a Spinco ultracentrifuge and all operations were carried out at 4°C. The mixture was centrifuged for 30 min at 10,000 rpm in a #30 head. The supernatant was poured through 4 layers of cheesecloth and centrifuged as before for 30 min at 20,000 rpm. The supernatant was discarded and any lipid adhering to the tubes was wiped away. The pellets were suspended in 53% sucrose containing 0.1 M sodium phosphate buffer, pH 7.1, by using a 50 ml Dounce homogenizer with an A size pestle. Any dark brown material at the bottom of the pellet was discarded. The final concentration of sucrose was adjusted to 42.7% by means of a Bausch and Lomb Abbe 3L refractometer (Bausch and Lomb Incorporated, Rochester, N.Y.). The next step involved zonal centrifugation. All sucrose solutions were adjusted to the proper density with the refractometer and the pH was adjusted to 7.1 with NaOH. The step-gradient was prepared with 16.0% and 37.3% sucrose and a Spinco gradient-maker was programmed to give the following series of sucrose concentrations in the rotor: 230 ml of 16.0%, 120 ml of 23.9%, 120 ml of 34.5%, and 180 ml of 37.3% sucrose. The gradient was placed in the B-14 rotor while it was spinning at 5000 rpm. The sample (40–50 ml in 42.7% sucrose containing 0.1 M sodium phosphate, pH 7.1) was introduced into the periphery of the rotor, followed by 100 ml of 53% sucrose as a cushion. The gradient was centrifuged for a total of 45 min at 35,000 rpm and the rotor

was then unloaded from the center by pumping 55% sucrose into the periphery. Fractions of 20 ml each were collected at a rate of 10–20 ml per minute. After mixing, the concentration of sucrose was determined with the refractometer and protein by the Lowry procedure (10). Fractions were combined as indicated in the individual experiments. The combined fractions and a portion of the sample placed on the gradient (R_2 fraction) were diluted with an equal volume of cold distilled water and centrifuged for 60 min at 30,000 rpm. The pellets were finally suspended in 0.25 M sucrose in small Potter-Elvehjem homogenizers.

A similar procedure was used to prepare a Golgi-rich fraction from bovine epididymis. The epididymis from two bulls was stripped of connective tissue and ground in a small meat grinder. The yield of ground tissue was about 70 g. The tissue was processed as described above for bovine liver. After centrifugation at 10,000 rpm for 30 min in the #30 rotor, a pellet was obtained which contained a white portion (mainly sperm) overlaid with a brown layer (residual mitochondria) and a copious yellowish-white fluff. The fluff was collected, combined with the supernatant and centrifuged at 20,000 rpm for 30 min. The resultant yellowish-white pellet was suspended in 50% sucrose containing 0.35 M NaCl, and the density was adjusted to 42.7%. The fraction (14 ml) was placed in a Spinco tube and overlaid sequentially with the following solutions of sucrose: 15 ml of 37.3%, 10 ml of 34.5%, 10 ml of 23.9%, and 6 ml of 16% sucrose, all containing 0.34 M NaCl. The tube was centrifuged at 25,000 rpm for 60 min in a Spinco SW 25.2 rotor. The Golgi fraction, which was found at the interface between the 23.9 and 34.5% sucrose layers, was collected, diluted 2-fold with cold distilled water, and centrifuged at 30,000 rpm for 60 min. The pellet (3 mg protein) was finally suspended in 0.25 M sucrose.

Other Subcellular Fractions

Plasma membranes, rough and smooth microsomes, nuclei, and mitochondria from bovine liver were prepared as described previously (11). Smooth and rough microsomes from bovine pancreas were prepared by the same procedure used for preparing these fractions from bovine liver.

Enzymic Assays

Glucose -6- phosphatase, Mg^{++}-stimulated ATPase, 5'-nucleotidase, rotenone- or antimycin-insensitive DPNH-cytochrome c reductase, and succinate cytochrome c reductase activities were determined as described previously (11). TPNH-cytochrome c reductase was measured in the presence of antimycin in the same manner as DPNH-cytochrome c reductase. Thiamine pyrophosphatase was estimated as described by Yamazaki and Hayaishi (12), except that the reaction was terminated by the addition of 2 ml silicotungstic acid and the P_i liberated was estimated by the method of Martin and Doty as modified by Lindberg and Ernster (13).

Galactosyl transferase was determined by a modification of the method of Babad and Hassid (14). The assay mixture (75 μl) contained the following compounds (micromoles): sodium cacodylate (pH 6.75), 6; $MnCl_2$, 3; 2-mercaptoethanol, 3; uridine diphospho-^{14}C-galactose (UDP-^{14}C-gal), uniformly labeled in the sugar moiety (specific activity 3 $\times$ 10^6 cpm/μmole), 0.15; N-acetyl glucosamine, 3; and approximately 50 μg protein. Incubation was carried out for 1 hr at 37° C. At this time, the reaction was stopped with the addition of 17 μl of 0.3 M ethylenediaminetetraacetic acid (EDTA), neutralized to pH 7.4 with NaOH, and the mixture was chilled. The mixture was then passed through a column of Dowex-2 X8, 200–400 mesh, in the Cl$^-$ form, 0.5 cm diameter and 2 cm high; the column had previously been washed with distilled water. Unreacted UDP-gal remains bound to the column, while galactose which has been transferred to N-acetyl glucosamine to form lactosamine, as well as free galactose, was washed directly onto a tared planchet by two washes with 0.5 ml distilled water. The planchets were dried with an infra-red lamp and radioactivity was determined with a Nuclear-Chicago gas-flow counter Model No. 4312 with a micromil window (Nuclear-Chicago Corporation, Des Plaines, Ill.). The dried planchets were weighed and corrections were made for self-absorption. For each assay, a control tube was run in which all ingredients were present except N-acetyl glucosamine. This value represents galactose released. The tube in which acceptor is present represents combined hydrolysis and transferase activity. The difference between these values represents transferase activity. For each set of assays a control sample without enzyme was included to correct for nonenzymic

hydrolysis or for contamination of the substrate with free radioactive sugar. Under these conditions, the amount of transferase activity was linear with time and with the quantity of enzyme preparation added. In purified Golgi complex preparations, the ratio of hydrolysis to transferase activity was variable but was usually less than 0.25.

Electron Microscopy

Negative staining with phosphotungstic acid (PTA) was carried out as described previously (15). For sectioning, samples were fixed in 1% OsO_4 in 0.1 M veronal-acetate buffer, at pH 7.4, containing 2.4 mM $CaCl_2$ and 0.06 M NaCl, block stained in 0.5% uranyl acetate, and dehydrated in a series of increasing ethanol concentrations. Ethanol was removed with propylene oxide and the samples were embedded in Araldite (15). Osmication was carried out by the method of Friend and Murray (8). Particulate fractions (a total of about 1 mg protein) were centrifuged to form a small pellet, fixed with 1% OsO_4 as described previously (15) for 1 hr, then treated with unbuffered 2% OsO_4 at 40°C for 40 hr. They were then block stained, dehydrated, and embedded by the same procedures used for normally fixed samples. Counterstaining of the sections was not used for heavily osmicated samples.

Chemical Assays

Protein was determined by the procedure of Lowry et al. (10) and phosphorus by modification of the method of Chen (16).

Uridine diphospho galactose (UDP-gal) was obtained from Calbiochem, Los Angeles, Calif. UDP-gal uniformly labeled with ^{14}C in the sugar moiety was obtained from New England Nuclear Corp., Boston, Mass.

The RNA content of the fractions was determined by extraction with 5% trichloracetic acid (TCA) and analysis with orcinol (17). Type III RNA from yeast (Sigma Chemical Co., St. Louis, Mo.) was used as standard.

The products of the galactosyl transferase assay were characterized in two ways:

(1) High voltage electrophoresis was carried out with a Savant pressure-plate type electrophoresis unit. After incubation and addition of EDTA, the entire reaction mixture was applied as a band to Whatman 3 MM paper saturated with 1% sodium borate. Electrophoresis was run at 2,500 v for 1 hr, with 1% sodium borate in the reservoirs. Water

at 5°C was circulated continuously through one plate during the run. The paper was cut into 1 cm wide strips, which were placed in 15 ml toluene containing 42 ml Liquifluor (Fisher Scientific Company, Pittsburgh, Pa.) per liter, and radioactivity was determined in a Packard Tri Carb Liquid Scintillation Spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.).

(2) Chromatography of the reaction products was carried out on DEAE paper (Whatman #DE 81) sheets. The entire incubation mixture after addition of EDTA was applied as a bar 1″ wide at the origin. The developing solvent was n-butanol, n-propanol, water (3:1:1 v/v/v) and chromatography was continued for 48 hr. At this time, the paper was air-dried overnight and the distribution of radioactivity was determined as described for the electrophoretic separation.

Sugars were visualized after chromatography by spraying with analine phthalate (260 mg/10 cc water-saturated n-butanol) and heating for 5–10 min at 105°C. N-acetyl lactosamine was the kind gift of Dr. Mary C. Glick, Department of Therapeutic Research, School of Medicine, U. of Pennsylvania, Philadelphia, Penn.

RESULTS

In our preliminary studies, we obtained a heavy microsome fraction (R_2) from beef liver, enriched in Golgi membranes, by differential centrifugation essentially according to Morré et al. (4) except that Ca^{++} and glutaraldehyde were not used in the original homogenate. The R_2 fraction was further fractionated by centrifugation for 1 hr in a discontinuous sucrose gradient in a manner similar
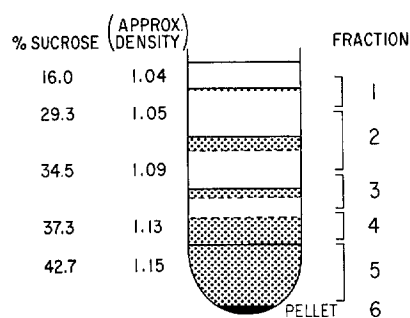

FIGURE 1 Fractionation of crude Golgi fraction (R_2) from beef liver (4) according to Kuff and Dalton (1). Fractions 1–3 appear colorless. Fraction 4 appeared yellow-pink while Fraction 5 was dark pink. Fraction 6 was a colorless pellet.

TABLE I

Subfractionation of Beef Liver Crude Golgi Fraction (R_2)

Fraction* R_2	Total protein 181	Glucose-6-phosphatase 0.29	Galactosyl transferase 3.9
1	0.08	—	—
2	2.0	0.08	55.9
3	3.0	0.07	23.6
4	9.3	0.13	8.5
5	172.0	0.33	2.6
6	18.0	0.22	0.5
% Recovery	112	119	104

* Fractions obtained as outlined in Fig. 1. Glucose-6-phosphatase expressed as μmoles P_i released/min/mg protein at 32°C. Galactosyl transferase was measured using Dowex-2 method described in text, and is expressed as mμmoles galactose transferred to N-acetyl glucosamine/hr/mg protein at 37°C. Total protein in mg.

to that described by Kuff and Dalton for the preparation of Golgi membranes from rat epididymis homogenate (1). The R_2 fraction was adjusted to 42.7% sucrose by the addition of 50% sucrose, and 15 ml of this fraction was placed in a tube. The sample was overlayed with 15 ml 37.3%, 10 ml 34.5%, 10 ml 29.3%, and 5 ml 16% sucrose. After centrifugation for 1 hr at 25,000 rpm in a Spinco SW 25.2 rotor, fractions were obtained as shown diagramatically in Fig. 1. The fractions were collected as indicated, diluted, and recovered by centrifugation. Table I summarizes the distribution of protein, glucose 6-phosphatase activities and galactosyl transferase activities of fractions obtained in this manner. Fraction 1 was of such negligible quantity that it could not be characterized. Fractions 2 and 3 showed low glucose-6-phosphatase activity and high transferase activity. Fraction 4 appeared to contain significant amounts of endoplasmic reticulum. Fraction 5, the bulk of the material, was very high in microsomal content. The pellet, fraction 6, was also predominantly microsomal. Figure 2 shows representative electron micrographs of these fractions after negative staining. The starting material, R_2, is shown in Fig. 2 a. Five elements commonly seen in this mixture are large sacs; smooth tubular profiles, often with bulging ends; medium-sized vesicles with irregular, electron-opaque edges; glycogen aggregates; and small fragments and vesicles. These elements are partially resolved in the gradient to give large sacs and tubules in the upper two fractions (Fr. 2 and

3, Fig. 2 b and c), medium-sized irregular vesicles and tubules in the intermediate fraction (Fr. 4, Fig. 2 d), irregular medium-sized and small vesicles and glycogen in the bulk microsome fraction (Fr. 5, Fig. 2 e), and mostly small vesicles and glycogen in the pellet (Fr. 6, Fig. 2 f).

In order to obtain enough material for more extensive studies and a more reproducible method of subfractionation, the separation of the R_2 fraction described above was scaled up by using the B-14 zonal rotor. The gradient used was modeled after that shown in Fig. 1. The distribution of protein and sucrose obtained after 45 min at 35,000 rpm is shown diagrammatically in Fig. 3. Fractions of 20 ml each were collected from the inner portion of the rotor (which corresponds to the top of a gradient made in a centrifuge tube). Peak a appeared colorless while peak b was yellowish-pink in color. Peak c was reddish brown and corresponded to the bulk of the original R_2 fraction. The fractions collected in tubes 4–11 were combined, diluted, and centrifuged to give fraction 1, while tube 12 was taken separately as fraction 2. Analysis of these fractions is shown in Table II. It can be seen that material with high galactosyl transferase activity and low glucose-6-phosphatase can be obtained by this procedure. About 20 mg protein was recovered in this fraction from about 400 mg R_2 protein obtained from 330 g wet weight of liver. The specific activity of galactosyl transferase in the purified fraction was increased about 10-fold over that of the fraction isolated by differential centrifugation.

Table III summarizes the galactosyl transferase activities of purified organelles of beef liver. It can be seen that the Golgi-rich fraction isolated by the zonal method is highly enriched with respect to this activity. Nuclei, mitochondria, plasma membranes, and rough microsomes show little or no activity. Smooth microsomes show some activity but it may merely be an index of the amount of Golgi membranes present in this heterogeneous fraction.

The enzymic activities of purified Golgi fractions as compared to plasma membranes and smooth and rough microsomes prepared from bovine liver are shown in Table IV. The Golgi fraction is distinct from plasma membrane in its low ATPase, 5'-nucleotidase and galactosidase activities. It also has a much higher phosphorus content, and rotenone- or antimycin-insensitive DPNH-cytochrome c reductase and TPNH-cyto-
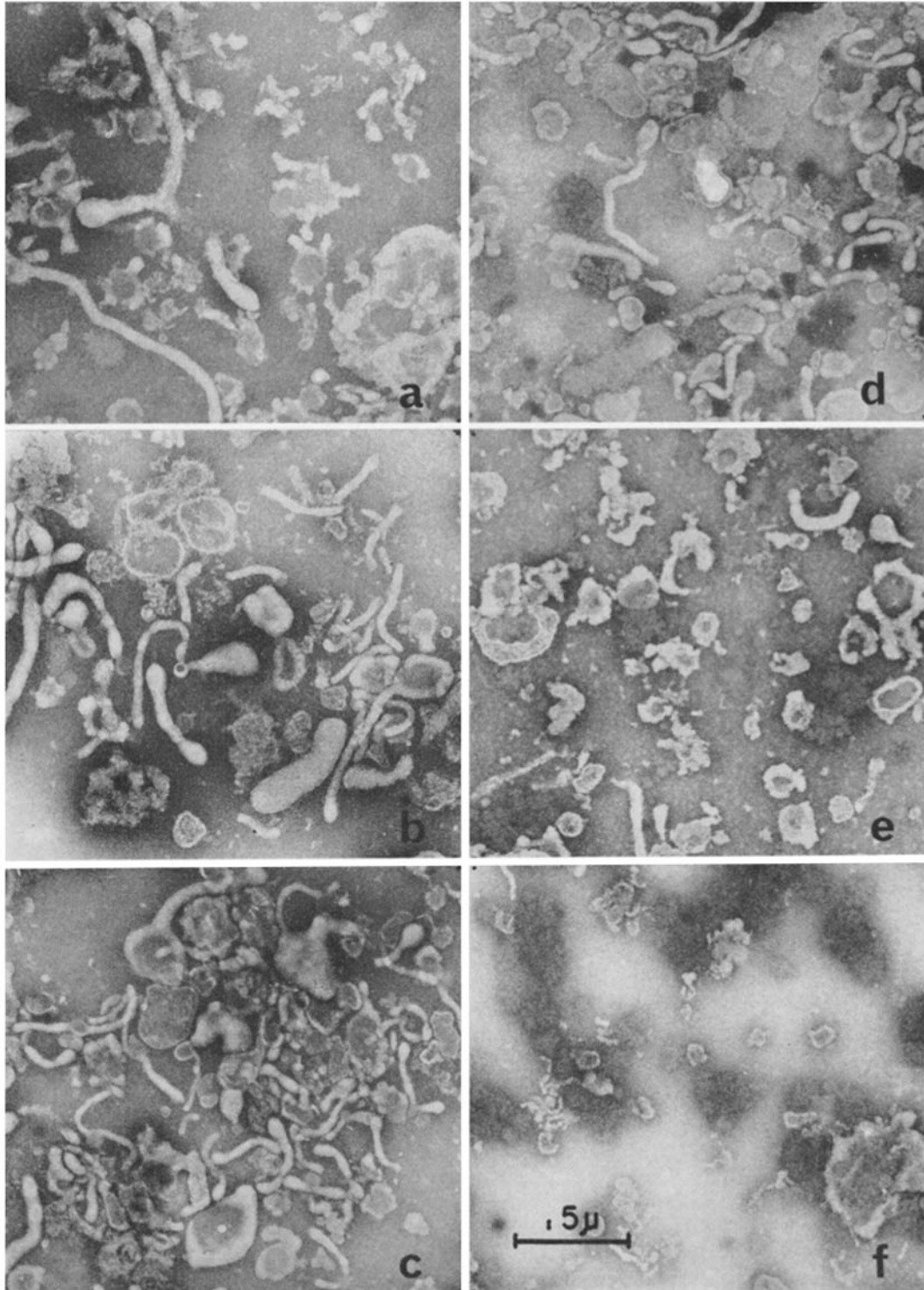
FIGURE 2 Electron micrographs of fractions isolated as indicated in Fig. 1. All fractions negatively stained with 2% potassium phosphotungstate, pH 7.2. *a*, Fraction R$_2$; *b*, fraction 2; *c*, fraction 3; *d*, fraction 4; *e*, fraction 5; *f*, fraction 6.
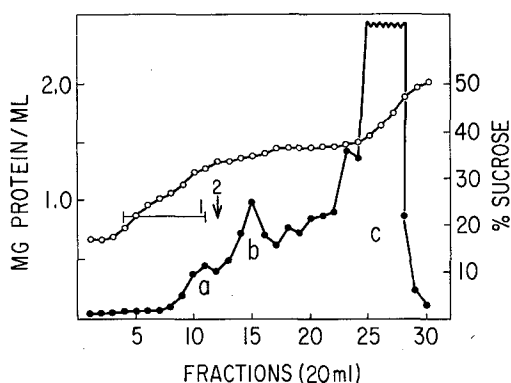
FIGURE 3 Fractionation of crude Golgi fraction (R_2) from beef liver by zonal ultracentrifugation. 20 ml fractions were collected. Fraction 1 = tubes 4–11, fraction 2 = tube 12; O–O, % sucrose; ●—●, mg protein/ml.

TABLE II

Specific Activity of Galactosyl Transferase in Bovine Liver Fractions

Fraction*	Total protein	Galactosyl transferase	Glucose-6-phosphatase
Homogenate	35,640	1.9	—
S_1	16,200	1.1	—
R_2	419	9.6	—
S_2	15,900	0.4	—
Fraction 1	18.1	80	0.035
Fraction 2	5.2	32	0.12

* S_1 = supernatant after centrifugation for 10,000 rpm × 30 min. Spinco #30 rotor. R_2 = residue, S_2 = supernatant after centrifugation 20,000 rpm × 30 min.
Fractions 1 and 2 are the subfractions of R_2 collected by zonal centrifugation in the experiment shown in Fig. 3. Specific activity is expressed as in Table I. Total protein in mg.

chrome c reductase activities. It can be differentiated from total smooth microsomes, most of which is probably derived from smooth endoplasmic reticulum, by its low content of glucose-6-phosphatase activity. The only characteristic enzymic property found as yet in this fraction is galactosyl transferase activity.

We decided to study the galactosyl transferase activity further, both to check the validity of the assay procedures used as well as to characterize more fully the products formed in the reaction. Fig. 4 shows the amount of radioactive product

formed when purified Golgi fraction is incubated with UDP-^{14}C-gal in cacodylate buffer of varying pH in the presence and absence of added N-acetyl glucosamine. In the absence of acceptor, the major product formed which will pass through Dowex-2 is free galactose. The pH optimum for this galactosidase activity is 7.2 and the curve is identical to the pH curve for the galactosidase found in purified bovine liver plasma membranes (11). The most likely source of this activity is contaminating plasma membrane in the preparation. A contamination of 10–15% plasma membrane in the preparation would account for this level of activity. In the presence of N-acetyl glucosamine the pH optimum is 6.75; the difference in radioactivity released from UDP-^{14}C-gal presumably is due to the formation of lactosamine. The addition of 5.1

TABLE III

Galactosyl Transferase Activities in Isolated, Purified Organelles of Beef Liver

Fraction	Specific activity*
Nuclei	0.9
Mitochondria	0.8
Rough microsomes	2.6
Smooth microsomes	15
Plasma membranes	0.0
Golgi-rich fraction	80

* Expressed as in Table I.

μmoles EDTA to the reaction mixture before incubation at pH 6.5 and 37°C for 1 hr caused 89% inhibition of total activity. Glucose will not substitute for glucosamine as an acceptor for the galactosyl moiety. UDP-glucose, however, can be utilized by this fraction as a glycosyl donor to N-acetyl glucosamine.

The effect of added Mn^{++} or Mg^{++} on the galactosyl transferase activity of the Golgi fraction is shown in Fig. 4 b. Optimum stimulation occurs with 0.04 M Mn^{++}. Some activity is present with no addition of Mn^{++}, the basal rate being stimulated about 5-fold by the addition of 3 μmoles Mn^{++} per 75 μl of assay mixture. Mg^{++} does not substitute for Mn^{++} in this stimulation. The preparation used in this study had an unusually high specific activity for galactosyl transferase, about 3-fold greater than any observed previously. The total homogenate and R_2 fraction also had specific activities much higher than previously found, in-

TABLE IV

Enzyme Profiles of Plasma Membranes, Golgi Fractions, and Smooth and Rough Microsomes

Assay	PM	Golgi fractions	Microsomes	
			Smooth	Rough
ATPase	2.5	0.21	0.39	0.074
5'-Nucleotidase	0.81	0.16	0.074	0.029
Galactosidase*	410	55	57	18
Glu-6 P'ase	0.048	0.023	0.27	0.30
TPPase*	0.20‡	0.25	0.26	0.17
Acid P'ase*	0.0039	0.016	0.022	0.026
DPNH-cyt. c reductase§	0.01	1.4	2.4	2.3
TPNH-cyt. c reductase§	0.0046	0.055	0.094	0.089
Succinate-cyt. c reductase	0.009	0.002	0.009	0.005
μg P/mg Pr.	16.0	40.0	40.0	34.9
mg RNA/mg Pr.	—	0.044	0.069	0.250

All phosphatases are expressed as μmoles P_i released/min/mg protein.

Cytochrome c reductases are expressed as μmoles cyt. c reduced/min/mg protein.

Galactosidase is expressed as mμmoles galactose released/hr/mg protein.

* Assayed at 37°C, all others assayed at 32°C.

‡ No ATP added since plasma membranes have a very active ATPase activity.

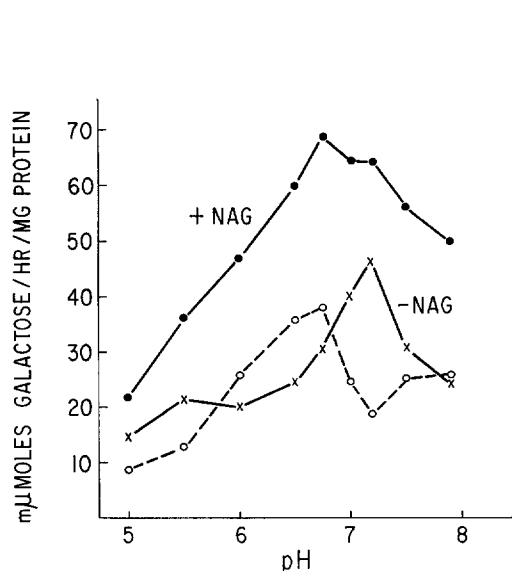§ 4 μg rotenone or 8 μg antimycin added as 1 mg/ml ethanol solution before addition of substrate.

FIGURE 4 a Release or transfer of galactose-^{14}C from UDP-^{14}C-gal by purified Golgi fraction of beef liver as a function of pH. X—X, in the absence of added N-acetyl glucosamine; ●—●, in the presence of added N-acetyl glucosamine; O----O, difference between the two curves which represents transferase activity.
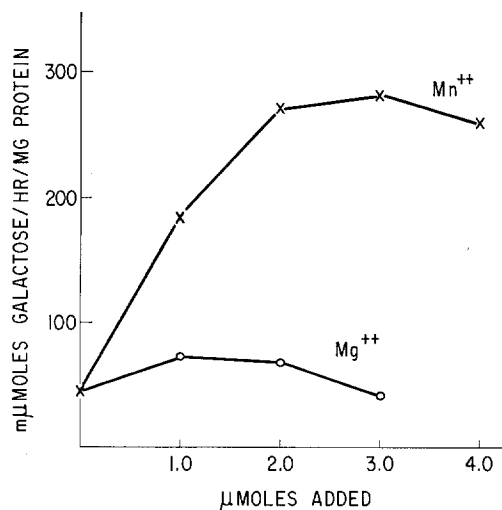
FIGURE 4 b Effect of added $MnCl_2$ or $MgCl_2$ on galactosyl transferase activity of Golgi fraction from beef liver. The pH of the assay was 6.75 and the final volume was 75 μl. Maximum activation occurred when 3.0 μmoles Mn^{++} were added per assay volume of 75 μl (i.e., 0.04 M Mn^{++}). It should be noted that the liver homogenate and Golgi membranes from this cow had unusually high specific activities compared with previous preparations.
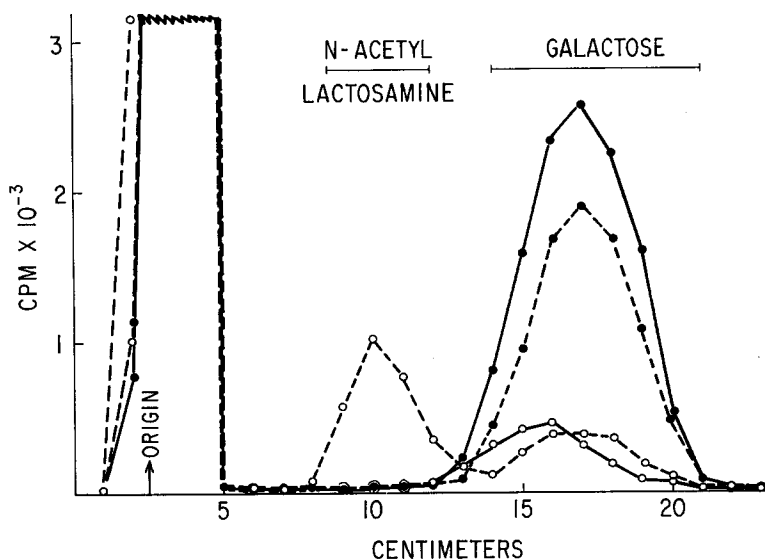
FIGURE 5 Chromatography of reaction products after incubation of plasma membranes or Golgi-rich fractions from beef liver with UDP-^{14}C-gal. ●—●, Plasma membranes without added N-acetyl glucosamine (NAG); ●---●, Plasma membrane with added NAG; O—O, Golgi-rich fraction without added NAG; O---O, Golgi-rich fraction with added NAG. Galactose (1.5 μmoles) and N-acetyl lactosamine (3 μmoles) were added to the reaction mixture before chromatography. After chromatography, they were located by spraying with analine phthalate dissolved in water-saturated n-butanol and heating, and their migration is indicated at the top of figure.

dicating some variability in the amount of enzyme present in individual animals.

The products of the reaction were characterized further by chromatography. Golgi fractions and purified plasma membrane fractions were incubated with UDP-^{14}C-gal as described in Methods. The reaction was stopped by adding EDTA and the entire reaction mixture was spotted on DEAE paper. After chromatography, the distribution of ^{14}C was determined on 1 cm sections of the paper. The results are summarized in Fig. 5. When plasma membranes are incubated with UDP-^{14}C-galactose, with or without added N-acetyl glucosamine, a single product is formed which migrates as a monosaccharide. Beef liver Golgi fraction, when incubated in the absence of N-acetyl glucosamine, gave a single product migrating in the same area as the plasma membrane product. When N-acetyl glucosamine is added to the assay, a second product is formed which migrates as a disaccharide. The amount of monosaccharide formed is not diminished by the production of disaccharide. Carrier galactose or N-acetyl lactosamine, added to the reaction mixture before spotting on the paper, has the same mobility as the

radioactive products formed from the Golgi fraction.

The products were also separated by high-voltage paper electrophoresis. When plasma membranes were incubated with UDP-^{14}C-gal, in the presence or absence of N-acetyl glucosamine, a single product was formed which migrated, on paper electrophoresis, slightly slower than unreacted UDP-^{14}C-gal. When fraction 2 of the step-gradient separation illustrated in Fig. 1 was incubated with UDP-^{14}C-gal in the presence of N-acetyl glucosamine, and the mixture was separated by electrophoresis, two radioactive products were formed, one which migrated in the same area as the plasma membrane product, and a second which migrated closer to the origin.

In order to see if the transferase enzyme was present in Golgi-rich fractions from other bovine tissues we isolated total smooth microsomes from pancreas which are known to be predominantly of Golgi origin (5). In addition, we applied the procedure used for beef liver as indicated in Fig. 1 to isolate similar fractions from beef epididymis. Table V summarizes the results found with Golgi-rich fractions of bovine liver, pancreas, and epi-

Table V

Galactose-C^{14} Released or Transferred by Golgi-Rich Fractions from Bovine Tissues

| | | | mμmoles galactose | |
Fraction	Assay method	NAG* added	released	trans- ferred‡
Liver	Dowex-2	—	26.9	—
		+	—	42.0
	Chromatog-	—	33.8	0.0
	raphy	+	34.9	54.9
Pancreas				
rough	Dowex-2	—	0.0	—
micro-		+	—	0.8
somes				
smooth	Dowex-2	—	9.2	—
micro-		+	—	15.1
somes	Chromatog-	—	13.4	0.0
	raphy	+	12.0	17.9
Epididymis	Dowex-2	—	94.0	—
		+	—	666
	Chromatog-	—	95.1	0.0
	raphy	+	45.6	689

* NAG = 3 μmoles N-acetyl glucosamine added per 75 μl of assay mixture.

‡ In the Dowex-2 assay, galactose released is obtained by measuring the amount of radio-activity which passes through Dowex-2 when the sample is incubated with UDP-^{14}C-gal in the absence of NAG. Galactose transferred is taken to be the increase in the radioactivity under the same conditions when NAG is included in the assay mixture. Liver fractions were assayed at pH 6.5 in the presence of 3 μmoles Mn^{++}. Pancreas and epididymis fractions were assayed at pH 6.0 with added Mn^{++}. These conditions are optimum for transfer of galactose rather than for release.

didymis. Pancreas rough microsomes had neither galactosidase nor transferase activity whereas the smooth fraction had a significant amount of both activities compared to the Golgi fraction from liver. The epididymis fraction obtained in the same manner as the liver fraction had a high galactosidase activity and a very high transferase activity. Chromatography of the reaction mixture, to separate, identify, and estimate the products formed by both pancreas smooth microsomes and epididymis Golgi-rich fraction, gave very similar values as the Dowex-2 method of assay. In addition, all products migrated as expected. The epi-

didymis fraction isolated was about four times as active in transferring galactose as the crude Golgi fraction (R$_2$) from which it was prepared.

We attempted to characterize further what we believed to be Golgi-rich fractions from bovine liver, pancreas and epididymis by using electron microscopic techniques. Fig. 6 illustrates the most characteristic features found in the liver "Golgi-rich" preparations. By negative staining (Fig. 6 a) large sacs are often seen with attached tubules. Enclosed lipoprotein particles (small light spheres) are seen occasionally in our preparations (Fig. 6 c). Very small particles sometimes appear, by negative staining with PTA, to line the surface of both the sacs and the tubules, but not as regularly as seen in bovine liver plasma membrane preparations (11). Thin sections of OsO$_4$-fixed and embedded samples (Fig. 6 b) also show characteristic tubular profiles and large sacs. The tubules appear full of a granular material. The sacs have a network of material inside them as well. A more extensive view of this preparation is shown in Fig. 6 d. In order to obtain a representative view of the entire preparation, a small sample (<1 mg protein) was pelleted, fixed, and dehydrated in the standard manner. The thin pellet was cut in pie-shaped sections and embedded in an oriented manner in a flat mold. Sections were cut from the top, middle and bottom of the pellet. Representative fields are shown in Fig. 7 a(top), b(middle), and c(bottom). It can be seen that the top of the pellet is covered by a thin layer of large membranous material which very likely originates from plasma membrane, although it is not possible to make a definite identification. The middle portion is rich in tubules and smooth vesicles, while the bottom region has an increased amount of vesicles with attached ribosomes. This particular preparation was contaminated with about 10% plasma membrane and 10% endoplasmic reticulum as estimated enzymically from the content of galactosides and glucose-6-phosphatase activities.

Osmication of the Golgi apparatus of beef liver, epididymis, and mouse pancreas in situ is illustrated in Fig. 8 a, b, and c. It can be seen that large amounts of reduced osmium are concentrated in the Golgi areas of these tissues. Plasma membrane, smooth and rough endoplasmic reticulum and mitochondria do not take up appreciable amounts of osmium. The entire Golgi apparatus, however, is not stained in any of the tissues, an observation which has been made also by others and which

FIGURE 6   Electron microscopy of Golgi-rich fractions from beef liver. *a*, negative staining with 2% PTA (× 140,000). Characteristic features are large sacs with connected tubular profiles. *b*, Golgi-rich fractions fixed in OsO₄, embedded, and sectioned. Numerous sacs often associated with tubular profiles are seen. Dense granular material appears to be present in the tubules whereas the sacs contain a light network of granular material (× 140,000). *c*, An occasional feature seen in negatively stained preparations of Golgi-rich fractions are spherical inclusions which appear light by negative staining (× 45,000). *d*, A larger field of material prepared as in *b* (× 35,000).
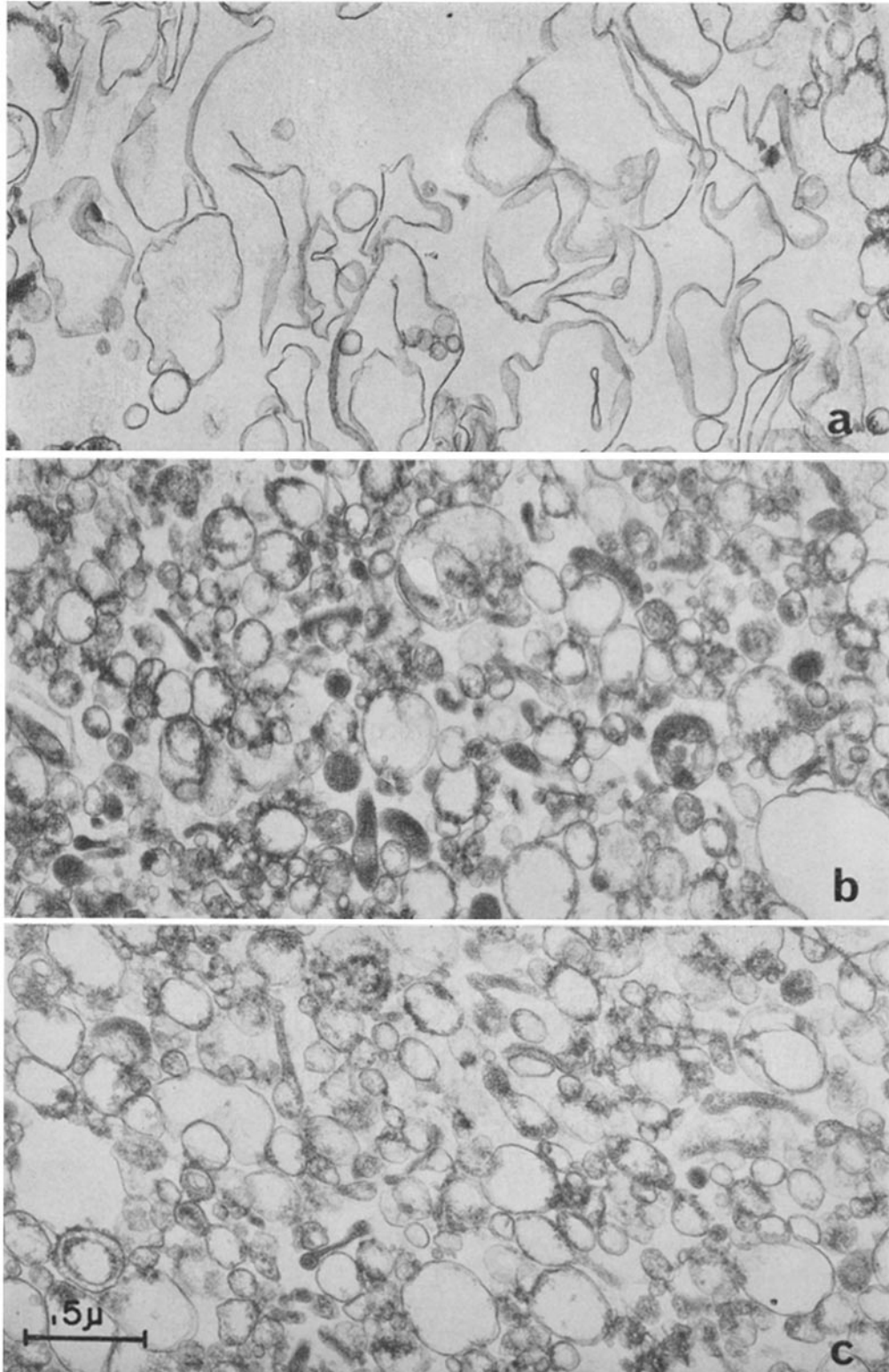
FIGURE 6

FIGURE 6

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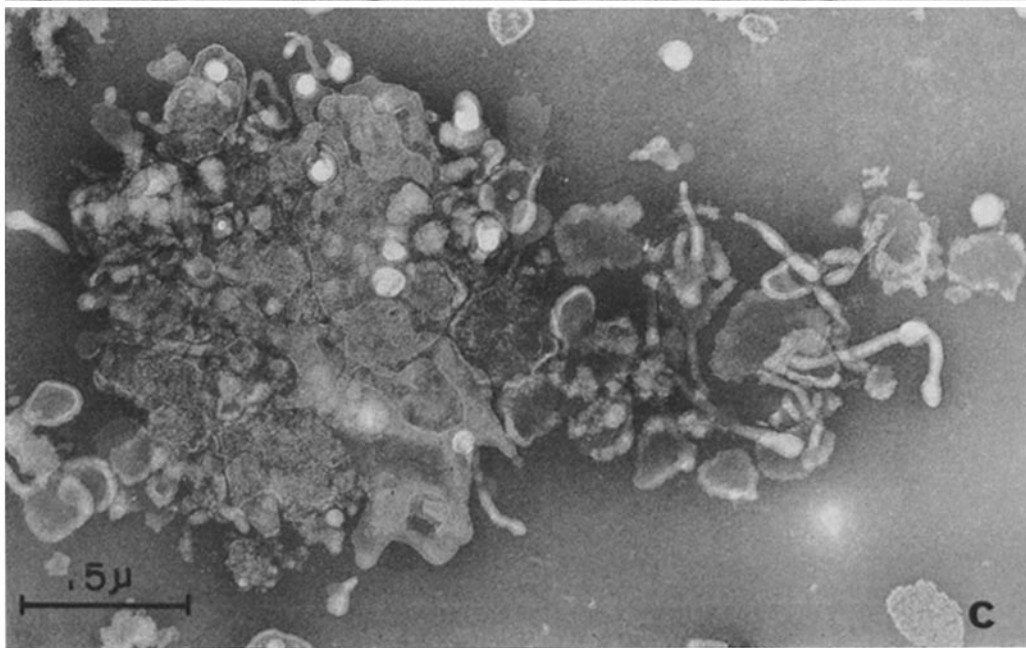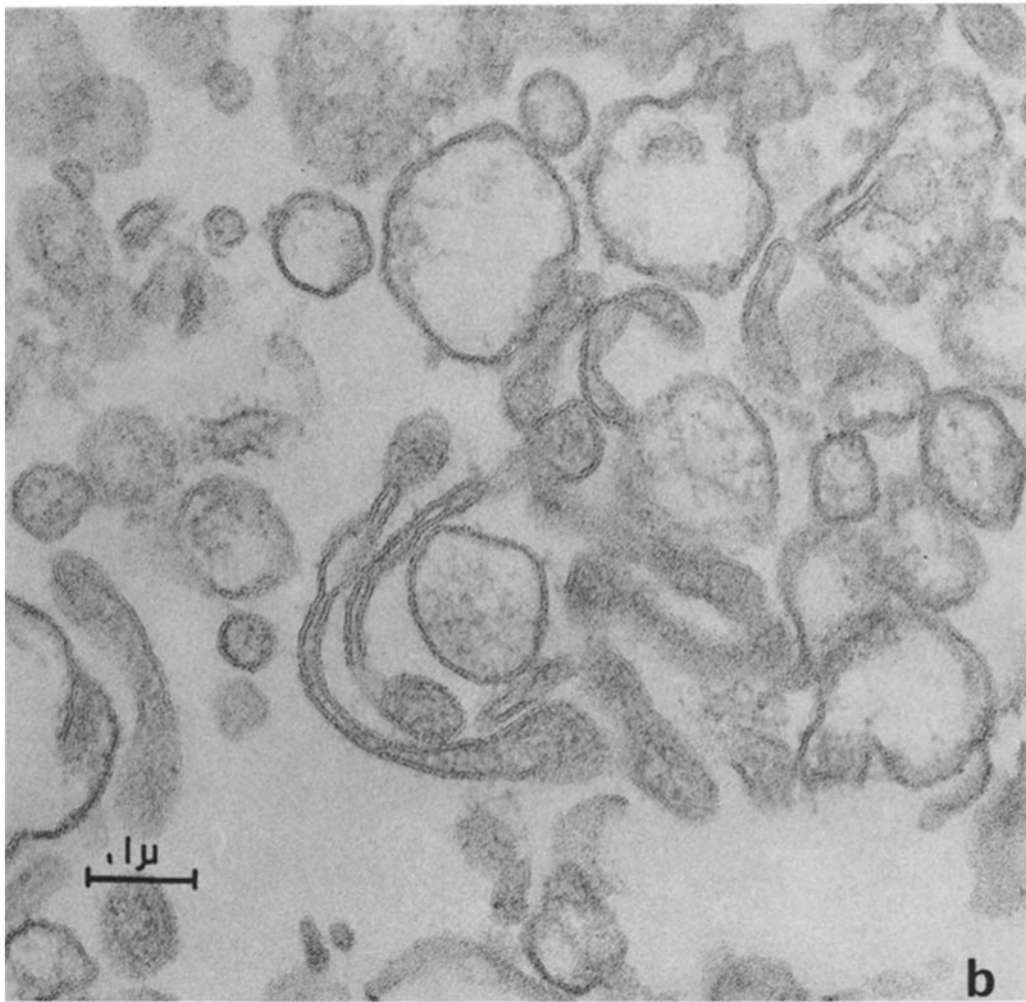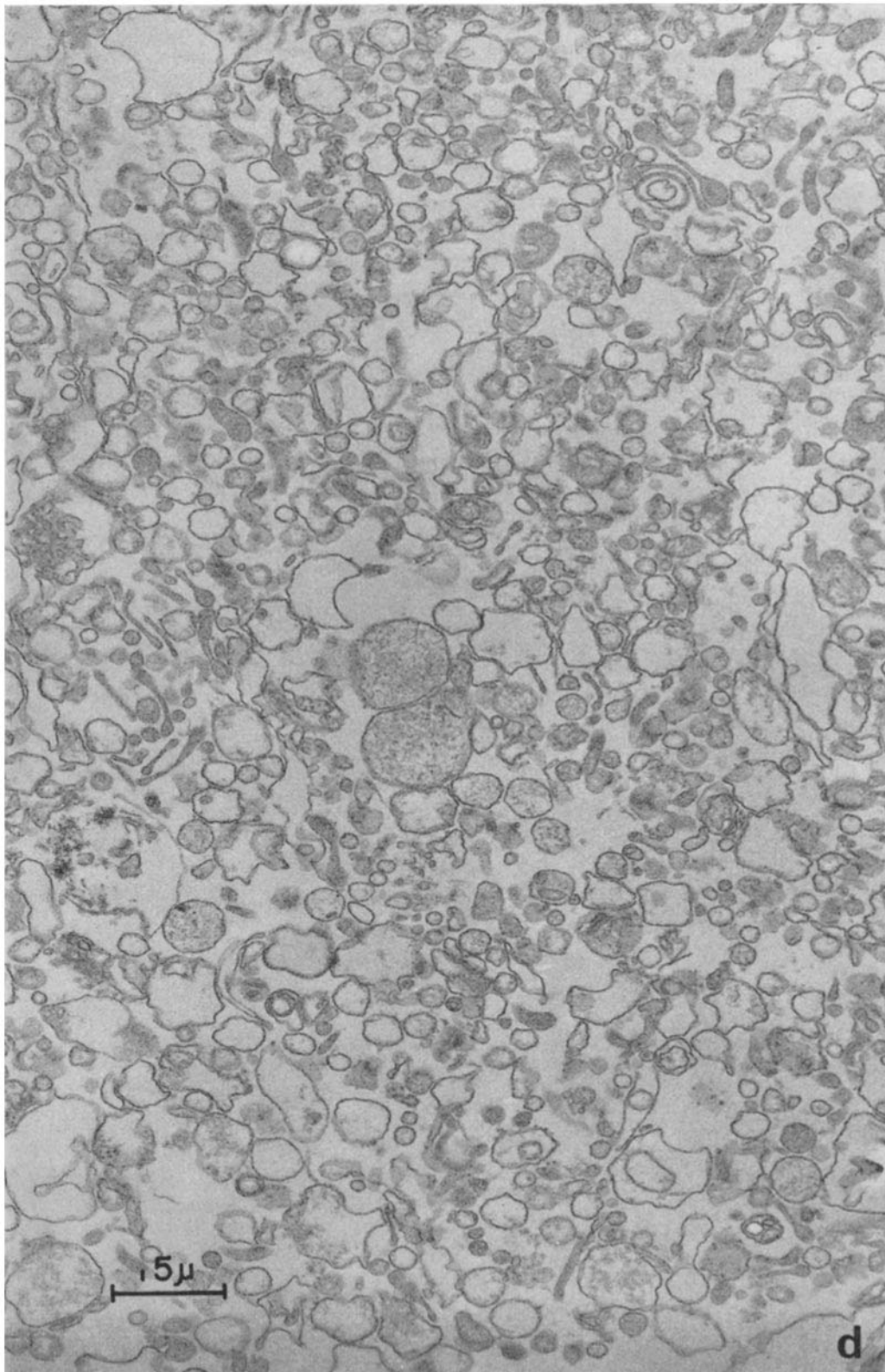
FIGURE 7   Pellet of Golgi-rich fraction of beef liver sectioned in an oriented manner. *a*, section from top of pellet, *b*, section from middle of the pellet, *c*, section taken from bottom of the pellet (all × 35,000).
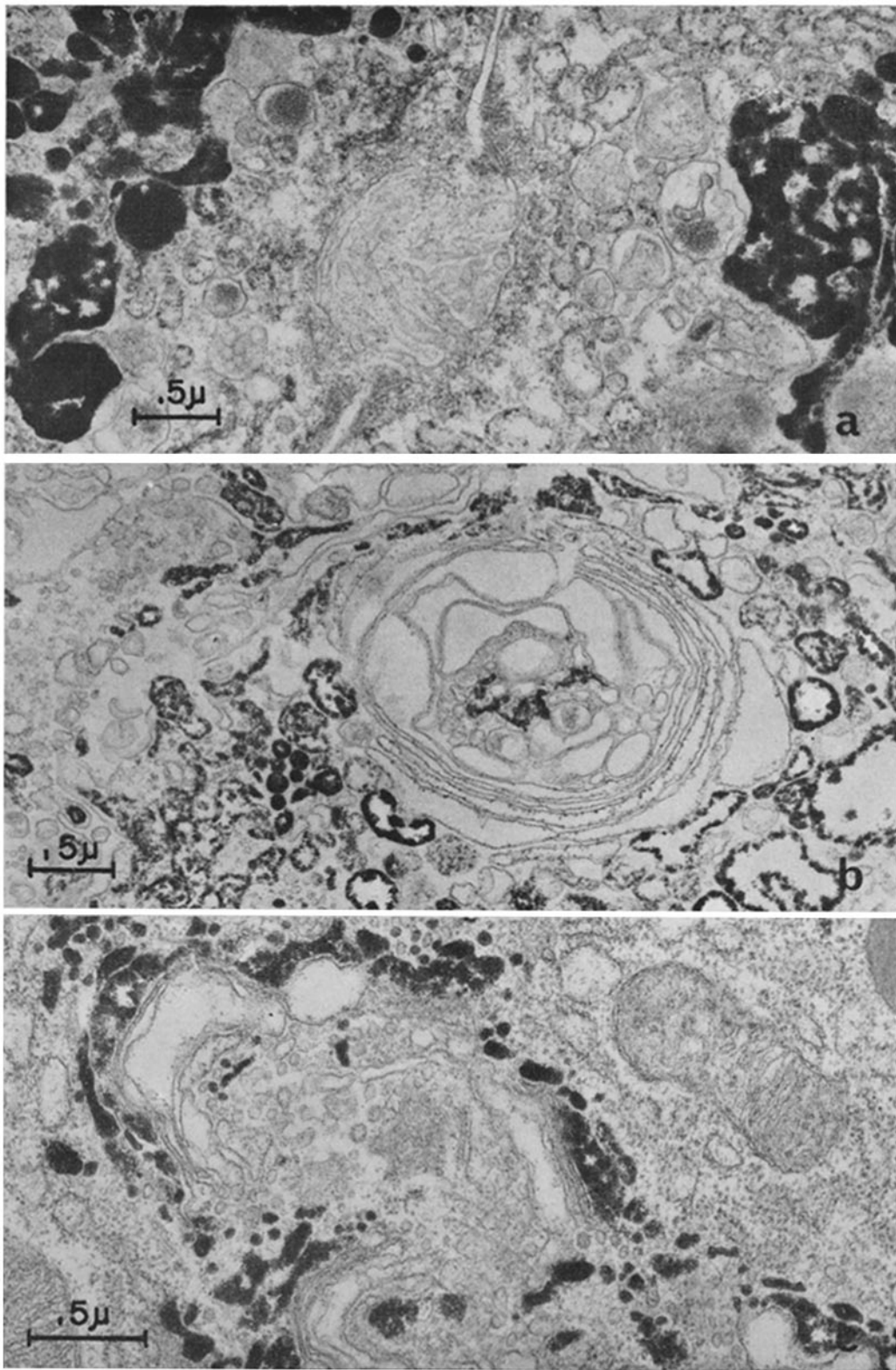
FIGURE 8 Osmication of Golgi apparatus in situ. *a*, bovine liver (× 26,200), *b*, bovine epididymis (× 26,200) and *c*, rat pancreas (× 35,000). Osmication carried out as described by Friend and Murray (8).
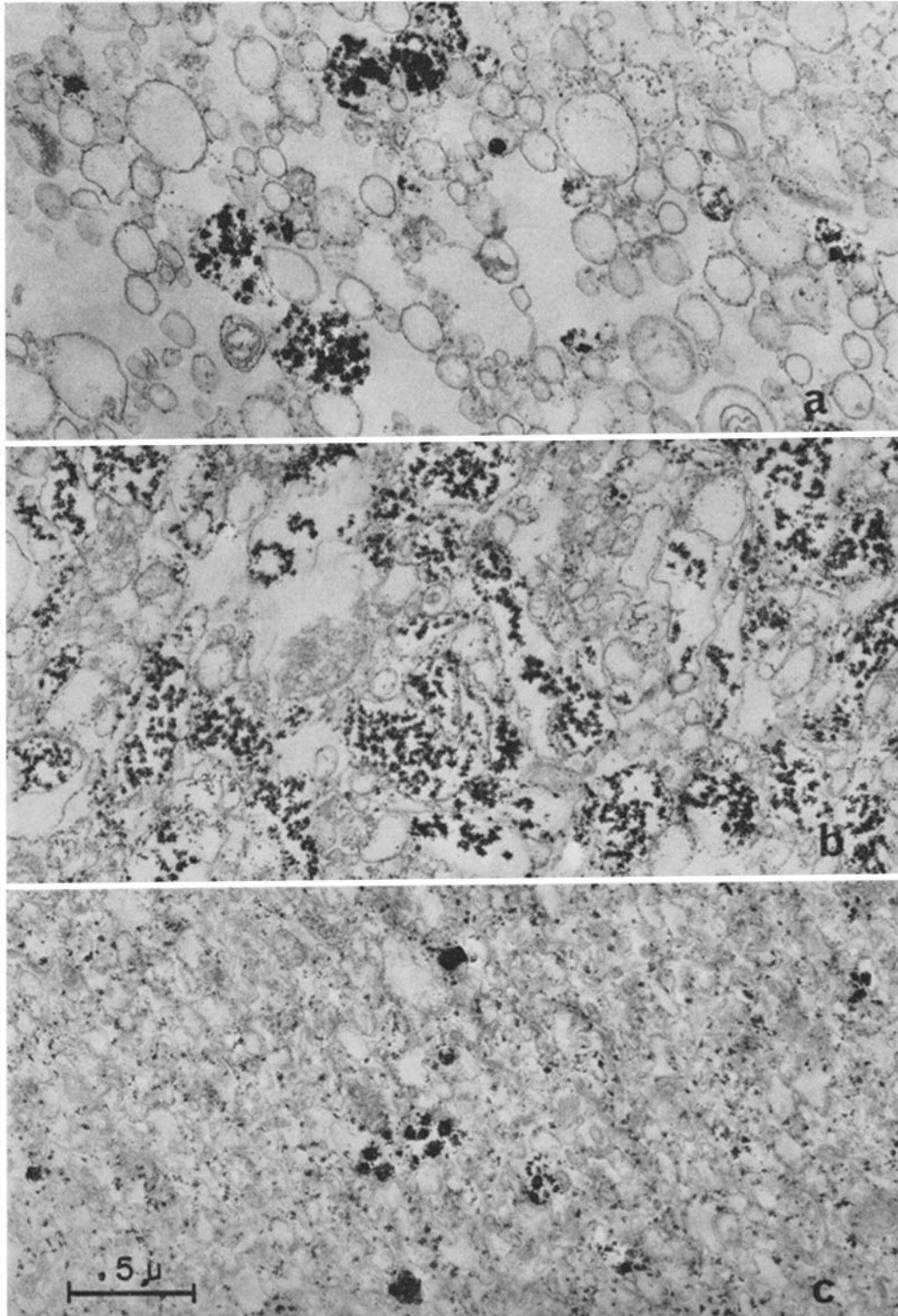
FIGURE 9   Osmication of isolated fractions from beef liver. *a*, crude Golgi fraction (R₂), *b*, purified Golgi fraction 1 (Fig. 3 and Table II), *c*, under Golgi fraction 2 (Fig. 3 and Table II) (all × 35,000). Osmication is much more extensive in *b* compared to *a* and *c*.
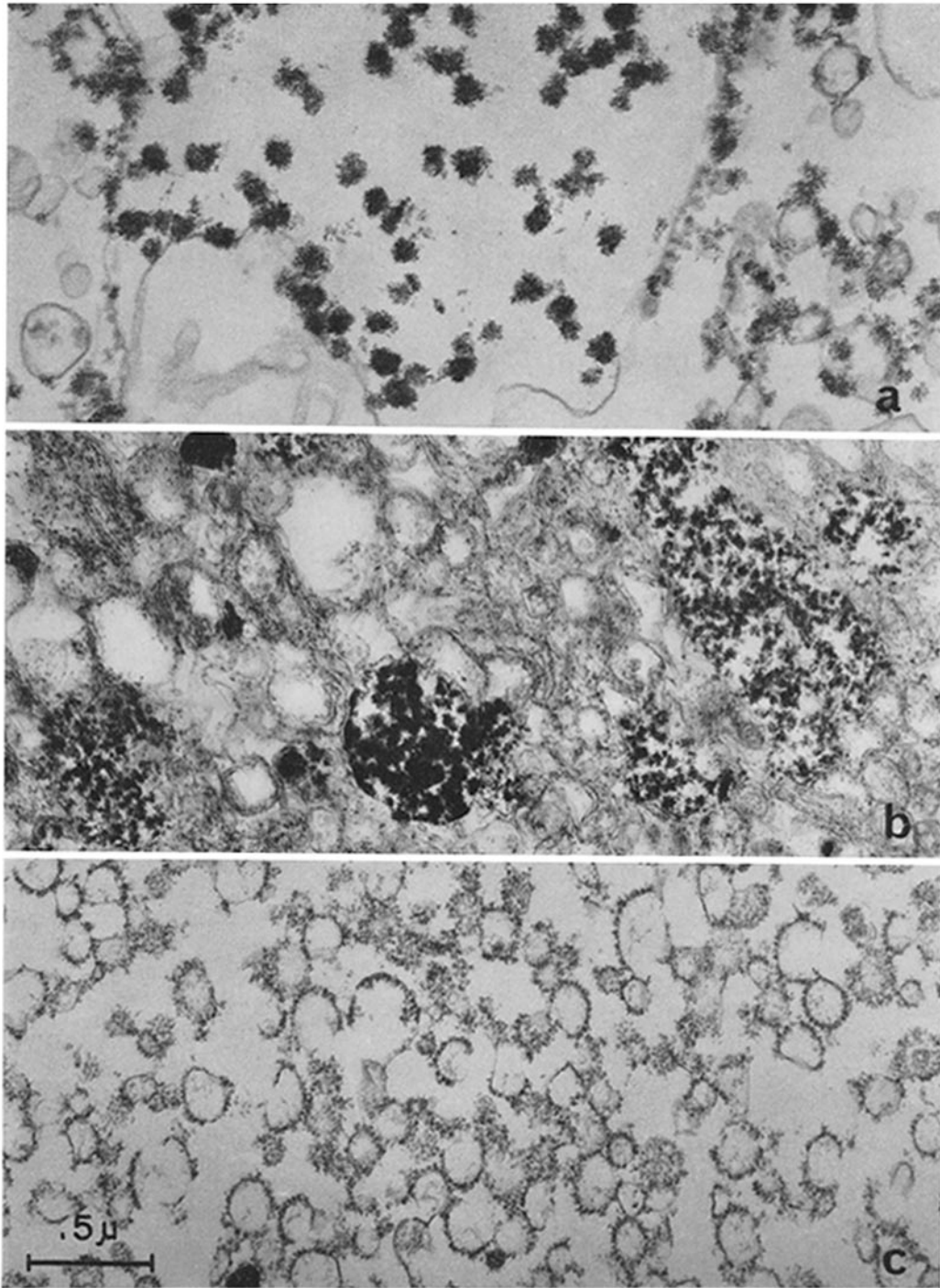
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FIGURE 10 Osmication of isolated fractions from bovine tissues. *a*, pancreas smooth microsomes, *b*, epididymis Golgi-rich fraction, and *c*, pancreas rough microsomes (all × 35,000). Extensive osmication is seen in *a* and *b* but little or none is seen in *c*.

argues for heterogeneity within the Golgi apparatus.

Our next experiment was done to see if isolated fractions which we believed to be rich in Golgi apparatus would also osmicate under the same conditions used for whole tissue. Figs. 9 a–c summarize the results. Little osmication is seen in fractions such as crude Golgi fraction R_2 from beef liver (Fig. 9 a). Fraction 1, isolated by zonal centrifugation of R_2 and shown to be high in galactosyl transferase activity (Fig. 3 and Table II), shows increased osmication over the crude fraction (Fig. 9 b). Fraction 2, which has increased glucose-6 phosphatase activity and decreased galactosyl transferase activity compared to fraction 1 (Fig. 3 and Table II), also shows marked decrease in osmication (Fig. 9 c). Osmication was also observed in total smooth microsomes from beef pancreas (Fig. 10 a) and Golgi-rich fractions from beef epididymis (Fig. 10 b). Pancreas rough microsomes, on the other hand, showed no osmication (Fig. 10 c). Since the entire Golgi area does not osmicate even in situ, and our preparations from beef liver are probably only about 80% pure, it is not surprising that more osmication is not seen in the purified Golgi fractions. Vesicles osmicate more often than the tubular, dense structures seen in the purified liver Golgi fractions.

DISCUSSION

Isolation of the Golgi apparatus or membranes derived from it as morphologically identifiable entities from bovine liver has the inherent difficulty that the tissue is much tougher than rat liver and contains much more connective tissue. We have developed relatively mild procedures which make possible the isolation of reasonable quantities of a fraction which has morphological features compatible with its possible origins in the Golgi apparatus. The method involves breaking up the tissue first with a small meat grinder and then brief homogenization with a loose homogenizer. The Golgi preparation isolated from such a homogenate is rich in sacs with tubular extensions often with the appearance of networks, and occasional spherical inclusions which appear light by negative staining and could be lipoprotein particles. Comparable morphological features have been ascribed to Golgi apparatus in situ (6) in rat liver and in partially purified preparations from rat liver (3, 4).

The fraction is biochemically unique. It can readily be distinguished from mitochondria by its lack of succinate-cytochrome c reductase, from endoplasmic reticulum by its lack of high glucose-6-phosphatase, and from plasma membrane by its low levels of ATPase, 5'-nucleotidase, and glycosidase. It contains negligible acid phosphatase. The preparation does show substantial rotenone- or antimycin-insensitive DPNH-cytochrome c reductase and TPNH-cytochrome c reductase activities. The level of these enzymes is about 60–80% of the levels found in smooth or rough microsomes, a level too high to be accounted for by contamination with microsomes.

We have demonstrated that this fraction has the unique ability to transfer radioactive galactose from UDP-gal to free N-acetyl glucosamine, which further differentiates it from smooth endoplasmic reticulum. Glucose will not substitute for N-acetyl glucosamine as an acceptor in this system. The incorporation of sugars into glycoproteins has been shown to proceed by the stepwise addition of sugars each mediated by a specific glycosyl transferase (9, 18, 19). One of the most common terminal sequences of sugars found in glycoproteins is sialic acid $\rightarrow$ galactose $\rightarrow$ N-acetyl glucosamine attached to protein or to an inner core of sugars (9, 18, 19). Highly purified galactosyltransferase from calf thyroid has been shown capable of using free N-acetyl glucosamine as an acceptor for galactose from UDP-gal. The Km found was 1.9 $\times$ 10^{-2} M compared to a Km of 3.3 $\times$ 10^{-3} M for a glycopeptide with terminal N-acetyl glucosamine (19), indicating that the natural acceptor probably is a glycopeptide rather than free N-acetyl glucosamine. The natural substrates for the galactosyl transferases found in our Golgi-rich fractions of bovine liver, pancreas, and epididymis may be glycopeptides, and this step may be part of a sequence of steps leading to the biosynthesis of glycoproteins.

Some controversy exists as to the subcellular localization of biosynthesis of the carbohydrate side chains of glycoproteins. The incorporation of ^{14}C-glucosamine from UDP-N-acetyl ^{14}C-glucosamine into rat liver microsomal protein in vitro has been shown to occur in smooth rather than rough microsomes (20). Other workers, however, feel that in vivo the site of incorporation of the first glucosamine residue on the polypeptide backbone takes place in ribosomes, while the polypeptides are still associated with tRNA(21). In both Ehrlich ascites cells and HeLa cells, however, polypep-

tidyl:N-acetyl galactosaminyl transferase activity as well as glycoprotein:galactosyl transferases appears to be localized in the smooth internal membranes of the cell, presumably partly of Golgi origin (22). Furthermore, it has been shown by radioautography (7) that radioactivity accumulates in the Golgi region of pancreas, liver, and epididymis of rats 5–15 min after injection of the intact animal with galactose-H^3. The activity was not removed by treatment of the tissue with amylase. All three tissues may be active secretors of glycoproteins. Liver is active in synthesizing and secreting serum glycoproteins (23), a few of the enzymes found in zymogen granules of pancreas are glycoproteins (24, 25), and epididymis could be involved in secreting the glycoproteins or mucoproteins found in seminal plasma. Complex carbohydrates have been detected by specific staining in the Golgi apparatus of all three cell types in the rat in situ (26). Heterogeneity was seen in the Golgi apparatus, the mature face generally being more heavily stained than the immature face. The opposite polarity has been seen by ordinary osmium staining (27) as well as heavy osmication (8).

It has been proposed that the A protein of lactose synthetase (isolated from bovine milk), when assayed alone, is actually a UDP-galactose.N-acetyl glucosamine galactosyl transferase. In the presence of added α-lactalbumin the specificity of the transferase reaction is changed to allow synthesis of lactose in the presence of added glucose (28). It has also been shown that α-lactalbumin, when added to rat liver homogenates, modifies the transferase reaction in an analogous manner. On the basis of these observations it has been suggested that the A protein of lactose synthetase is identical to the N-acetyl lactosamine synthetase found in particulate fractions of rat liver by McGuire et al. (9). The possibility arises, therefore, that the enzyme found membrane "bound" in our Golgi fraction of bovine liver could be a secreted form of the A protein of lactose synthetase and not necessarily be involved in glycoprotein synthesis in the liver. This interpretation is compatible with the known secretory functions of the Golgi apparatus. Clarification of this point must await purification of the enzyme from the Golgi fraction and comparison of its properties to the A protein of lactose synthetase.

The overall recovery of the galactosyl transferase activity in the purified Golgi fraction was about 2% of the total activity present in the homogenate. The procedures used in the isolation, however, were designed to yield the greatest purity in the Golgi membrane fraction rather than to maximize the yield of activity. About 50% of the activity is lost in the first residue, probably due to the mild homogenization procedure used which leaves a large percentage of cells unbroken. Of the galactosyl transferase activity released by the homogenization, about 25% is recovered in the crude Golgi membrane pellet (R_2). This loss is probably referable to fragmentation of the Golgi complex during homogenization, with loss of the fragments into the supernatant. Further fractionation of the R_2 pellet concentrates the activity about 8-fold, with about 25% recovery of the activity. Assuming that the preparation of Golgi complex with a specific activity of 80 mμmoles galactose transferred per hr per mg protein is about 80% pure, and that the activity is localized exclusively in the Golgi complex, Golgi membranes would account for about 2% of the total protein of the homogenate.

Further evidence that the membranes isolated by the procedures outlined in the present work are actually derived from the Golgi apparatus of beef liver is provided by the ability of these fractions to reduce osmium tetroxide in a manner analogous to Golgi apparatus in tissue sections. The chemical basis of the reaction is not known except that it is not enzymatic in nature and does not occur if the OsO_4 used is buffered at pH 7 (8). In situ the osmication occurs in a polarized fashion, the outer, "immature" face being heavily stained whereas the inner, "mature" face is not stained. Staining for complex carbohydrates also shows polarization, but in the opposite direction. The "mature" or inner face is more heavily stained. Heavy osmication in the isolated fraction is probably not due to glycogen. Glycogen is present in the crude Golgi fractions which show little osmication. In addition, purified Golgi fractions which do osmicate have little or no glycogen present when viewed directly by negative staining with phosphotungstate. Nucleoproteins also do not appear to give the reaction, since neither rough endoplasmic reticulum nor nuclei show heavy osmication under these conditions. It is not likely that phospholipid is the cause of the reaction since smooth endoplasmic reticulum is as rich in phospholipid but does not appear to osmicate. In their composition of the lipids, the Golgi fractions do

not appear to be grossly different from endoplasmic reticulum (unpublished observations of B. Fleischer, S. Fleischer, and G. Rouser). In isolated fractions, heavy osmication appears to occur in the lumen of the sacs rather than on the membrane, or in the tubular elements which also occur in the preparation. These sacs are probably portions of the flattened cisternae of the Golgi apparatus which are swollen during the isolation procedure. The osmication of the isolated fractions appears more diffuse than in situ, which would be understandable if the Golgi vesicles were swollen during the isolation procedure.

Thiamine pyrophosphatase (TPPase) has been used extensively to reveal the Golgi complex histochemically in tissues such as rat liver (29). Our results show about equal specific activities for isolated plasma membranes, smooth microsomes, and Golgi membranes. This is consistent with the work of Yamazaki and Hayaishi (12), who isolated nucleoside diphosphatase from bovine liver microsomes, and showed the purified enzyme to be an active thiamine pyrophosphatase at pH 9.0 in the

presence of small amounts of added ATP. Histochemically, nucleoside diphosphatase activity is present in endoplasmic reticulum, nuclear membrane, and plasma membrane of rat liver (29). Since our assay conditions for TPPase were identical to those used by Yamazaki and Hayaishi, it might be expected that isolated bovine endoplasmic reticulum and plasma membrane would show this activity. Further investigation will be necessary to elucidate whether the TPPase found in isolated Golgi membranes is the same as that found in endoplasmic reticulum.

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