The Site of Incorporation of Sialic Acid Residues into Glycoproteins and the Subsequent Fates of These Molecules in Various Rat and Mouse Cell Types as Shown by Radioautography after Injection of [^{3}H] *N*-Acetylmannosamine

I. Observations in Hepatocytes

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ABSTRACT To study the site of incorporation of sialic acid residues into glycoproteins in hepatocytes, we gave 40-g rats and 15-g Swiss albino mice a single intravenous injection of [^{3}H] *N*-acetylmannosamine (8 mCi) and then sacrificed them after 2 and 10 min. To trace the subsequent migration of the labeled glycoproteins, we injected 40-g rats with 4 mCi of [^{3}H] *N*-acetylmannosamine and sacrificed them after 20 and 30 min, 1, 4, and 24 h, and 3 and 9 d. Concurrent biochemical experiments were carried out to test the specificity of injected [^{3}H] *N*-acetylmannosamine as a precursor for sialic acid residues of glycoproteins. In radioautographs from rats and mice sacrificed 10 min after injection, grain counts showed that over 69% of the silver grains occurred over the Golgi region. The majority of these grains were localized over the trans face of the Golgi stack, as well as over associated secretory vesicles and possibly GERL. In rats, the proportion of grains over the Golgi region decreased with time to 37% at 1 h, 11% at 4 h, and 6% at 24 h. Meanwhile, the proportion of grains over the plasma membrane increased from 4% at 10 min to 29% at 1 h and over 55% at 4 and 24 h; two-thirds of these grains lay over the sinusoidal membrane, and the remainder were equally divided over the lateral and bile canalicular membranes. Many silver grains also appeared over lysosomes at the 4- and 24-h time intervals, accounting for 15–17% of the total. At 3 and 9 d after injection, light microscope radioautographs revealed a grain distribution similar to that seen at 24 h, with a progressive decrease in the intensity of labeling such that by 9 d only a very light reaction remained. Because our biochemical findings indicated that [^{3}H] *N*-acetylmannosamine is a fairly specific precursor for the sialic acid residues of glycoproteins (and perhaps glycolipids), the interpretation of these results is that sialic acid is incorporated into these molecules in the Golgi apparatus and that the latter then migrate to secretion products, to the plasma membrane, and to lysosomes in a process of continuous renewal. It is possible that some of the label seen in lysosomes at later time intervals may have been derived from the plasma membrane or from material arising outside the cells.

During the past several years, radioautographic studies have been carried out in our laboratory to determine the intracellular sites at which sugar residues are added to the carbohydrate side chains of glycoproteins. These molecules, although diverse in structure, always consist of a polypeptide chain and one or more carbohydrate side chains. In the case of side chains linked

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to asparagine residues, the core sugars located close to the polypeptide chain, such as mannose, are seen to be added in the rough endoplasmic reticulum, whereas more peripheral sugars, such as galactose and fucose, are added only after the newly synthesized glycoprotein has migrated to the Golgi apparatus (7).

Sialic acid residues are common in the carbohydrate side chains of secretory and membrane glycoproteins (54, 94, 99), as well as glycolipids (53, 63, 92). These sialic acid residues have been implicated in a number of diverse biological functions (53, 54, 88, 94). In liver, the role of sialic acid residues in determining the life span of circulating blood cells and glycoproteins has been well documented (1, 16). Furthermore, the hepatocyte plasma membrane receptor for asialoglycoproteins is itself a sialoglycoprotein whose own own sialic acid residues are essential for its functioning (97).

Radioautographic studies on the site of incorporation of sialic acid residues into glycoproteins have been hindered by the fact that exogenously administered sialic acid does not easily enter intact cells (22, 27, 29, 43, 49). Various biochemical studies, however, have shown that *N*-acetylmannosamine can serve as a fairly specific precursor for sialic acid residues of glycoproteins and glycolipids (39, 43, 65, 81, 86, 87, 114).

We have therefore injected [^{3}H]*N*-acetylmannosamine into young rats and mice, and examined radioautographs of various tissues from animals killed 2 or 10 min later. The subsequent fate of the newly labeled glycoproteins was investigated in radioautographs of rats killed 20 min to 9 d after injection. We carried out concurrent biochemical studies to determine the specificity of injected *N*-acetylmannosamine as a precursor for the sialic acid residues of glycoprotein molecules in our experimental animals. The present paper describes our findings in the hepatocyte. Some of the results of the present study have been published in preliminary form (7, 9, 60).

MATERIALS AND METHODS

Radioautographic Studies

Young (40 g) male Sherman rats and Swiss albino mice (15 g), fed ad lib were anesthetized with Nembutal and given a single injection, via the external jugular vein, of 8 mCi of [^{3}H]*N*-acetylmannosamine (New England Nuclear, Boston, Mass.; spec act 2.2 Ci/mM) in 0.2 ml saline solution. After 2 or 10 min, the animals were killed by intracardiac perfusion for 15 min at room temperature with a solution of 2.5% glutaraldehyde in 0.05 M Sorensen's buffer, following a 30-s prewash with lactated Ringer's solution. Other young male rats (40 g) were injected as above with 4 mCi of [^{3}H]*N*-acetylmannosamine and killed after 20 and 30 min, 1, 4, and 24 h, and 3 and 9 d.

Samples of liver were removed and immersed in the above perfusion fixative solution for 2 h at 4°C. The samples were then washed in 0.15 M Sorensen's buffer, postfixed in 1% OsO_4 in 0.1 M Sorensen's buffer for 1 h at 4°C, dehydrated in graded ethanol solutions, passed through propylene oxide, and then embedded in Epon.

For light microscope radioautography, 1-μm sections were stained with iron-hematoxylin before coating with Kodak NTB2 emulsion. Radioautographs were developed in Kodak D-170.

For electron microscope radioautography, thin sections were placed on celloidin-coated glass slides and stained with uranyl acetate before carbon coating and coating with Ilford L-4 emulsion. Radioautographs were developed in Kodak D-19b (1:10), and the sections placed on 300-mesh copper grids and poststained with lead citrate.

Analysis of Electron Microscope Radioautographs

A modification of the direct scoring method of evaluation was used as follows: grains over hepatocytes were recorded as being over one of a selected list of hepatocyte organelles, i.e. Golgi saccules, secretory vesicles, lysosomes, mitochondria, bile canalicular plasma membrane, lateral plasma membrane, and sinusoidal plasma membrane. Any grain which was not located over any of the selected organelles (i.e. whose center was further away than the diameter of the grain) was listed as being over the remainder of the cell. Grains located over the sinusoidal lumen or over sinusoidal lining cells at a distance greater than their own diameter from a hepatocyte surface were not included in the evaluation.

Incubation of Liver Tissue to Identify Lysosomes and GERL Elements

In previous studies by Novikoff and Novikoff (71) and by Hand and Oliver (42), when tissues were incubated for acid phosphatase activity using cytidine monophosphate (CMP) as a substrate, both lysosomes and GERL were found to react positively. To reveal these elements in the present study, our liver tissue was incubated in the same fashion. Young (40 g) rats were anesthetized with Nembutal and then received an intracardiac perfusion with lactated Ringer's solution for 30 s, followed by fixative solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and 0.05% calcium chloride for 15 min at room temperature. Pieces of liver were removed and immersed in the same fixative for an additional 2 h at 4°C. For incubation, a modification of the procedure of Novikoff (70) was carried out as follows: The pieces were washed for several hours at 4°C in 0.1 M sodium cacodylate buffer (pH 7.4) containing 4% sucrose (CS buffer), and then embedded in agar (7% solution). Thick 60–75-μm sections were prepared using a Sorvall TC-2 tissue chopper (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.); these were washed several times in cold CS buffer, rinsed twice in 40 mM sodium acetate buffer (pH 5.0) containing 5% sucrose, and then incubated with agitation for 1 h at 37°C in an incubation medium consisting of: 4 mM CMP, sodium salt (Sigma Chemical Co., St. Louis, Mo.); 40 mM sodium acetate buffer (pH 5.0); 4 mM lead citrate (Suprapur, BDH Chemicals Ltd., Poole, England); 10 mM manganese chloride; and 5% sucrose. Control sections were incubated for 1 h at 37°C in a duplicate medium containing no substrate. After incubation the sections were washed several times in 40 mM sodium acetate buffer (pH 5.0) containing 5% sucrose, rinsed several times in CS buffer, and postfixed in an aqueous solution of 1% OsO_4 and 1.5% potassium ferrocyanide for 1 h at 4°C. After Epon embedding, sections were cut and stained with uranyl acetate and lead citrate.

Biochemical Analysis of Radioactivity Distribution in Rat Liver and Duodenum after Injection of [^{3}H]*N*-Acetylmannosamine

Young rats (40 g) were injected, via the external jugular vein, with [^{3}H]*N*-acetylmannosamine. After 10 min or 4 h the rats were perfused through the left ventricle for 30 s with lactated Ringer's solution. Samples of liver and duodenum were then removed and fractionated into "small molecular," lipid, and protein fractions. Two different methods of fractionation were used. In two animals (injected with 1 mCi of [^{3}H]*N*-acetylmannosamine, and killed at 10 min and 4 h), each sample was homogenized in 19 ml/g of 2:1 chloroform:methanol (C-M) (34) and centrifuged for 20 min at 10,000 rpm to separate proteins from lipids and small molecules. The pellet, containing the proteins, was washed in 2 ml of 2:1 C-M and the washing was added to the original supernate. The supernate was then partitioned by the method of Folch et al. (34) using 0.05% $CaCl_2$. This resulted in an upper phase containing small molecules (and perhaps gangliosides [82]) and a lower phase containing lipids. Aliquots of the upper and lower phases and of the pellet (after digestion with NCS tissue solubilizer) were then counted for radioactivity using a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The second method of fractionation was used on tissues of an animal injected with 2 mCi of [^{3}H]*N*-acetylmannosamine and killed 4 h later. Each sample was homogenized in 5 ml/g of 0.25 M sucrose at 4°C, and then an equal volume of 10% trichloroacetic acid:2% phosphotungstic acid (TCA-PTA) was added (103). After 20 min, the homogenate was centrifuged at 1,000 *g* for 10 min to separate macromolecules from small molecules. The pellet, containing the macromolecules, was washed twice in 0.5 ml 5%: 1% TCA-PTA, and these washings were added to the original supernate. The pellet was extracted with 2:1 C-M to separate lipids from proteins. To separate any remaining small molecules from the lipids, the extract was partitioned by the method of Folch et al. (34).

Aliquots of the TCA-PTA supernate, Folch upper and lower phases, and the pellet (after digestion) were counted for radioactivity.

To determine the proportion of label in the protein fractions associated with sialic acid residues, we hydrolyzed aliquots of the final pellets from each of the above experiments, using 0.1 N H_2SO_4 at 80°C for 1 h to selectively remove sialic acid residues from the glycoprotein molecules (3, 58, 95, 106). The suspension was allowed to cool to 4°C and was centrifuged at 1,000 *g*. The pellet was washed twice in 0.1 N H_2SO_4, and the washings were added to the original supernate. Aliquots of the supernate and the pellet (after digestion) were then counted for radioactivity.

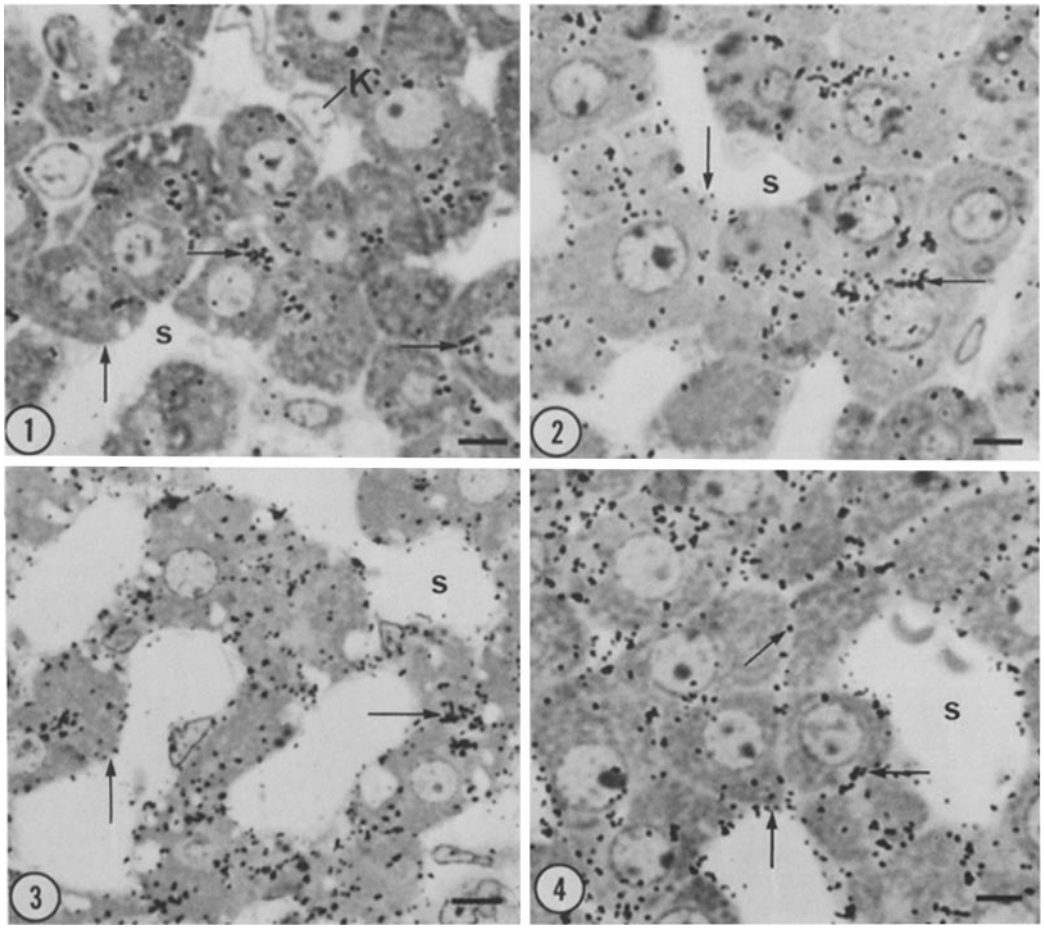
RESULTS

Light Microscope Radioautographic Studies

In liver tissue from rats and mice killed 2 and 10 min after [^{3}H]*N*-acetylmannosamine injection, the hepatocytes exhibited reactions in which the silver grains were localized over the bile canalicular region (containing the Golgi apparatus) (Fig. 1). No significant reaction appeared over the sinusoidal cell surfaces or over cells lining the sinusoids (i.e., endothelial and Kupffer cells). In rats killed 20 min after injection the reactions were similarly localized, but by 30 min after injection some reaction began to appear over the sinusoidal cell surfaces (Fig. 2). By 4 h, the sinusoidal surfaces exhibited a substantial reaction (Fig. 3), and at 24 h, lines of grains could also be distinguished over lateral cell surfaces (Fig. 4). By this latter time interval, only light reactions remained over the bile canalicular regions. In addition, Kupffer cells lining some sinusoids exhibited heavy reactions over their cytoplasm. At 3 and 9 d after injection, light microscope radioautographs revealed a grain distribution similar to that seen at 24 h but with a progressive decrease in the intensity of labeling such that by 9 d only a very light reaction remained.

Electron Microscope Morphologic and Histochemical Studies

In electron micrographs of hepatocytes, Golgi stacks were most frequently found in the cytoplasm near to bile canaliculi (Figs. 5 and 6). When seen in cross-section, they exhibited a definite polarity. At the cis face, which usually faced the bile canaliculus, the saccules were somewhat dilated and contained lipoprotein particles in a lightly stained matrix (Figs. 6–8).



FIGURES 1–4 Light microscope radioautographs of Epon sections of liver tissue from rats injected with [^{3}H] *N*-acetylmannosamine. Stained with iron hematoxylin. × 1,200. Bar, 5 μm.

FIGURE 1 10 min after injection. Exposed 5 mo. The silver grains are seen to be localized over the bile canalicular (Golgi) region of hepatocytes (horizontal arrows). No significant reaction is observed over the remainder of the cytoplasm of the hepatocytes, over their sinusoidal surface (vertical arrow), over Kupffer cells (*K*), or over endothelial cells. (*S*, sinusoid).

FIGURE 2 30 min after injection. Exposed 2 mo. Many grains remain over the bile canalicular region of hepatocytes (horizontal arrow), but by this time, some grains also appear over the sinusoidal surface (vertical arrow). (*S*, sinusoid).

FIGURE 3 4 h after injection. Exposed 2 mo. By this time interval many grains are seen over the sinusoidal surface of the hepatocytes (vertical arrow). Clusters of silver grains also remain over the bile canalicular region of the hepatocytes (horizontal arrow). (*S*, sinusoid).

FIGURE 4 24 h after injection. Exposed 5 wk. Many silver grains are seen over sinusoidal surfaces of hepatocytes (vertical arrow), and lines of reaction can also be distinguished over the lateral surfaces (oblique arrow). Some grains remain localized over the bile canalicular region (horizontal arrow). (*S*, sinusoid).

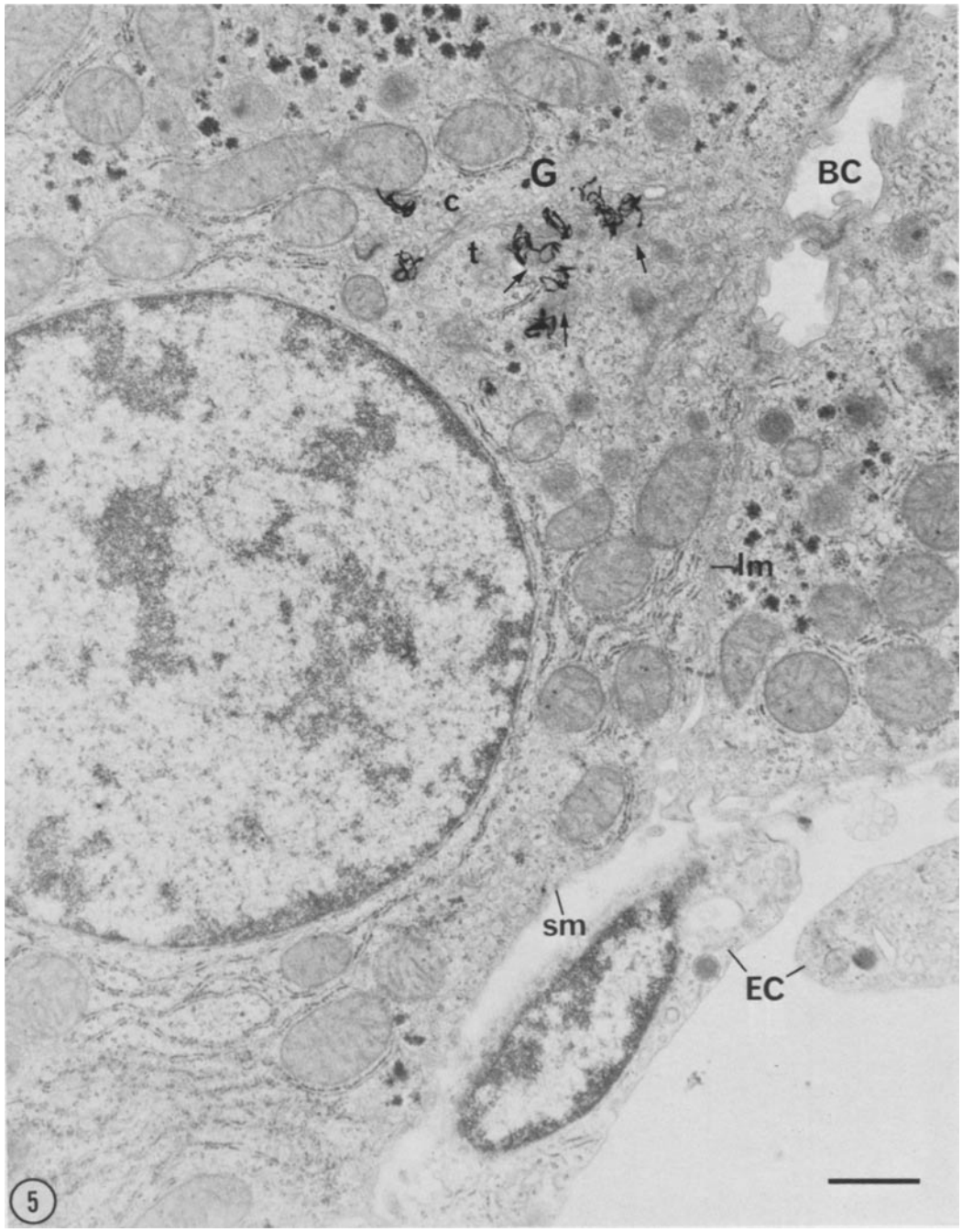


FIGURE 5 Electron microscope radioautograph of liver tissue from a rat sacrificed 10 min after injection of [^{3}H]*N*-acetylmannosamine. Stained with uranyl acetate and lead citrate. Exposed 8 mo. All of the silver grains in this photograph are localized over the region of a hepatocyte Golgi apparatus (*G*). The latter exhibits a cis face (*c*) and a trans face (*t*). Some of the grains lie directly over Golgi saccules, but others are associated with secretory vesicles (arrows) which are found adjacent to the trans face of the Golgi stack. No reaction occurs over the remainder of the cytoplasm, the plasma membrane of the bile canaliculus (*BC*), the lateral plasma membrane (*lm*), the sinusoidal plasma membrane (*sm*), or the adjacent endothelial cells (*EC*). × 13,000. Bar, 1 μm.

Towards the trans face of the stack the saccules became narrower and had a somewhat denser matrix. The ends of these saccules were expanded, contained many lipoprotein granules, and appeared to be forming secretory vesicles (Fig. 8). Secretory vesicles were routinely located adjacent to the trans face of the Golgi stack.

In sections of liver tissue that had been incubated with CMP at pH 5 to detect acid phosphatase activity, an irregular cisterna was often seen along the trans face of the Golgi stack (Figs. 7 and 8). This cisterna exhibited a heavy reaction and is presumed to be part of GERL as described in this site in normal rat liver cells by Novikoff and Yam (72). The dilated portions of this cisterna had the same dimensions as secretory vesicles and contained several light areas the size of lipoprotein particles. Secretory vesicles in the Golgi area and the Golgi saccules themselves exhibited occasional light deposits of reaction product (Figs. 7 and 8).

Electron Microscope Radioautographic Studies

Electron microscope radioautographs of hepatocytes from rats and mice killed 10 min after [^{3}H]*N*-acetylmannosamine

injection revealed that most silver grains were localized over the Golgi stacks and adjacent secretory vesicles (Figs. 5 and 6). Grain counts showed that ~70% of the total grains occurred over these structures (Table I).

The distribution of silver grains over the Golgi stacks was not uniform. Close examination of grains occurring over transversely sectioned Golgi stacks showed that a majority of the grains were associated with the trans face of the Golgi stack and the adjacent secretory vesicles (Figs. 5 and 6). Counts of these grains in two different animals showed that 63–70% occurred over the trans face and adjacent secretory vesicles; 9–15% occurred over the expansions of saccules at the ends of the Golgi stacks; 15% occurred over the central portions of saccules; and only 7% occurred over the cis face (Table II).

In rats killed 1 h after [^{3}H]*N*-acetylmannosamine injection, some silver grains remained over the Golgi stacks and adjacent secretory vesicles, but now many grains appeared over the plasma membrane (Fig. 9). In addition, a significant number of grains were associated with secretory vesicles in the cytoplasm outside of the Golgi region, often just beneath the sinusoidal plasma membrane (Fig. 9, inset). Counts showed that ~37% of the total grains remained over the Golgi region at the 1-h time interval; 19% were associated with secretory vesicles elsewhere in the cytoplasm, and ~29% were over the plasma membrane (Table I). In the latter group, the majority of the grains were over the sinusoidal portion of the plasma membrane, whereas smaller numbers occurred over the bile canalicular and lateral portions.

Radioautographs from rats killed 4 h after injection showed that most of the grains now occurred over the plasma membrane (Figs. 10 and 11). Grain counts revealed that over 15% of the label was now associated with lysosomes (Table I).

In rats killed 24 h after injection, the grain distribution was similar to that seen at 4 h, with the majority of grains occurring over the plasma membrane (Figs. 12 and 13, Table I). At both the 4- and 24-h time intervals an increasing amount of reaction was seen over the lysosomes of Kupffer and endothelial cells lining the sinusoids (Figs. 10, 12, and 13).

Biochemical Studies

When samples of liver and duodenal tissue from a rat killed 10 min after [^{3}H]*N*-acetylmannosamine injection were fractionated into small molecular, lipid, and protein fractions, 72.0% of the label in liver and 70.0% of that in duodenum was recovered as small molecules (Folch upper phase) (Table III). Label in the lipid fraction (Folch lower phase) amounted to only 0.3% in liver and 0.4% in duodenum. Protein molecules contained 27.7% of the label in liver and 29.6% in duodenum.

When rats were killed 4 h after [^{3}H]*N*-acetylmannosamine injection, and samples of liver and duodenal tissue were fractionated by two different methods into small molecular, lipid, and protein fractions (see Materials and Methods), 98.0–99.0% of the label in liver and 90.0–99.8% in the duodenum was recovered in the protein fraction (Table III). The lipid fraction contained from 0.0 to 0.3% of the label in liver, and from 0.2 to 1.2% in duodenum. The small molecular fraction contained

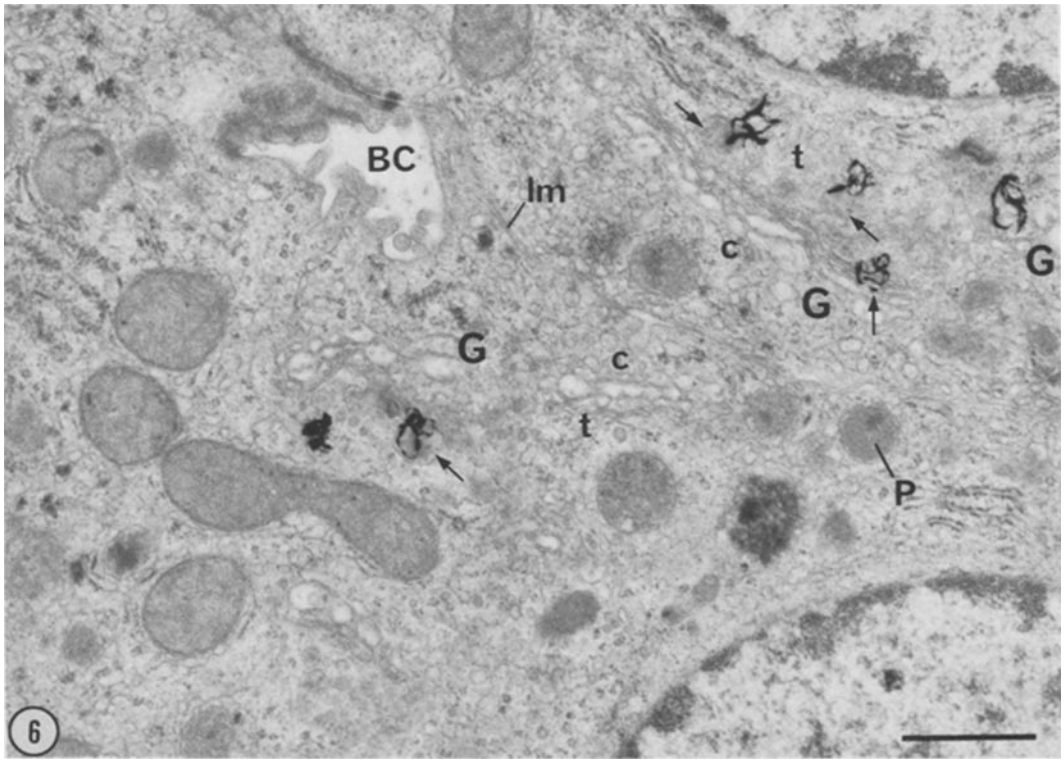
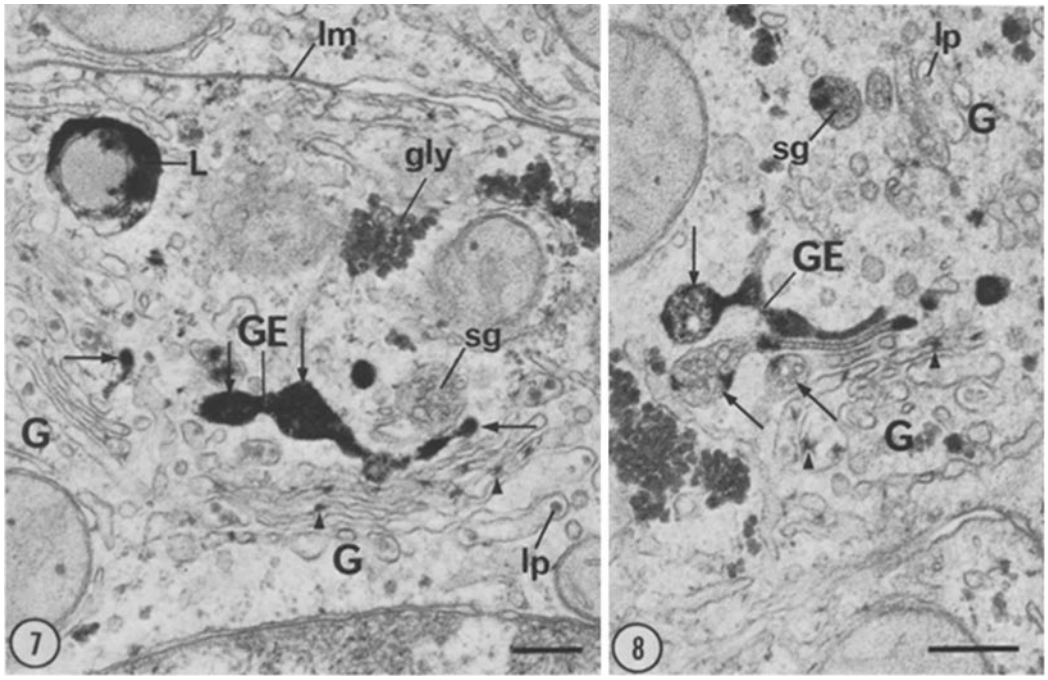


FIGURE 6 Electron microscope radioautograph of the bile canalicular region of two hepatocytes from a rat sacrificed 10 min after injection of [^{3}H] *N*-acetylmannosamine. Stained with uranyl acetate and lead citrate. Exposed 8 mo. Two Golgi stacks (*G*) are present, each exhibiting a cis face (*c*) and a trans face (*t*). In each of the Golgi stacks, the cis face is directed toward the bile canaliculus (*BC*), while the trans face is directed away from it. This was found to be the usual pattern in these cells. All of the silver grains in this photograph are associated with the Golgi stacks. Some grains occur directly over Golgi saccules (vertical arrow), but most of the grains are found over the trans face where they are often associated with secretory vesicles (oblique arrows). No reaction occurs over the plasma membrane of the bile canaliculus or the lateral plasma membrane (*lm*). (*p*, peroxisome). × 18,400. Bar, 1 μm.



FIGURES 7 and 8 Electron micrographs of rat liver tissue incubated with CMP at pH 5 for 1 h to detect acid phosphatase activity. Each figure shows a Golgi stack (*G*) which exhibits a cis face (where the *G* is located) and an opposing trans face. Expanded regions of saccules on the cis face contain lipoprotein particles (*lp*). At the trans face of each Golgi stack, an elongated irregular cisterna exhibits a heavy reaction, and is presumed to be part of GERL (*GE*). Small dilations of this cisterna have a fuzzy coating and probably represent coated vesicles either leaving or joining the GERL (Fig. 7 horizontal arrows). Near the trans face of the Golgi stack are secretory vesicles (*sg*) which contain numerous lipoprotein particles. The secretory vesicle in Fig. 7 exhibits no reaction product, but some is present in the secretory vesicle in Fig. 8. Dilations of Golgi saccules near the trans face in Fig. 8 (oblique arrows) presumably represent forming secretory vesicles and also exhibit some reaction product. The large dilations of GERL (vertical arrows) exhibit circular light areas the size of lipoprotein particles. Heavy reaction product occurs in a lysosome (*L*) in Fig. 7, and occasional light deposits of reaction product occur in the Golgi saccules in both figures (arrowheads). (*gly*, glycogen; *lm*, lateral plasma membrane). × 30,000 (Fig. 7), × 40,000 (Fig. 8). Bar, 0.3 μm.

only 1.0–1.7% of the label in liver and 0.0–0.8% in duodenum.

When aliquots of the final pellets of the above experiments were hydrolyzed in 0.1 N H_2SO_4 at 80°C for 1 h to selectively remove sialic acid residues from glycoprotein molecules, the hydrolysis released 95.3–96.0% of the label in liver, and 91.3–98.0% in duodenum (Table IV).

DISCUSSION

Suitability of [^{3}H]N-Acetylmannosamine as a Precursor of Sialic Acid Residues of Glycoproteins in Radioautographs

In the biochemical investigations of the present study, samples of fresh liver and duodenum from young rats killed at 10 min and 4 h after [^{3}H]*N*-acetylmannosamine injection were fractionated into a small molecular fraction, a macromolecular lipid fraction, and a macromolecular protein fraction. In all cases, the macromolecular label was found to occur very largely in the protein fraction and interpreted to be glycoprotein in nature (Table III).

The lipid fraction accounted for only 0–1.2% of the label, a finding in accord with that of Harms and Reutter (43) who reported that after injection of [^{14}C]*N*-acetylmannosamine into rats, <5% of the incorporated label in liver was in the form of glycolipid. It should be noted, however, that extraction of protein pellets with 2:1 CM, as carried out in the present study, does not quantitively remove all very polar gangliosides from the pellet (100). Thus, some of the label observed in the macromolecular protein fraction may be caused by gangliosides. Similarly, in the partitioning method of Folch et al (34), a portion of the gangliosides present may remain in the small molecular Folch upper phase even when 0.05% (0.005 M) $CaCl_2$ is used (82). Thus, some of the label observed in the small molecular fraction may also be the result of gangliosides. In liver, the relative amount of ganglioside present is low, as indicated by the finding of Benedetti and Emmelot (5) that at least 95% of the sialic acid in rat liver plasma membrane is protein bound. This is not the case in all tissues, however, and in such instances a substantial portion of the injected [^{3}H]*N*-acetylmannosamine label may be incorporated into glycolipids.

In the Epon-embedded tissue used for radioautography, only macromolecular label would be retained; thus many labeled gangliosides in the small molecular fraction may be lost. In addition, some lipids may be lost in the organic solvents used for embedding. Thus, in radioautographic sections of liver and duodenum, most of the label could be expected to reside in glycoproteins.

When the protein fractions from fresh tissues in the present experiments were hydrolyzed in 0.1 N H_2SO_4 at 80°C for 1 h, over 95% of the label was released in liver, whereas 91–98% was released in duodenum. This mild acid hydrolysis procedure is considered to cleave specifically sialic acid residues from glycoproteins (3, 58, 95, 106). When radioactivity was released from protein fractions under identical circumstances by various workers and subsequently analyzed by chromatography, at least 90% of the radioactivity was characterized as sialic acid (24, 27, 48, 114).

From the above results, it may be concluded that in Epon-embedded sections in liver and duodenal tissues used for radioautography, the [^{3}H]*N*-acetylmannosamine label is attached to sialic acid residues, and, although these may not reside exclusively in glycoproteins, the latter molecules probably account for most of the label. In other tissues, especially those rich in gangliosides, it is possible that even in radioautographic sections a significant portion of the label may reside in glycolipids.

Analysis of Electron Microscope Radioautographs

In the past, the simplest method used for evaluating the relative content of radioactive label amongst the structures seen

TABLE II

Distribution of Silver Grains Associated with Transversely Sectioned Golgi Stacks in Hepatocytes of Rats Sacrificed 10 min after [^{3}H]N-Acetylmannosamine Injection

		Distribution of grains, %			
Animal	Total grains counted	Cis face of Golgi stack	Central portion of Golgi saccules	Expansions of saccules at ends of Golgi stack	Trans face of Golgi stack and adjacent secretory vesicles
1	230	7	15	15	63
2	304	7	15	9	70

TABLE I

Distribution of Silver Grains over Structures in Hepatocytes at Various Time Intervals after [^{3}H]N-Acetylmannosamine Injection

		Distribution of grains over organelles, %							
					Plasma membrane				
Time after injection	Total grains counted	Golgi stacks and adjacent secretory vesicles	Secretory vesicles elsewhere in cytoplasm	Lysosomes	Bile canaliculus	Lateral	Sinusoidal	Mitochondria	Remainder of cell
10 min (rat)	1,257	69.0	5.0	1.5	0.7	1.2	2.0	10.0	10.6
10 min (mouse)	512	72.0	3.1	0.9	1.4	2.1	1.1	8.7	10.7
1 h (rat)	300	37.2	19.1	5.6	7.2	3.9	17.7	4.1	5.2
4 h (rat)	1,092	11.3	6.3	15.4	6.9	8.2	40.2	6.8	4.9
24 h (rat)	635	6.6	0.6	17.3	15.4	7.2	41.4	6.3	5.0

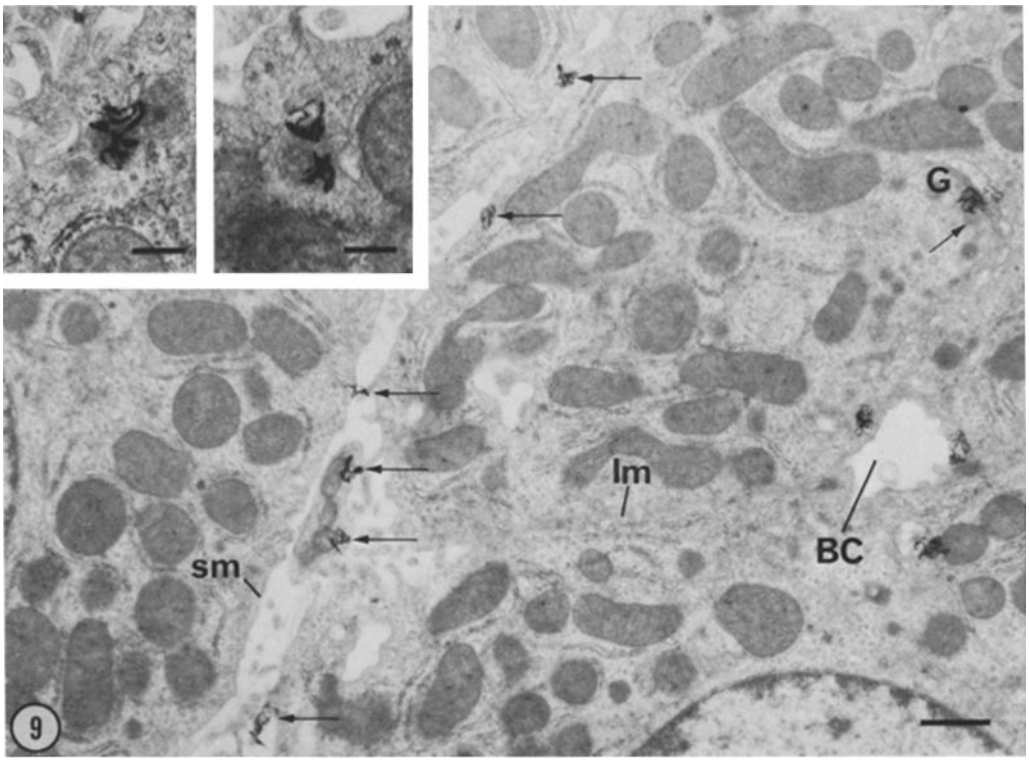
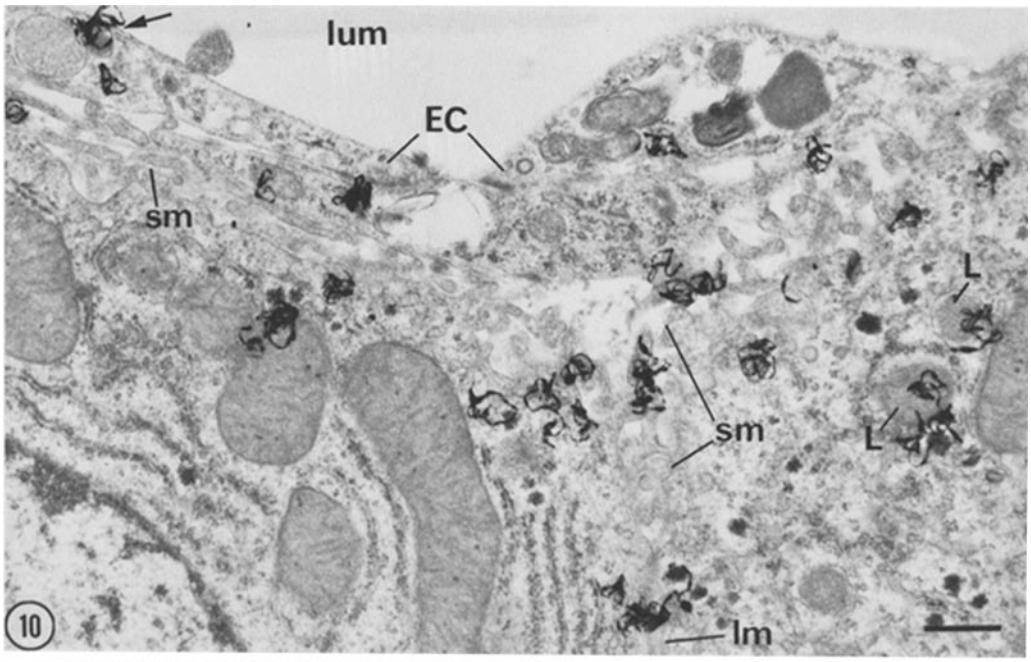
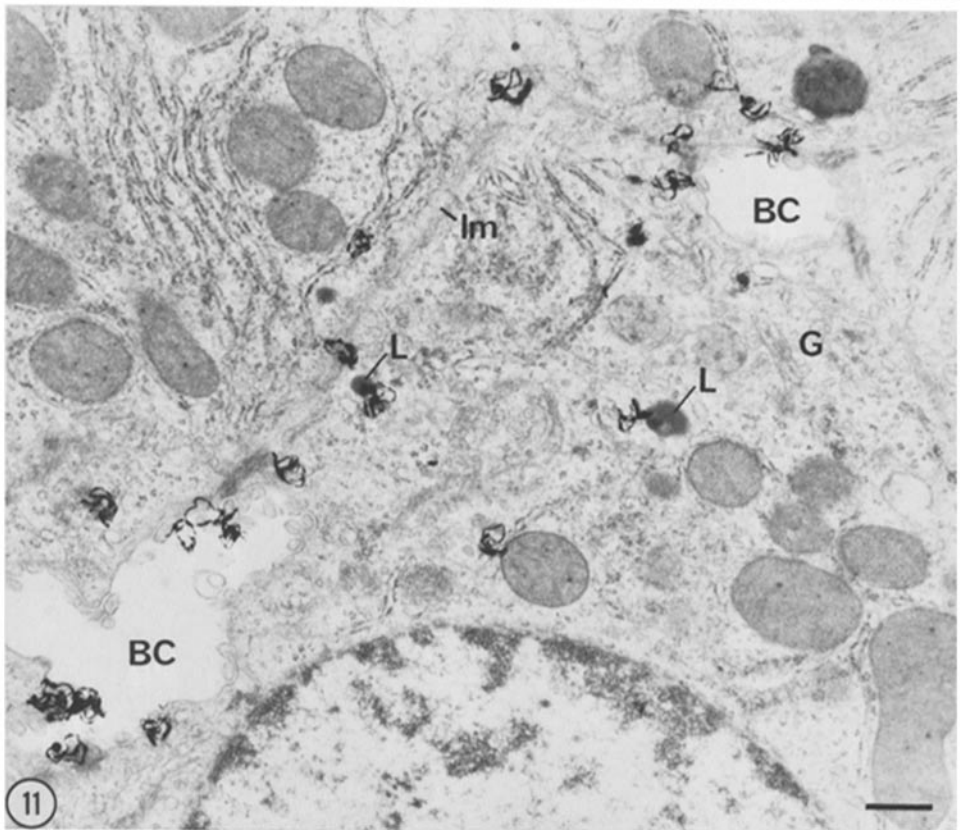


FIGURE 9 Electron microscope radioautograph of hepatocytes from a rat sacrificed 1 h after injection of [^{3}H] *N*-acetylmannosamine. Stained with uranyl acetate and lead citrate. Exposed 8 mo. One silver grain lies over the edge of a secretory vesicle (oblique arrow) adjacent to the trans face of a Golgi stack (G). All but one of the remaining grains are found over either the plasma membrane of the bile canaliculus (*BC*) or the sinusoidal plasma membrane (*sm*, horizontal arrows). Some grains are associated with secretory vesicles located in the cytoplasm just beneath the sinusoidal membrane (*inset*). (*lm*, lateral plasma membrane.) × 9,300. Bar, 1 μm. × 23,000 (*inset*). Bar, 0.3 μm.

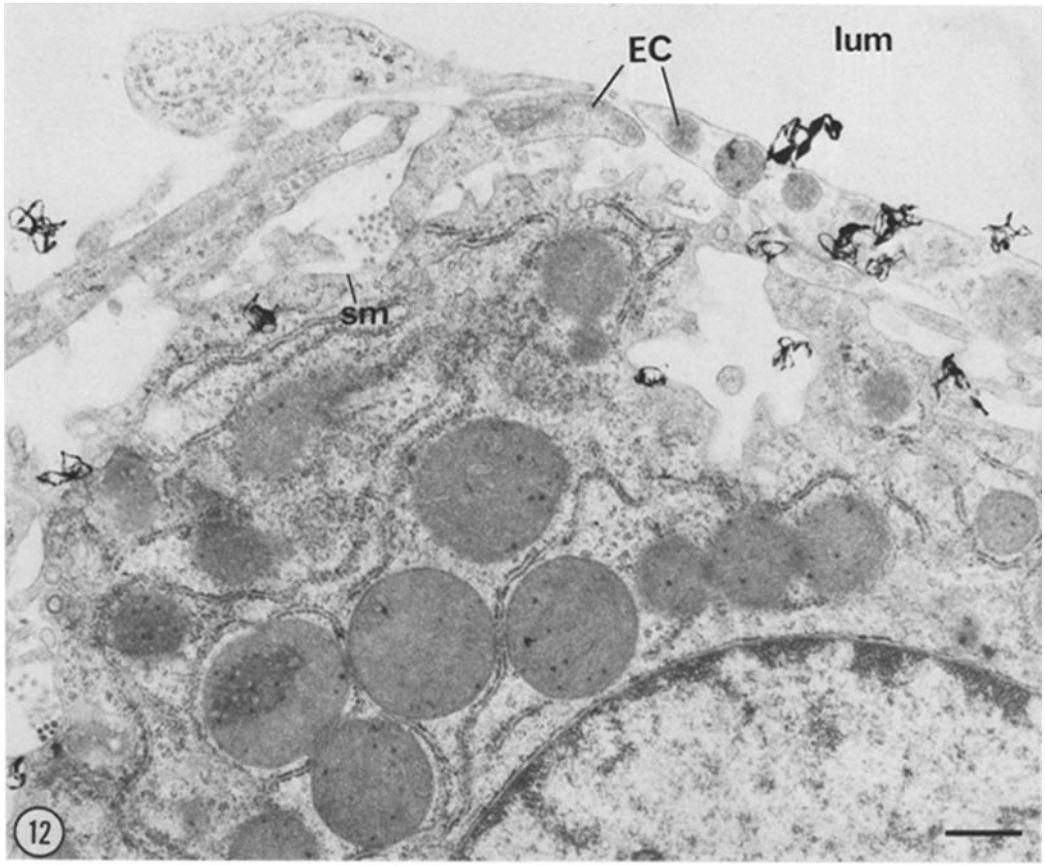


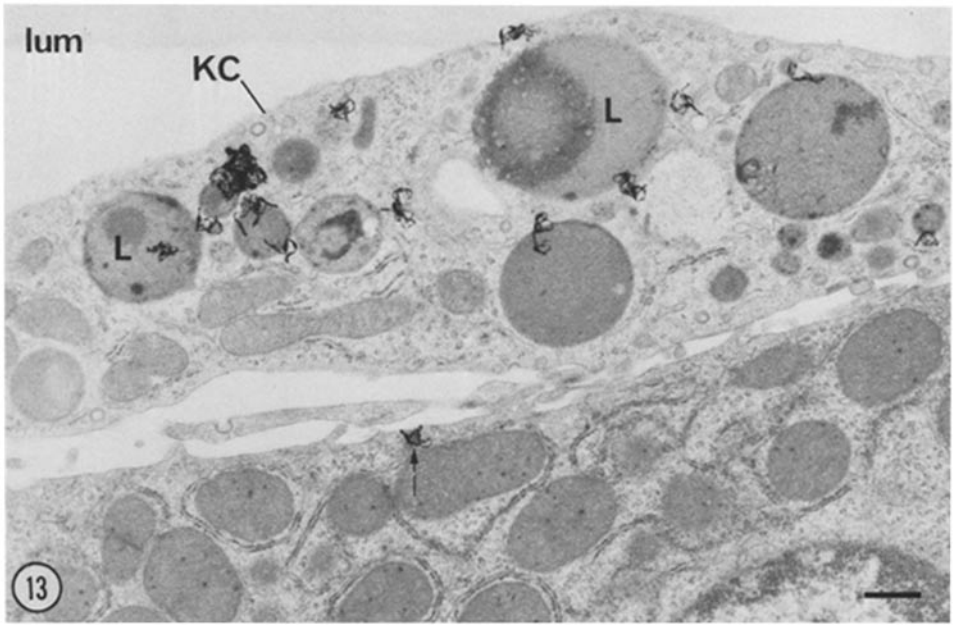


FIGURES 10 and 11 Electron microscope radioautographs of liver tissue from a rat sacrificed 4 h after injection of [^{3}H]*N*-acetylmannosamine. Stained with uranyl acetate and lead citrate. Exposed 8 mo.

FIGURE 10 Portions of two hepatocytes are seen. These border on a sinusoidal lumen (*lum*) which is lined by endothelial cells (*EC*). In the hepatocytes, most of the silver grains are associated with the sinusoidal plasma membrane (*sm*) or lateral plasma membrane (*lm*). Some grains occur over lysosomes (*L*). Similarly, in endothelial cells, some of the silver grains are associated with lysosomes (arrow). × 19,200. Bar, 0.5 μm.

FIGURE 11 Portions of three hepatocytes are seen, separated by lateral plasma membranes (*lm*) and bile canaliculi (*BC*). Most of the silver grains in the photograph are associated with the plasma membrane of the bile canaliculi or with the lateral plasma membranes. Two grains occur over lysosomes (*L*). A Golgi stack (G) is unlabeled. × 17,500. Bar, 0.5 μm.





FIGURES 12 and 13 Electron microscope radioautographs of liver tissue from a rat sacrificed 24 h after injection of [^{3}H]*N*-acetylmannosamine. Stained with uranyl acetate and lead citrate. Exposed 8 mo.

FIGURE 12 A portion of a hepatocyte is seen bordering on a sinusoidal lumen (*lum*) which is lined by endothelial cells (*EC*). In the hepatocyte, all of the silver grains are localized over the sinusoidal plasma membrane (*sm*). In the endothelial cells the grains lie over the plasma membrane and lysosomes. × 20,000. Bar, 0.5 μm.

FIGURE 13 A portion of a hepatocyte is seen bordering on a sinusoidal lumen (*lum*) which is lined by a Kupffer cell (*KC*). Most of the silver grains are localized over the numerous lysosomes (*L*) of the Kupffer cell. One grain overlies the plasma membrane of the hepatocyte (arrow). × 14,300. Bar, 0.5 μm.

TABLE III

Distribution of Label in Small Molecular, Lipid, and Protein Fractions of Tissues from Rats Injected with [^{3}H]N-Acetylmannosamine

Tissue	Time of sacrifice	Treatment	Total radioactivity	Percentage of total radioactivity		
				Small* molecular	Lipid	Protein‡
			dpm/g			
Liver	10 min	C-M	59,350,700	72.0	0.3	27.7
	4 h	C-M	69,777,000	1.7	0.3	98.0
	4 h	TCA-PTA	231,313,900	0.8	0.0	99.2
Duodenum	10 min	C-M	10,525,400	70.0	0.4	29.6
	4 h	C-M	19,628,000	8.8	1.2	90.0
	4 h	TCA-PTA	221,511,200	0.0	0.2	99.8

Fractions obtained as described in Materials and Methods. Radioactivity is expressed as disintegrations per minute per gram wet weight of tissue before homogenization. Efficiency was determined by channels ratio method.

* May contain some gangliosides (82).

‡ May also contain some gangliosides (100).

TABLE IV

Release of Label from Protein Fractions by Acid Hydrolysis*

Tissue	Treatment	Total radioactivity	Percentage of radioactivity remaining in protein fraction	Percentage of radioactivity released by hydrolysis
		dpm/aliquot		
Liver	C-M	698,800	4.0	96.0
	TCA-PTA	345,270	4.7	95.3
Duodenum	C-M	258,370	2.0	98.0
	TCA-PTA	328,810	8.7	91.3

* From animals sacrificed 4 h after [^{3}H] N-acetylmannosamine injection.

in electron microscope radioautographs, has been that of direct scoring, i.e., the structure underlying the center of a silver grain is considered to be the radioactive source of that grain. An objection to this method is that a silver grain observed in the electron microscope does not necessarily lie directly over its radioactive source. It has been shown experimentally that silver grains can be developed at distances >2 μm from a known tritium source (23). In attempts to take into consideration this relatively poor radioautographic resolution in the evaluation of electron microscope radioautographs, Nadler (40) designed an analytical method requiring the drawing of a resolution boundary circle around every silver grain in photographs of radioautographs to enclose with some degree of probability the expected source of radioactivity in the tissue that was responsible for that grain. When two or more structures occurred within the resolution boundary circle of one silver grain, that grain was considered to be shared, and the method required a special analysis to determine what proportion of shared grains should be assigned to each of the structures involved. Further, Blackett and Parry (14, 15) designed an analytical method using the known distribution of grains about a point source to construct a hypothetical distribution of silver grains over the radioautographs being analyzed, assuming a certain amount of activity in the various structures of the radioautographs. The hypothetical grain distribution was then compared with the real distribution of grains, and the activities in the various structures changed until a statistically good fit was obtained.

A recent study by Nadler (69), however, has compared the results obtained by direct scoring with those obtained by the methods of Nadler and of Blackett and Parry, both on hypothetical composites and on a number of sets of experimental radioautographs. In all cases, the direct scoring method provided results which were not statistically different from those of the latter two methods. Nadler concludes from this study that the direct scoring method is therefore acceptable, especially if it can be shown that in the system under evaluation, fewer than 50% of all grains would be shared using a 230-nm radius resolution boundary circle. In the present investigation, preliminary studies showed that the percentage of shared grains using a 230-nm radius resolution boundary circle varied from 15 to 25% depending on the time interval studied.

In the present studies, therefore, we have applied a modification of the direct scoring method as outlined in Materials and Methods.

Addition of Sialic Acid Residues to Glycoproteins in the Golgi Region

Biochemical studies have shown that the *N*-acetylglucosamine and mannose containing core portion of asparagine-linked oligosaccharide side chains is added to glycoproteins by way of a lipid intermediate during or soon after completion of synthesis of the polypeptide on bound ribosomes (75). Thus, radioautographic studies in thyroid follicular cells have indicated that mannose is incorporated into glycoproteins in the rough endoplasmic reticulum (112). The peripheral sugars, galactose and fucose, on the other hand, are added only after the glycoprotein has migrated to the Golgi apparatus (40, 112). Radioautographic studies on a variety of other cell types have also shown that incorporation of galactose and fucose occurs in the Golgi apparatus (7). Complimentary evidence has been provided by the finding of high levels of galactosyltransferase (11, 33, 67, 89) and fucosyltransferase activity (68) in the Golgi fractions of various cell types.

In the case of sialic acid, no radioautographic evidence localizing the intracellular site of incorporation has been available before the present work. Biochemical evidence suggesting that this sugar also is incorporated in the Golgi apparatus has been provided by the finding of high levels of sialyltransferase enzymes catalyzing the addition of sialic acid residues to glycoproteins (32, 38, 68, 89 [and glycolipids (31, 32, 85]) in Golgi fractions of rat liver and kidney cells. Supporting immunochemical evidence of Jamieson (56) has indicated that

the Golgi apparatus is the main site of addition of sialic acid residues to α_1-acid glycoprotein. Similarly, biochemical studies by Banerjee et al. (4) have shown that when [^{3}H]glucosamine is injected into rats, the incorporation of label into the sialic acid residues of serum glycoproteins occurs only in the Golgi fraction of hepatocytes.

In the present study, light microscope radioautographs of hepatocytes of animals killed 2 and 10 min after [^{3}H]*N*-acetylmannosamine showed that the label was localized to the Golgi region. The reaction observed at 2 min after injection was too light to permit electron microscope analysis, but in animals killed 10 min after injection, electron microscope radioautographs showed that nearly 70% of the total grains were localized over Golgi stacks and adjacent secretory vesicles (Fig. 5; Table I), thus establishing that the main site of addition of sialic acid residues to glycoproteins in these cells is the Golgi region.

The present results suggest, however, that this addition may not occur homogeneously throughout the Golgi stack and may not be limited to Golgi saccules; i.e. counts of silver grains occurring over transversely sectioned Golgi stacks showed that 63–70% of the total grains were found over the trans face and adjacent secretory vesicles, whereas the expansions of saccules at the ends of the Golgi stacks accounted for 9–15%, the central portions of saccules for 15%, and the cis face for only 7% (Table II). In previous radioautographic studies with [^{3}H]fucose and [^{3}H]galactose, the silver grains in hepatocytes of rats killed 2 and 10 min after injection had been fairly uniformly distributed over the Golgi stack (6, 98), a situation similar to that seen in thyroid follicular cells (40, 112) and in various other cell types (7, 60).

It is possible that the preferential localization of the [^{3}H]*N*-acetylmannosamine label over the trans face of the Golgi stacks and adjacent secretory vesicles in the present study was caused by a small amount of migration of the labeled glycoproteins between the time of their incorporation of the label and the time of sacrifice; i.e. in radioautographs of liver Golgi fractions, reaction was maximal over Golgi cisternae at 2 min after [^{3}H]fucose injection, but maximal over the network of tubules surrounding the cisternae at 10 min after injection (98). Examination of light microscope radioautographs in the present study showed, however, that the total amount of macromolecular label in liver tissue increased until 30 min after injection, indicating that new addition of labeled sialic acid residues to glycoproteins continued at least until that time. This fact argues against migration as a cause for the preferential localization of [^{3}H]*N*-acetylmannosamine label to the trans face of the Golgi stack at 10 min after injection, since a more homogeneous distribution would be expected because of new labeled residues still being added at that time. Thus, it appears likely that the localization seen in the present studies represents the site of addition of sialic residues to glycoprotein side chains, and may indicate that sialic acid residues are added later than fucose residues in hepatocytes.

Bretz et al. (20) have examined the distribution of galactosyltransferase, sialyltransferase and *N*-acetylglucosaminyltransferase activities in light, medium, and heavy Golgi fractions isolated from livers of ethanol-treated rats, and no significant distribution differences between the enzymes could be detected. All three enzymes, however, exhibited greater activity in the light and medium fractions (enriched in trans Golgi elements). Similarly, Hino et al. (47) examined galactosyltransferase activity in liver Golgi subfractions and reported the highest activity in a fraction believed to be enriched in trans Golgi elements. Such studies used exogenous substrates, and therefore showed the potential transferase activity of these enzymes at different sites. This may not necessarily represent the actual activity in vivo, because the latter may also depend on the levels of endogenous acceptor molecules present. Other evidence has also indicated that some sugars may be added to glycoproteins at the trans face, however, i.e. galactose, fucose and sialic acid residues are apparently added to the carbohydrate side chains of immunoglobulin M molecules in a very distal compartment of the Golgi apparatus in plasma cells (102), and fucose may be added to glycoproteins in frog bladder epithelial cells in trans Golgi elements or GERL (78). Finally, Merritt and Morré have reported a high level of galactosyltransferase activity in secretory vesicles from rat liver Golgi fractions (64).

The addition of sialic acid residues to glycoprotein side chains at the trans face of the Golgi stack in hepatocytes allows the possibility that some incorporation may take place in the GERL element whose presence at this site in normal adult rat hepatocytes has been demonstrated histochemically by Novikoff and Yam (72). In the present study, sections of liver tissue were incubated to detect acid phosphatase activity to determine whether a GERL element existed in the hepatocytes of our young animals. Such an incubation revealed, along the trans face of many hepatocyte Golgi stacks, an irregular cisterna which exhibited a heavy reaction for acid phosphatase and therefore could be considered to be a GERL element (Figs. 7 and 8), thus confirming the findings of Novikoff and Yam. In our animals, occasional deposits of reaction product were observed over Golgi saccules and some secretory vesicles (arrowheads in Figs. 7 and 8). The presence of such deposits over the innermost Golgi saccule and the forming secretory vesicle in Fig. 8 make it difficult to determine whether these elements are indeed part of the Golgi stack or whether they represent GERL elements. The studies of Hand and Oliver in rat lacrimal gland acinar cells (41, 42) have shown that the innermost saccule of the Golgi stack frequently exhibits some acid phosphatase reactivity, and suggest that this saccule may be in the process of conversion to a GERL element. It is possible that the innermost saccule seen in Fig. 8 represents a similar situation.

In many secretory cell types, the secretion granules appear to be formed from GERL elements rather than Golgi saccules (41, 42, 71). In hepatocytes, however, there is considerable evidence that secretory materials pass from the rough endoplasmic reticulum through saccules of the Golgi apparatus to secretory vesicles which then migrate to the sinusoidal cell surface (10, 25, 35, 36, 84), whereas there is no evidence for secretion of materials from GERL (72). As mentioned above, the work of Hand and Oliver in rat lacrimal gland (41) suggests that secretory material passes through the Golgi apparatus and reaches the GERL by way of conversion of the innermost Golgi saccules into GERL. If such circumstances pertained in the present study, the secretory vesicle-like elements of GERL could also be part of the secretory pathway.

Early Labeling of Structures Outside of the Golgi Region

A few silver grains (4% of total) appeared over various regions of the plasma membrane at 10 min after [^{3}H]*N*-acetylmannosamine injection, raising the possibility that some sialic acid residues may have been added to glycoproteins at this site.

There is evidence for the presence of sialytransferases, as well as other glycosyltransferases, at various animal cell surfaces (18, 24, 26, 50, 73, 76, 77, 80, 93, 105, 109, 110), although some of this evidence has been challenged (28, 48, 59, 76). The study of Munro et al. (68) showed no significant glycoprotein or glycolipid sialyltransferase activities in their liver plasma membrane fraction. The results of the present study indicate that in hepatocytes, under physiological conditions, no significant amount of sialic acid is added at the plasma membrane. A small amount of addition cannot be ruled out, but it is possible that much of the label observed at this site, even at the 10-min time interval, may represent glycoproteins which have quickly migrated from the Golgi region to the plasma membrane.

Finally, some grains (9% of total) appeared over mitochondria at 10 min after [^{3}H]*N*-acetylmannosamine injection (Table I). This proportion did not change significantly at later time intervals, suggesting an independent handling of the label. Bosmann and Hemsworth (19) have reported incorporation of monosaccharides into glycoproteins of isolated mitochondria and concluded that these organelles are capable of performing their own glycoprotein synthesis.

Migration of Labeled Glycoproteins

MIGRATION TO SECRETION PRODUCTS: As mentioned previously, there is considerable evidence that secretory materials pass from the rough endoplasmic reticulum through saccules of the Golgi apparatus to secretory vesicles which then migrate to the sinusoidal cell surface. With the exception of albumin, all of the numerous plasma proteins are glycoproteins, and nearly all contain sialic acid (113). Apart from immunoglobulin, most of these plasma proteins are synthesized in hepatocytes (94, 113). It is likely, therefore, that a substantial portion of the label observed in the Golgi region of hepatocytes at 10 min after [^{3}H]*N*-acetylmannosamine injection in the present study had been incorporated into secretory glycoproteins. This possibility is supported by the observation that many of the grains were located over secretory vesicles. At 1 h after injection, a significant number of grains appeared over secretory vesicles located in the cytoplasm outside of the Golgi region, especially in that region just beneath the sinusoidal plasma membrane (Fig. 9; inset). With the release of labeled secretory glycoproteins into the blood, the label would be lost from our system in terms of radioautographic analysis, particularly since the 30-s prewash with lactated Ringer's solution before perfusion fixation would have removed virtually all of the blood from the circulatory system. However, in certain sites, such as small veins in the lungs, pockets of intense labeling could be observed over the lumen, perhaps representing trapped labeled blood plasma proteins (see following paper). The turnover of various plasma proteins has been studied, and half lives were found to range from 2 to 12 d (113).

MIGRATION TO THE PLASMA MEMBRANE: With time, a large proportion of the label appeared over various regions of the hepatocyte plasma membrane (Table I). This migration was similar to that observed after [^{3}H]fucose injection in rat hepatocytes (8, 98), as well as in a variety of other cell types (8).

Because the hepatocyte plasma membrane has been shown to contain glycoproteins, both by histochemical (83) and by biochemical methods (54, 97), it is likely that the label seen at this site represents membrane glycoproteins. In recent years, many studies have provided evidence that membrane glycoproteins destined for the plasma membrane are synthesized on ribosomes which become bound to the endoplasmic reticulum, after which the glycoproteins travel within the membranous walls of the rough endoplasmic reticulum cisternae, Golgi elements, and vesicles or secretion granules to reach the plasma membrane (2, 17, 30, 55, 61, 62, 66, 74, 111). It is also possible that some of the plasma membrane label may represent membrane glycolipids, which are known to be present in liver plasma membranes (101).

The intensity of reaction over the plasma membrane of hepatocytes reached its peak at 4 h after [^{3}H]*N*-acetylmannosamine injection (Fig. 3), maintained this level of intensity until 24 h after injection (Fig. 4), and then decreased gradually such that by 9 d after injection only a very light reaction remained. These results are in accord with findings by Harms and Reutter (43) that when [^{14}C]*N*-acetylmannosamine was injected into 150–200-g rats, label in liver plasma membrane-bound sialic acid had a half-life of ~24 h.

At the 4-h time interval, when the proportion of total plasma membrane reaction associated with each region is considered, the sinusoidal membrane accounted for 72.5% of the grains, the lateral membrane for 15%, and the bile canalicular membrane for 12.5%. This may be compared with the relative surface area of these regions as calculated in hepatocytes from 8- to 9-wk-old rats by Weibel et al. (108); thus the sinusoidal membrane was found to account for 37% of the total surface area, the lateral membrane for 50%, and the bile canalicular membrane for 13%. If the same relative areas occur in our young rats, then it would appear that the sinusoidal membrane, and to a lesser extent the bile canalicular membrane, have more label per unit area than the lateral membrane. It should be noted, however, that the method of measurement employed in the above study did not account for surface enlargement by formation of microvilli in the case of bile canalicular and sinusoidal membranes.

The precise mechanism by which membrane glycoproteins travel from the Golgi apparatus to the plasma membrane remains unsettled. In the case of glycoproteins migrating to the sinusoidal plasma membrane, it is likely that they travel in the membranous wall of secretory vesicles. When, in the process of exocytosis, the walls of the vesicles fuse with the plasma membrane to release their secretory contents, the labeled glycoproteins would reach the sinusoidal plasma membrane either by conversion of secretory vesicle membrane to plasma membrane, or by lateral diffusion of the membrane glycoproteins out of the vesicle membrane into the plasma membrane during the period of their continuity (7, 12). Label in the lateral plasma membrane may represent labeled membrane glycoproteins that have migrated, by means of lateral diffusion, from the sinusoidal plasma membrane. Label in the plasma membrane of the bile canaliculus is less likely to be accounted for by this mechanism, however, because a tight junction always exists between the lateral plasma membrane and the plasma membrane of the bile canaliculus, and the work of Pisam and Ripoche (79) has suggested that membrane glycoproteins will not diffuse through a tight junctional area. The various constituents of bile pass from the hepatocyte into the bile canaliculus, but the mechanism by which this process is accomplished is not known. A recent study by Jones et al. (57) has provided some evidence that the Golgi apparatus may be involved. In addition, the bile contains small amounts of glycoprotein molecules (i.e. alkaline phosphatase, phosphodiesterase and 5′-nucleotidase), and it has been suggested that these arise by shedding off of the bile canalicular membrane under the

influence of bile salts (13). The means by which membrane glycoproteins would reach the bile canalicular plasma membrane remains unclarified, however.

MIGRATION TO LYSOSOMES: At later time intervals, after [^{3}H]*N*-acetylmannosamine injection, a considerable amount of reaction appeared over lysosomes (i.e., over 17% of the total grain count at 24 h, Table I). These results were similar to those observed after [^{3}H]fucose injection, in which hepatocyte lysosomes were found to incorporate considerable label (6). The label in lysosomes in the present study could represent labeled hydrolytic enzymes, many of which have been shown to be glycoproteins (37) or could represent constituents of the lysosomal limiting membranes which have been shown to contain membrane glycoproteins (44, 91) and glycolipids (45). Work of Tsuji et al. (104) has implicated the rat liver Golgi apparatus in the formation of lysosomal β-glucuronidase, although other evidence from the same group suggests that no significant amount of sialic acid is added to the enzyme at this site (46).

Novikoff and colleagues have claimed that, in many cell types, lysosomes derive from GERL (71). As mentioned above, it is possible that some label observed in the Golgi region at early time intervals may have been associated with GERL.

It should be noted that some lysosomes in the present study were labeled at early time intervals after [^{3}H]*N*-acetylmannosamine injection (Table I), but the most intense reaction was observed at 4 and 24 h after injection (Figs. 10 and 11). Thus, whereas some or all of the label observed in lysosomes may have migrated to this site from the Golgi apparatus, it is equally possible that some of the label, especially that observed at later time intervals, may have been derived from the plasma membrane or even from material arising outside of the cells. Hubbard and co-workers have shown, for example, that ^{125}I-asialoglycoproteins in the blood, having been bound to hepatocyte plasma membrane receptors, are internalized and migrate to lysosomes where they are degraded (51, 52). Although such molecules would have to have some exposed galactose residues in order to be bound to the hepatocyte receptor, they could retain sialic acid residues at other locations in their carbohydrate moieties and thus could account for lysosomal labeling in the present study. In addition, other mechanisms may exist whereby [^{3}H]sialic acid-labeled glycoproteins from the blood could gain access to the hepatic lysosomal compartment.

Appearance of Label in Sinusoidal Lining Cells

At the 4- and 24-h time intervals after [^{3}H]*N*-acetylmannosamine injection, substantial reaction appeared over cells lining the hepatic sinusoids (i.e. endothelial cells and Kupffer cells) where it was mostly associated with lysosomes (Figs. 10, 12, and 13). These cells did not incorporate label at early time intervals and therefore presumably did not themselves synthesize the labeled glycoproteins responsible for the reaction observed at later time intervals. It is likely that this reaction was the result of labeled glycoproteins that had been taken up from the blood, and were in the process of being degraded in the lysosomes of the lining cells (90, 107). The studies of Hubbard et al. (51, 52), as well as those of others (21, 96), have shown that glycoproteins in the blood with side chains terminating in mannose residues are bound and internalized by sinusoidal cells of the liver, and subsequently degraded in lysosomes. In the study of Harms and Reutter (43), the authors found that the half-life of total protein-bound sialic acid label (34 h) was considerably longer than that of the plasma membrane-associated label (24 h), and they concluded that sialoproteins with considerably longer half lives of sialic acid must exist in subcellular structures other than plasma membranes. Our present radioautographic results indicate that these structures are probably the lysosomes of the hepatocytes themselves and of sinusoidal lining cells.

The authors gratefully acknowledge the assistance of Dr. Charles Smith for his advice on the histochemical procedures used, and the assistance of Dr. Michael Lalli in carrying out those procedures. In addition, we would like to thank Dr. John Bergeron for his advice on the biochemical procedures, and Dr. Beatrix Kopriwa for her assistance in the radioautographic techniques.

This research was carried out with the assistance of a grant from the Medical Research Council of Canada.

Received for publication 2 January 1980, and in revised form 14 July 1980.

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