SITES OF GLYCOGEN SYNTHESIS IN RAT LIVER CELLS AS SHOWN BY ELECTRON MICROSCOPE RADIOAUTOGRAPHY AFTER ADMINISTRATION OF GLUCOSE-H^3

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ABSTRACT

Glycogen synthesis was investigated by giving tritium (H^3)-labeled glucose with carrier to fasted rats in vivo or incubating liver slices from fasted rats in vitro using a glucose-H^3-containing medium. After 15 min or 1 hr, pieces of liver were fixed and radioautographed for light and electron microscopy. In vivo and in vitro, radioautographic reactions appeared over "glycogen areas" and over zones transitional between these areas and ergastoplasm. Treatment of sections by alpha amylase removed all but about 5% of the radioactivity, so that about 95% of it consisted of glycogen (synthesized during the 15 min or 1 hr elapsing after administration of glucose-H^3). Within glycogen areas and transitional zones, most silver grains were over or very close to glycogen granules and smooth (or partly smooth) vesicles. Presumably, much of the label was added onto growing glycogen granules, in accord with the biochemical view that glycogen may serve as substrate for further glycogen synthesis. The few silver grains located far from glycogen granules—15% at the 15 min interval in vivo—approximated smooth (or partly smooth) vesicles of endoplasmic reticulum. This observation raised the possibility that smooth membranes play a role in glucose uptake at an early stage in de novo formation of glycogen granules.

INTRODUCTION

The glycogen present in the liver cells of the rat appears in the electron microscope as 150 to 400 A granules (32). Each granule is in fact a molecule of glycogen (26) composed of a large but variable number of D-glucosyl residues (28, 39). Ever since Claude Bernard's time, it has been known that liver glycogen is in a dynamic state. Under physiological conditions, the D-glucosyl residues present at the periphery of glycogen molecules are renewed rapidly and those in the central core are renewed rather slowly (39). When an animal is fasted, drastic breakdown takes place; thus, the liver glycogen of rats fasted 24 hr was found to decrease from 55 mg to about 1 mg per gram wet weight (38). Conversely, within 20 min after feeding 200 mg of glucose to fasted animals, the liver glycogen rose from 1.1 to 3.6 mg per gram wet weight (38). In agreement with these results the electron microscope revealed that most glycogen granules disappear from the liver during fasting, but new granules are rapidly added after refeeding (5, 25, 31).

The deposition of glycogen in the depleted liver may also be observed in vitro; thus, when

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slices of liver from fasted rats were incubated in a medium rich in glucose and potassium (11), 2 mg of glycogen per gram of liver were synthesized in 15 min (15). When, under similar conditions, Kugler and Wilkinson added C^{14}-labeled glucose to the medium and radioautographed the liver slices, they noted the presence of radioactivity mainly at the periphery of the lobules; and they assumed it to be in new glycogen (18).

Both in vitro and in vivo conditions were used in the present investigation to study glycogen synthesis by electron microscope radioautography. Since glycogen is the only substance which can be extracted from animal tissues by alpha amylase (10, 34), glycogen was identified in the light microscope as periodic acid (PA)–Schiff–stained material removable by alpha amylase treatment (40); in the electron microscope, it was recognized as the 150 to 400 A glycogen granules, which also proved extractable by the enzyme; and in radioautographs prepared after administration of tritium (H^3)-labeled glucose, glycogen was detected as amylase-labile radioactivity. The labeled substance was added to the glucose of the medium for in vitro experiments and to glucose injected or given by stomach tube for in vivo experiments. It was hoped that observations made on the sites of labile radioactivity in liver cells soon after glucose-H^3 administration would provide information on the site and mode of glycogen synthesis.

MATERIALS AND METHODS

Histological Technique

Good retention of glycogen was observed in the light microscope when tissues were fixed by immersion in Rossman fluid first at $-20°C$ for 6 hr and then at room temperature for 18 hr (9, 22, 30, 41), paraffin embedded and sectioned at 5 μ. Some of the sections were digested for 4 hr at 37°C in saliva (previously centrifuged for 30 min) or in a solution of twice crystallized alpha amylase (Worthington Biochemical Corporation, Freehold, New Jersey; protein concentration, 32 mg/ml; activity 712 μ/mg) diluted with 0.01 M phosphate buffer at pH 7.0 to obtain a 0.1% concentration of enzyme. Both treated and control sections were stained with hematoxylin and PA-Schiff.

Preservation of glycogen was also obtained when tissues were fixed for electron microscopy, either in osmium tetroxide (24) at pH 7.3 at 0–4°C for 2 hr or in 2.5% glutaraldehyde in 0.1 M phosphate buffer (36) at the same pH and temperature, followed by treatment with sucrose buffer overnight and postfixation in Millonig's osmium tetroxide fixative.

Embedding was in Epon 812 (occasionally in methacrylate). Some sections were cut at 1 to 3 μ and stained with hematoxylin and PA-Schiff (20 min in periodic acid and 2 hr in Schiff reagent, reference 14). Amylase digestion was ineffective on Epon sections. For routine electron microscopy, silver-to-gold sections were stained with lead hydroxide (16).

For study in the electron microscope after amylase digestion, pieces of liver (1 to 2 mm) which had been fixed in 2.5% glutaraldehyde, placed in sucrose buffer overnight, and washed in 0.01 M phosphate buffer were immersed in 1% alpha amylase for 1 or 2 hr at 37°C. After further washing in buffer, the pieces were postfixed in osmium tetroxide and embedded in Epon. Controls were processed similarly but kept in cold sucrose buffer instead of being subjected to enzyme digestion.

Experimental Design

EXPERIMENTS IN VITRO: The middle lobe of the liver from 300-g male Sprague-Dawley rats was investigated first in several animals referred to as fed controls. Similar rats were fasted for 24 hr and pieces from the middle liver lobe were fixed (fasted controls). Slices of the same liver lobe were cut by hand (3) and incubated at 37°C for 15 min or 1 hr in a shaken flask containing 1 ml of the medium of Hastings et al. (11) (K$^+$, 110 mM; Ca^{++}, 10 mM; Mg^{++}, 20 mM; HCO$_3^-$, 40 mM; Cl$^-$, 130 mM; gassed with a 95% O$_2$ to 5% CO$_2$ mixture giving a pH of 7.35 before incubation). In a first experiment, the liver slices from one rat were incubated with 100 μc of D-glucose-6-H^3 (specific activity, 225 mc/mmole, Radiochemical Centre, Amersham, England) with or without addition of 1% unlabeled glucose (Table I). In other experiments, the liver slices from three other animals were incubated with a higher dose, 1000 μc of labeled glucose (1300 mc/mmole), with 1% unlabeled glucose present in the medium.

EXPERIMENTS IN VIVO: Pieces of the middle lobe of the liver from a number of male Sprague-Dawley rats weighing approximately 100 g were investigated. Except for the "fed controls," all animals were fasted for 18 hr.

In a first series, 600 μc of D-glucose-6-H^3 (225 mc/mmole) without addition of unlabeled carrier glucose was dissolved in 0.6 ml of water and injected intraperitoneally into six rats. The animals were sacrificed in pairs after 15 min, 1 hr, and 4 hr (Table II).

Meanwhile, a pilot experiment revealed that, when glucose-H^3 was given by the intragastric route with 120 mg of carrier glucose, the liver uptake was about 30% higher than it was by the intraperitoneal route without carrier. Accordingly, in a second series, six rats were divided into three groups of two which received by stomach tube 600 μc of

the same D-glucose-6-H³ as in the first series, but now dissolved in 1.2 ml of 50% (two rats), 10% (two rats) or 2% (two rats) unlabeled D-glucose, so that the animals of each pair received 600, 120, and 24 mg of unlabeled D-glucose, respectively. One rat from each pair was sacrificed after 15 min, the other after 1 hr (Table III).

In a third series, two rats were each given by stomach tube 5000 μc of D-glucose-6-H³ (1300 mc/mmole) dissolved in 1.2 ml of a 10% solution of unlabeled D-glucose. Again, one rat was killed after 15 min, the other after 1 hr (Table IV). The livers of one fed and one fasted control were examined in each series along with those of the animals receiving the labeled glucose.

Radioautography

From each liver slice incubated in vitro and from the middle lobe of the liver of each animal given glucose-H³ in vivo, samples were processed for radioautography.

For light microscopy, paraffin and Epon sections were coated with Kodak NTB2 nuclear track emulsion after staining with hematoxylin-PA-Schiff or hematoxylin-eosin, exposed and processed (17). The number of silver grains in liver radioautographs was counted in 10 or 20 squares measuring 3249 μ^2 each. For Tables I, II, and IV (but not for Table III) the background was counted in 10 squares outside the section and subtracted from the liver grain count.

For electron microscopy, samples from the second in vitro series and the third in vivo series were examined. Silver-to-gold Epon sections were placed on 300-mesh copper grids previously covered with 0.4 to 0.5% Formvar film. The grids were attached to glass slides by the Formvar film and the slides dipped in Gevaert NUC-307 emulsion (42). After exposure, development was done in Kodak D-170, fixing in 24% sodium thiosulfate, and staining with lead (33).

The silver grain developed from the Gevaert 307 emulsion appears as a curved filament with a swollen portion often present at an extremity or along its length (Figs. 11 and 12). Each silver grain arises from a silver crystal hit by a beta ray and subsequently developed. The crystal is usually located above the source of the beta ray(s) which hit it, but may also be hit sideways. Furthermore, the silver grain developed from the crystal may spread outside to some extent; and, in fact, the mean diameter of the grain, 1300 A, is larger than that of the crystal, 670 A. It is difficult to relate a grain to the source of the radioactivity which hit the original crystal. One approach is to estimate the size of the area around the silver grain where the source is likely to be. Assuming that the effective path of 90% of the

beta particles of tritium does not exceed 0.1 μ (27), it follows that the maximum distance from the radioactive source to the latent image for 90% of the beta particles should be 0.1 μ, plus a distance equal to the crystal diameter (0.067 μ), that is, in all, 0.167 μ (27). Hence when a photograph is looked at, the source of radioactivity producing a silver grain should be within a circle with a diameter of 0.167 μ, drawn around the grain. Conversely, the silver grain should be within a circle with a 0.167 μ diameter drawn around the suspected source. To be on the safe side, it was decided to examine whether silver grains were within or without a circle with a diameter of 0.2 μ, from the suspected sources of radioactivity.
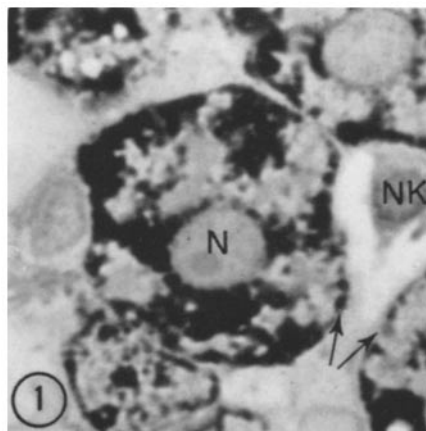
Key to Symbols

N, nucleus	Mb, microbody
NK, nucleus of Kupffer cell	L, lysosomes
M, mitochondria	$gn\ a$, glycogen area
G, Golgi complex	tr, transitional zone
rER, rough endoplasmic	BC, bile canalicule
reticulum	SD, space of Disse
sER, smooth endoplasmic	CM, cell membranes
reticulum	PV, portal vein
sv, smooth vesicle	CV, central vein
mv, mixed vesicle	r, ribosomes

FIGURE 1 Liver of fed adult rat. PA-Schiff–stained section fixed in Millonig's OsO₄ fixative and embedded in methacrylate.

Kupffer cells are unstained (NK). Parenchymal cells have an unstained nucleus (N) and patchily stained cytoplasm. In the centrally located cell, there are several heavily stained patches, the glycogen areas (the tiny light spots may be mitochondria). There are also unstained regions, which mainly contain ergastoplasm. It may be noted that the stained material often follows the edge of the cells (arrows). × 1400.
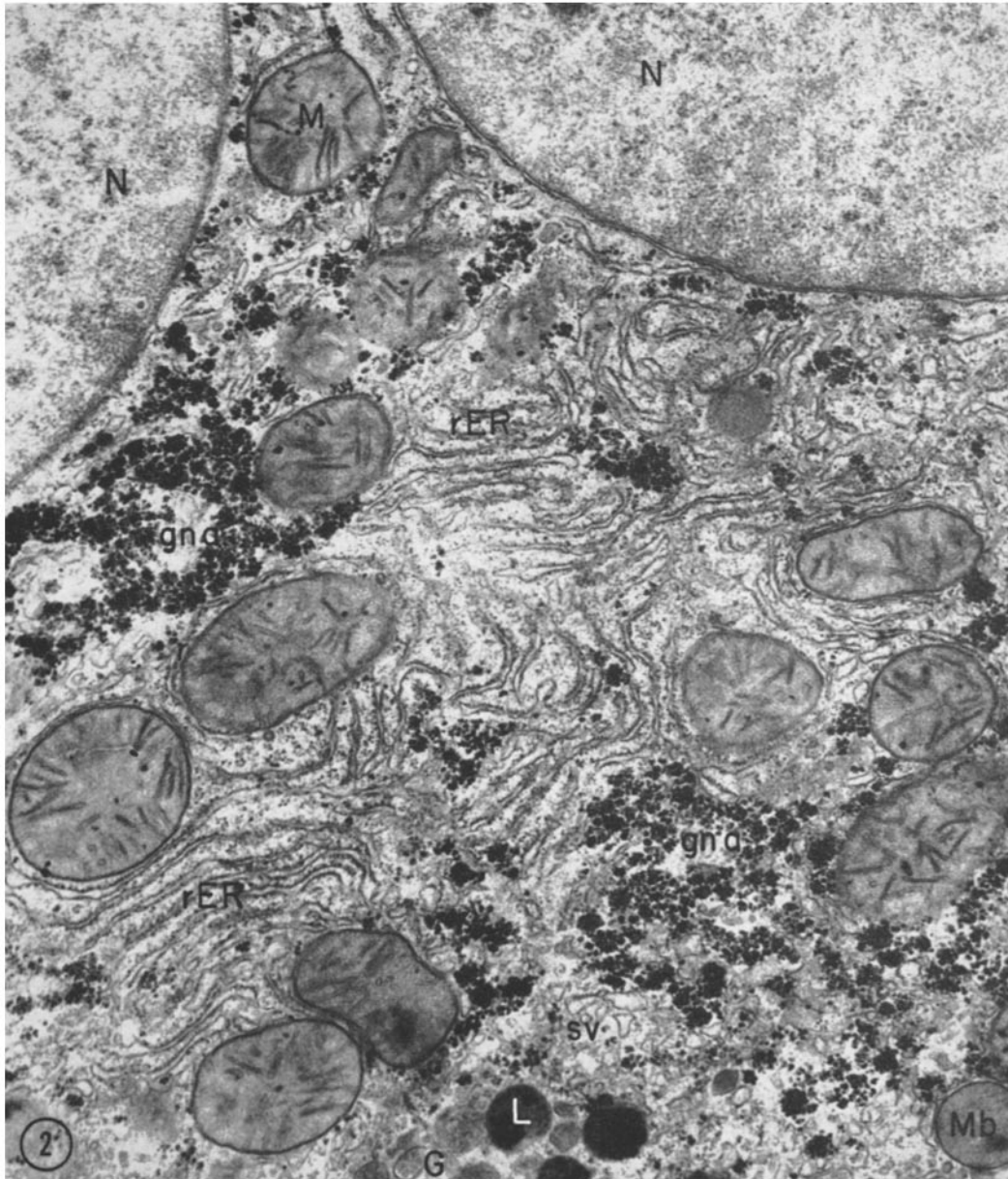
FIGURE 2 Liver of fed 300 g control. This and subsequent electron micrographs are from material fixed in Millonig's OsO₄ (except Figs. 3 and 4) and embedded in Epon.

The cytoplasm of this binucleate liver cell mainly shows mitochondria (M), ergastoplasm (composed of stacks of cisternae of rough endoplasmic reticulum, rER), and two intensely stained glycogen areas (gn a) with groups of vesicles of smooth endoplasmic reticulum (sv). Near the lower edge, a few lysosomes (L) are near a Golgi complex, only the edge of which appears in the micrograph (G). In addition to distinct glycogen areas, liver cells contain small glycogen deposits which are seen here scattered between groups of cisternae of rough endoplasmic reticulum. × 21,000.
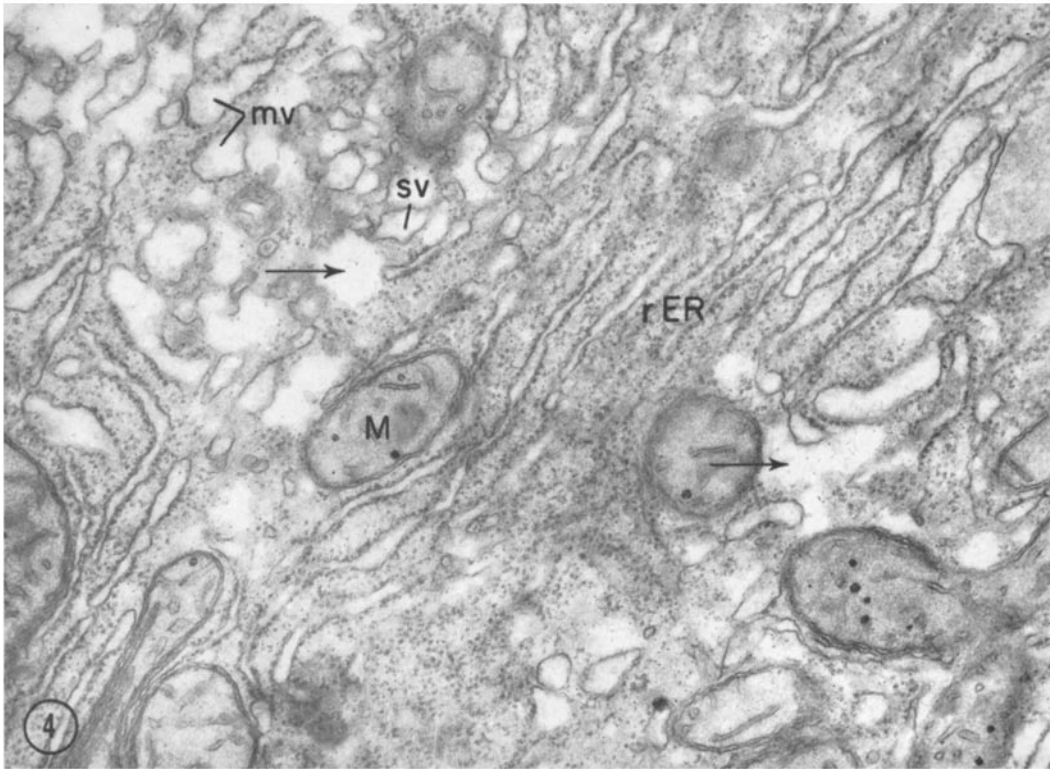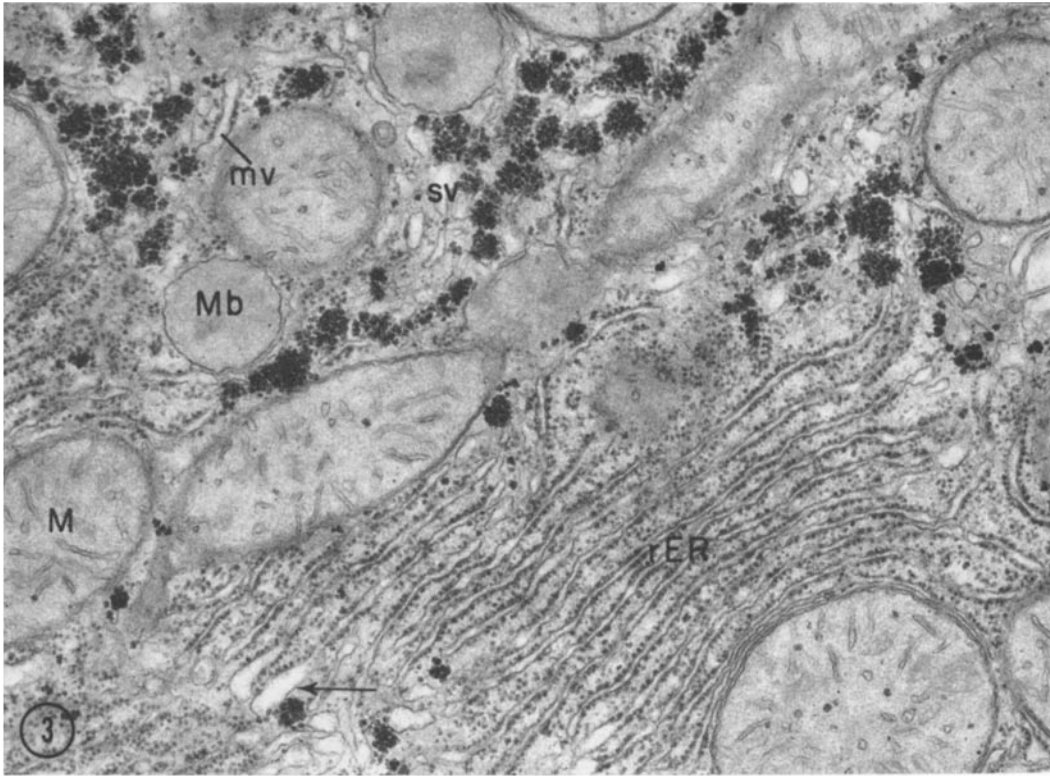
FIGURE 3   Liver of fed 200 g rat (glutaraldehyde fixation and postfixation in osmium tetroxide). The glycogen area at top center shows the rosettes (and component granules), and smooth vesicles (*sv*). Below is seen a stack of cisternae of rough endoplasmic reticulum (*rER*) in which ribosomes are located on the outer surface of the cisternae. The ribosomes stain less intensely than glycogen granules. Small deposits of glycogen may be associated with smooth vesicles, as shown in the upper right corner, or with distended portions of cisternae that are partially free of ribosomes, as shown at lower center (arrow). × 40,000.

FIGURE 4   Same as Fig. 3, but from piece treated with *alpha amylase* for 1 hr. Mitochondria (*M*) appear normal. Light spaces next to smooth vesicles (*sv*) indicate the sites from which glycogen has been extracted (horizontal arrows). × 40,000.

Another approach is to plot the distances between each silver grain and the possible source, to find out whether the distribution corresponds to the pattern expected for these distances on the basis of the work of Caro (2) and Granboulan (8). Two possible sources were considered for each grain, the nearest glycogen granule and the nearest smooth (or partly smooth) vesicle. The distance to both was measured from the most swollen portion found in each grain. (Unpublished experiments of Kopriwa suggested that this swollen portion is the first to appear during photographic development and may be the starting point of the growing filament.)

RESULTS

Experiments in Vitro and Controls

LIVER OF FED ADULT CONTROLS: In the light microscope (Fig. 1), the cytoplasm of liver cells showed patches of PA-Schiff-stained material alternating with basophilic areas. In the electron microscope, the cytoplasm showed, besides the usual organelles, an alternation of: (a) glycogen areas (5, 25, 32) packed with glycogen granules often grouped into distinct rosettes (Figs. 2 and 3) and containing scattered vesicles of smooth endoplasmic reticulum enclosed in ribosome-free membranes (Figs. 2 and 3, sv); and (b) ergastoplasmic areas composed of stacks of cisternae whose walls are covered with ribosomes (rough endoplasmic reticulum, rER, Figs. 2 and 3). Where cisternae abut on a glycogen deposit, they appear somewhat dilated and have few or no attached ribosomes (Fig. 3, arrow). Cisternal ends and vesicles whose walls are partly covered with ribosomes and partly free of them (referred to as "mixed vesicles": mv, Fig. 4) are characteristic of the transition between glycogen areas and ergastoplasm. Even the small groups of glycogen granules scattered outside glycogen areas (Fig. 2) are associated with smooth or mixed vesicles.

Treatment with alpha amylase eliminated glycogen granules and rosettes, leaving clear spaces (horizontal arrows, Fig. 4) which were lighter than the hyaloplasm present around smooth vesicles (sv, Fig. 4).

LIVER OF FASTED ADULT CONTROLS: The liver of adult rats fasted for 24 hr showed little PA-Schiff-stained material in the light microscope, a few discrete clumps at best, and infrequent glycogen granules in the electron microscope. Nevertheless, glycogen areas could be identified. The ergastoplasm was less well defined than in fed

animals, as the cisternae often appeared slightly separated from one another and distorted. The presence of more mixed vesicles at the periphery of glycogen areas made "transitional zones" appear larger than in fed controls.

EFFECT OF INCUBATION ON LIVER CELLS: The cells of incubated liver slices appeared histologically normal in the light microscope, except for the cells at the surface, which had shrunken or pyknotic nucleoli and a vacuolated cytoplasm (zone 1 in Fig. 5). Of the deep, normal-looking cells, only the more superficial ones (zone 2 in Fig. 5) contained PA-Schiff-stained material. The amount of this material increased between 15 min and 1 hr of incubation.

In the electron microscope, the cells of zone 2 showed normal mitochondria (Figs. 6, 7, 11, and 12), but the partition of the cytoplasm between glycogen areas and ergastoplasmic regions was no longer distinct (Fig. 6): only few glycogen areas and ergastoplasmic stacks were well defined (Figs. 6 and 7); and instead, transitional zones were so much enlarged that they occupied most of the cytoplasm (Fig. 7). Glycogen granules and rosettes were more numerous than in fed controls and appeared not only in glycogen areas but also in transitional zones (Fig. 7). All these changes were visible at 15 min and further accentuated at 1 hr of incubation.

RADIOAUTOGRAPHY OF LIVER SLICES INCUBATED IN VITRO: When at the early stages of this work liver slices were incubated with glucose-H^3 without unlabeled carrier glucose, radioautographs showed very little radioactivity in parenchymal liver cells (although amylase-resistant radioactivity was prominent in blood vessel walls, bile ducts, and Kupffer cells). However, with carrier glucose added to the glucose-H^3 of the medium, numerous silver grains appeared over those liver cells which exhibited PA-Schiff-stained material (zone 2, Fig. 5). The grains directly overlay this material and, like it, were diffusely distributed throughout the cytoplasm with predominance at the periphery (Fig. 8). Treatment of the sections with saliva removed much, and with alpha amylase nearly all, radioactivity along with the PA-Schiff-staining material (Table I; Figs. 8 to 10).

The number of silver grains seen in electron microscope radioautographs exposed for 1 to 3 months was less after 15 min' (Fig. 11) than after 1 hr' incubation (Fig. 12), but the location of the

FIGURE 5 Radioautograph of a cross-section of liver slice (from an animal fasted for 24 hr). The slice was incubated for 15 min in a high potassium medium containing 1000 μc of glucose-H^3 and 1% unlabeled glucose. Fixation in Rossman fluid. Paraffin embedding. PA-Schiff-hematoxylin.

Distinct cell layers may be distinguished in the slice:

zone 1 a peripheral layer made up of vacuolated cells with shrunken nuclei; they contain no PA-Schiff-stained material and do not take up radioactivity;

zone 2 the subjacent layer, composed of histologically normal cells which contain PA-Schiff-stained material and take up radioactivity; their dark appearance is due to the silver grains which overlie the cytoplasm;
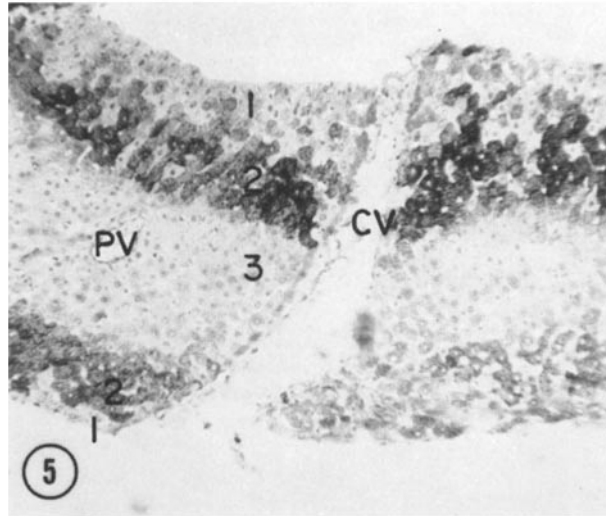
zone 3 the core, made up of histologically normal cells, most of which are not stained with PA-Schiff and do not take up radioactivity.

Thus, the production of PA-Schiff-stained material in the slices is conditioned by the distance between cells and surface rather than by the relation of the cells to portal vein (PV) or central vein (CV). $\times$ 116.

grains was about the same at both times. Grains were not seen over the few stacks of rough cisternae encountered, and only rare grains over mitochondria and nuclei. Nearly all grains were over glycogen areas and transitional zones. The distribution of these regions throughout most of the cytoplasm explained the diffuseness of the reactions observed in the light microscope (Fig. 8). In glycogen areas (Fig. 11) most silver grains were found over or next to glycogen granules and rosettes as well as over or next to smooth vesicles. Similarly, in transitional zones (Fig. 12), most silver grains were close to glycogen and to smooth or mixed vesicles.

The distance between the swelling of the silver grain and the nearest glycogen granule or the nearest vesicle was measured for 83 grains at the 15 min interval and for 155 grains at the 1 hr interval. The results (Fig. 13) confirmed that most grains were located next to both glycogen granules and vesicles. Only rare grains were located at or more than 0.2 μ beyond either of those structures.

Experiments in Vivo and Controls

CONTROLS: The liver cells of fed 100-g rats were similar to those of adults. However, glycogen areas were usually smaller and glycogen deposits more scattered than in the adult controls examined in relation to the in vitro experiment. The loss of glycogen granules from the liver cells of young rats fasted for 18 hr (Fig. 14) was more complete than in adults fasted for 24 hr.

EFFECT OF GLUCOSE: When glucose was administered to fasted 100-g rats, PA-Schiff-staining material appeared in the cells (Fig. 15). The light microscope showed small isolated clumps of this material at 15 min and denser, larger clumps at 1 hr after administration (Fig. 16). The electron microscope showed glycogen areas with small scattered rosettes (Fig. 17). These were usually in contact with smooth vesicles, which may appear loosely (Fig. 21) or tightly interconnected (Fig. 23). At the periphery of glycogen areas, the smooth vesicles were often anastomosed with rough cisternae, thus making up transitional zones. A Golgi complex was frequently seen nearby (Figs. 18 to 20) and was occasionally connected with smooth vesicles that seemed to be part of a glycogen area.

At the 1 hr interval there was a greater number of rosettes within glycogen areas, which looked better defined and larger than at the 15 min interval. The transitional border of mixed vesicles at
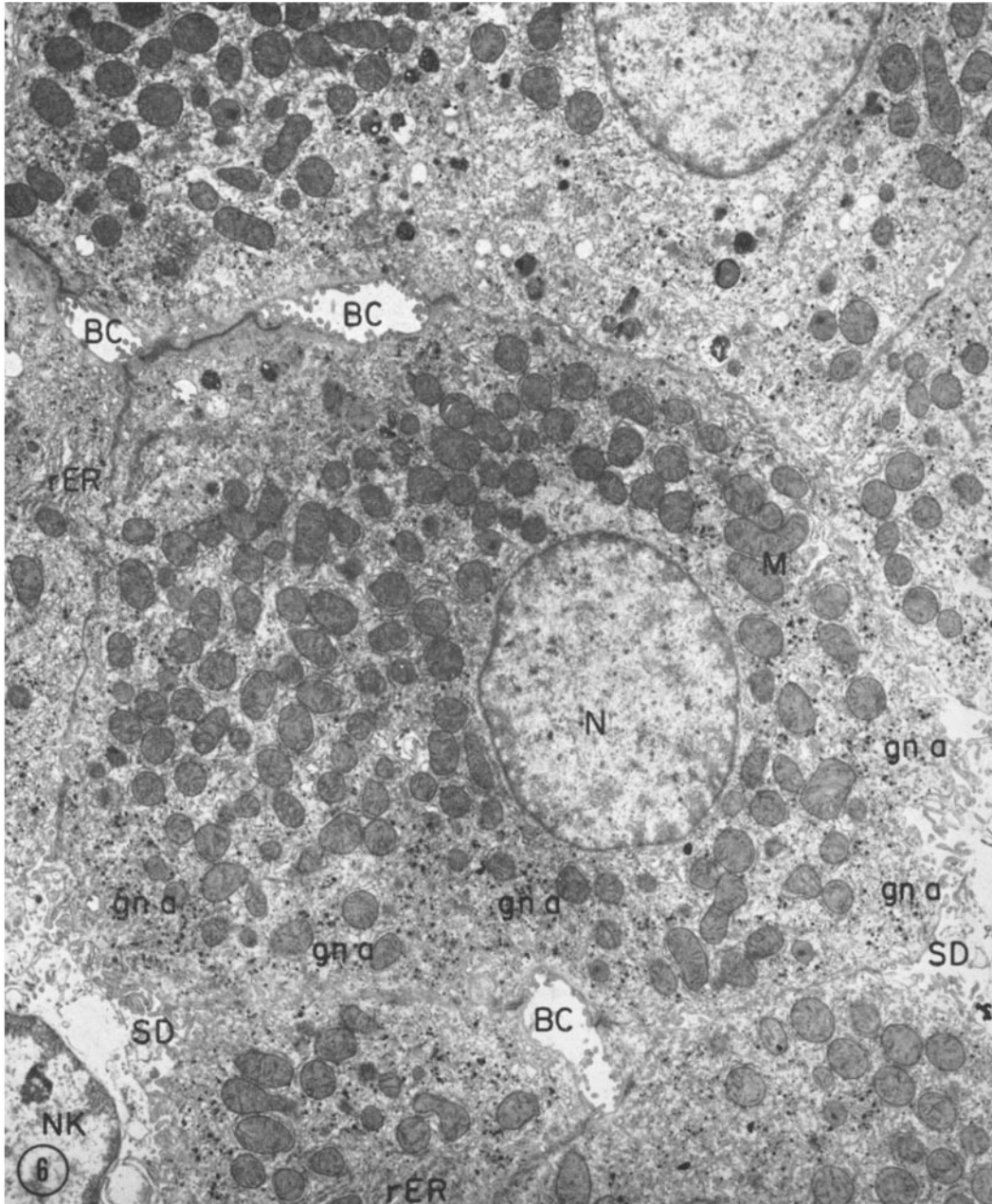
Figure 6   *Incubated* liver slice from fasted adult (15 min incubation). In the center a liver cell with nucleus (*N*) and numerous mitochondria (*M*) is associated with 3 bile canalicules (*BC*) and 2 sinusoids with space of Disse (*SD*). The lower left corner contains part of a nucleus of Kupffer cell (*NK*). Ergastoplasm is scarce; one stack is seen below the bile canalicule at left (*rER*). There are poorly defined glycogen areas, mainly at the periphery of the cell (*gn a*); the rest of the cytoplasm seems to be composed of regions transitional between glycogen areas and rough endoplasmic reticulum. Glycogen granules appear in these transitional regions as well as in glycogen areas proper. × 8000.
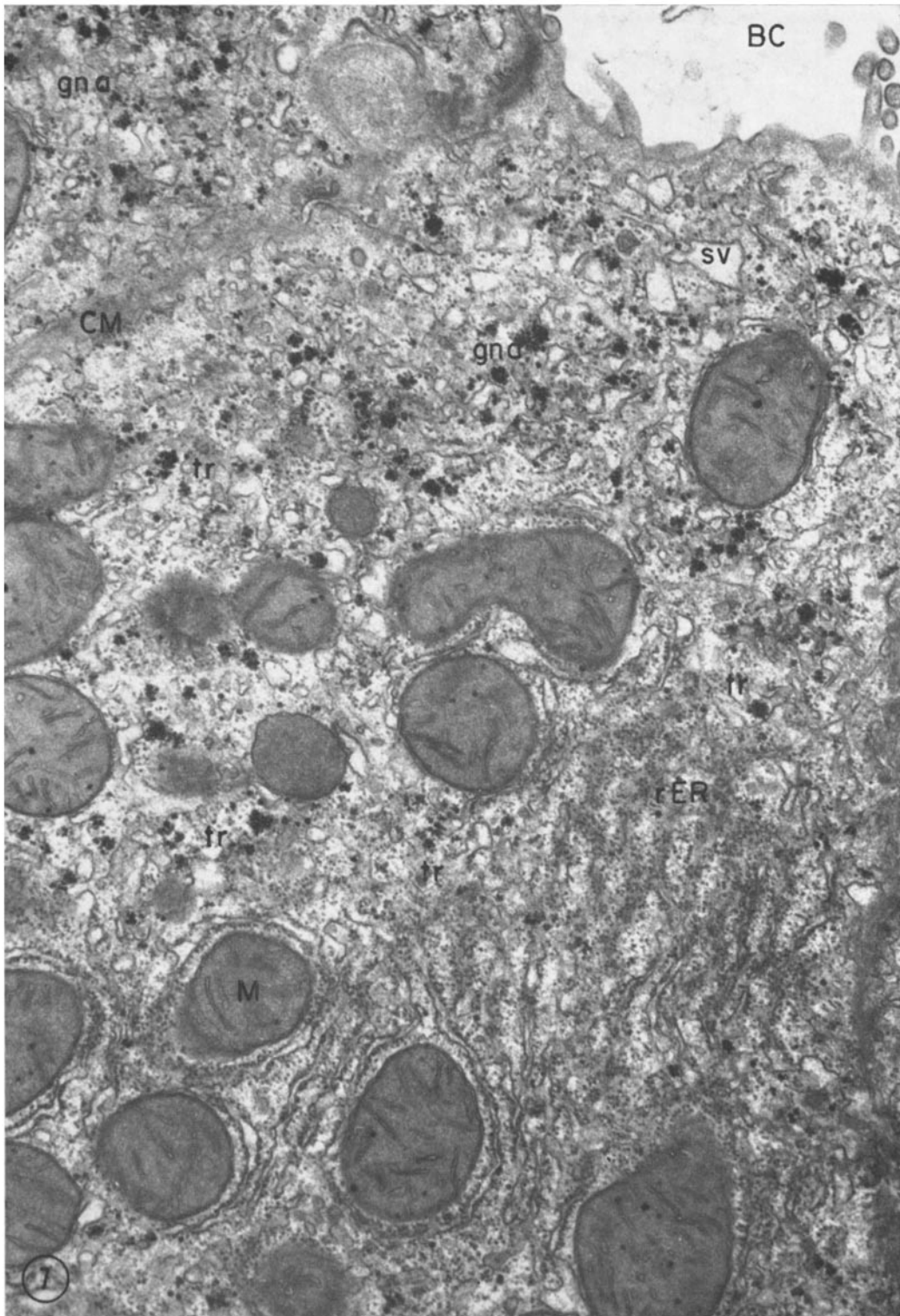
FIGURE 7 *Incubated* liver slice from fasted adult (15 min incubation). Two cells are shown, which are separated by cell membranes (*CM*) and show glycogen areas in the upper part of the figure. Even though the incubation has lasted only 15 min, more glycogen is present than in most areas from fasted controls (Fig. 14). The glycogen rosettes and granules are located between smooth vesicles. Ergastoplasm is rare but one stack is seen at lower right (*rRE*). Most of the rest of the field consists of transitional regions(*tr*) containing smooth and mixed vesicles as well as numerous free ribosomes; glycogen granules appear in these regions. × 35,000.
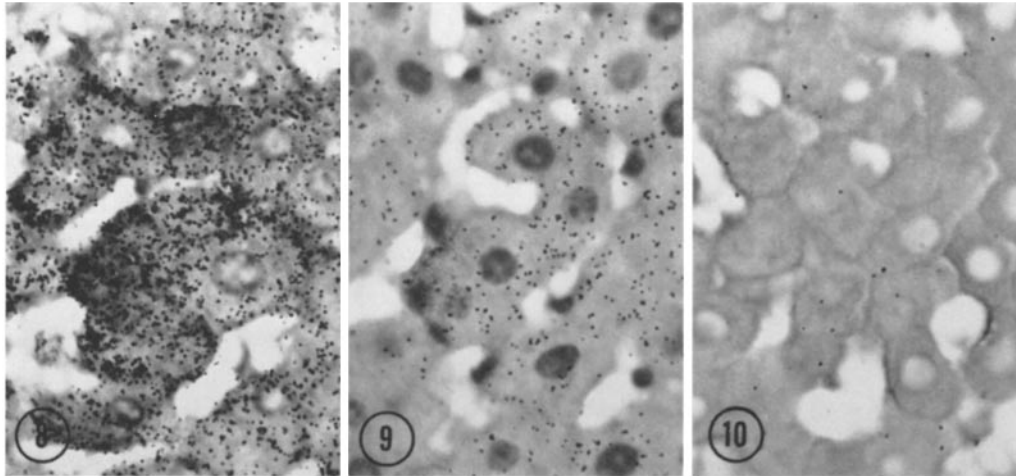
FIGURES 8 to 10 Three radioautographs of sections from the same liver slice, one used as control (Fig. 8), one treated with saliva (Fig. 9), and one with alpha amylase (Fig. 10). Paraffin sections. PA-Schiff–hematoxylin.

In Fig. 8, the cells show the dense PA-Schiff–stained material overlaid by numerous silver grains. Saliva removes most (Fig. 9) and amylase nearly all PA-Schiff–stained material and silver grains (Fig. 10). (Treatment with alpha amylase decreased nuclear staining.) × 680.

TABLE I

Influence of Enzymatic Digestion on Grain Counts in Radioautographs of Liver Incubated in Glucose-H³–containing Medium

Experiment in vitro; first series; 1% unlabeled glucose added

	Incubation	
	15 min	1 hr
Grain count per 1000 μ²	100	264
Per cent remaining after		
Saliva	38	18
α-Amylase	4	2

Each figure is the mean of 10 counts in one specimen.

their periphery was also more extensive and showed more glycogen rosettes than at 15 min.

RADIOAUTOGRAPHIC OBSERVATIONS: After injection of labeled glucose, radioactivity appeared in liver and most other tissues, but except for that in liver, muscle, and a few other locations, the radioactivity was usually not affected by alpha amylase treatment. Thus, when sections of tongue epithelium and lymph node were treated by the enzyme, only traces of radioactivity were removed.

TABLE II

Influence of Enzymatic Digestion on Grain Counts in Radioautographs of Liver from Rats Injected Intraperitoneally with Glucose-H³ without Added Carrier

Experiment in vivo; first series

	After glucose-H³		
	15 min	1 hr	4 hr
Grain count per 1000 μ²	41	61	103
Per cent remaining after			
Saliva	28	34	19
α-Amylase	21	27	15

Each figure is the mean of 20 counts in one animal.

When labeled glucose was given without carrier, the liver radioactivity was about three quarters removed by alpha amylase (Table II). When 24 to 600 mg of carrier glucose was added, over 90% of the radioactivity was removed by the enzyme (Table III). This conclusion was confirmed in the experiment in which 120 mg of glucose was added to a large dose of radioactivity for electron microscope radioautography, since about 95% of the liver radioactivity was extracted by the enzyme (Table IV).

TABLE III

Influence of the Carrier Dose on the Uptake of Glucose-H^3 as Liver Glycogen, as Measured in Radioautographs after Intragastric Administration of 600 μc of Glucose-H^3

Experiment in vivo; second series

		After glucose-H^3				
		15 min			1 hr	
Carrier dose (mg)	24	120	600	24	120	600
Grain count per 1000 μ^2	43	42	19	71	74	72
Per cent remaining after						
Saliva	24	14	16	9	18	7
α-Amylase	9	7	10	8	7	4

Each figure is the mean of 10 counts in one animal. The background was not subtracted.

TABLE IV

Influence of the Time after Administrations on the Uptake of Glucose-H^3 as Liver Glycogen, as Measured in Radioautographs after Intragastric Administration of 5000 μc of Glucose-H^3 in 120 mg of Carrier

Experiment in vivo; third series

		After glucose-H^3		
		15 min		1 hr
Type of sections	paraffin*	Epon‡	paraffin*	Epon‡
Grain count per 1000 μ^2	52	82	80	166
Per cent remaining after				
Saliva	11	8	12	6
α-Amylase	5	3	6	6

* Each figure is the mean of 20 counts in one animal.
‡ Each figure is the mean of 10 counts in one animal (the same animal as used for paraffin sections).

When the location of radioactivity was examined in radioautographs, the concentration was found to be greater at the periphery than in the central region of the liver lobule (Fig. 15), in agreement with a previous report (18). At a higher magnification, the radioactivity was traced to the PA-Schiff–staining clumps present in liver cells (Fig. 16).

Electron microscope radioautographs at 15 min and 1 hr after glucose-H^3 injection showed most silver grains over glycogen areas (Fig. 17) and a few over the mixed vesicles making transition with the surrounding rough cisternae (Fig. 18). In a transitional zone shown in Fig. 19 at high magnification, glycogen was scarce, and many of the silver grains were not close to glycogen but over the walls of vesicles which were either devoid of ribosomes (grains at lower left and center right) or partly coated with ribosomes (grains at upper center and left). Usually, however, most silver grains were close to both glycogen granules and smooth or mixed vesicles, particularly at the 1 hr interval (Figs. 20 to 23), as was indeed demonstrated by the distribution of the distances between them and the silver grains (Fig. 24). The diagram showed that the number of silver grains located more than 0.2 μ from glycogen granules was not negligible, especially at the 15 min interval, but remained rather low. On the other hand, very nearly all grains were close to smooth or mixed vesicles (Fig. 24), even when the network of vesicles was rather loose, as in Fig. 21.

DISCUSSION

Treatment of liver with the enzyme alpha amylase removed the PA-Schiff–staining patches seen in light microscopy (Figs. 8 to 10) and the lead-staining rosettes and granules seen in electron microscopy (Fig. 4). Since the enzyme extracts glycogen from animal tissue without affecting the other substances present (10, 34), these results confirm the glycogen nature of the PA-Schiff–staining patches
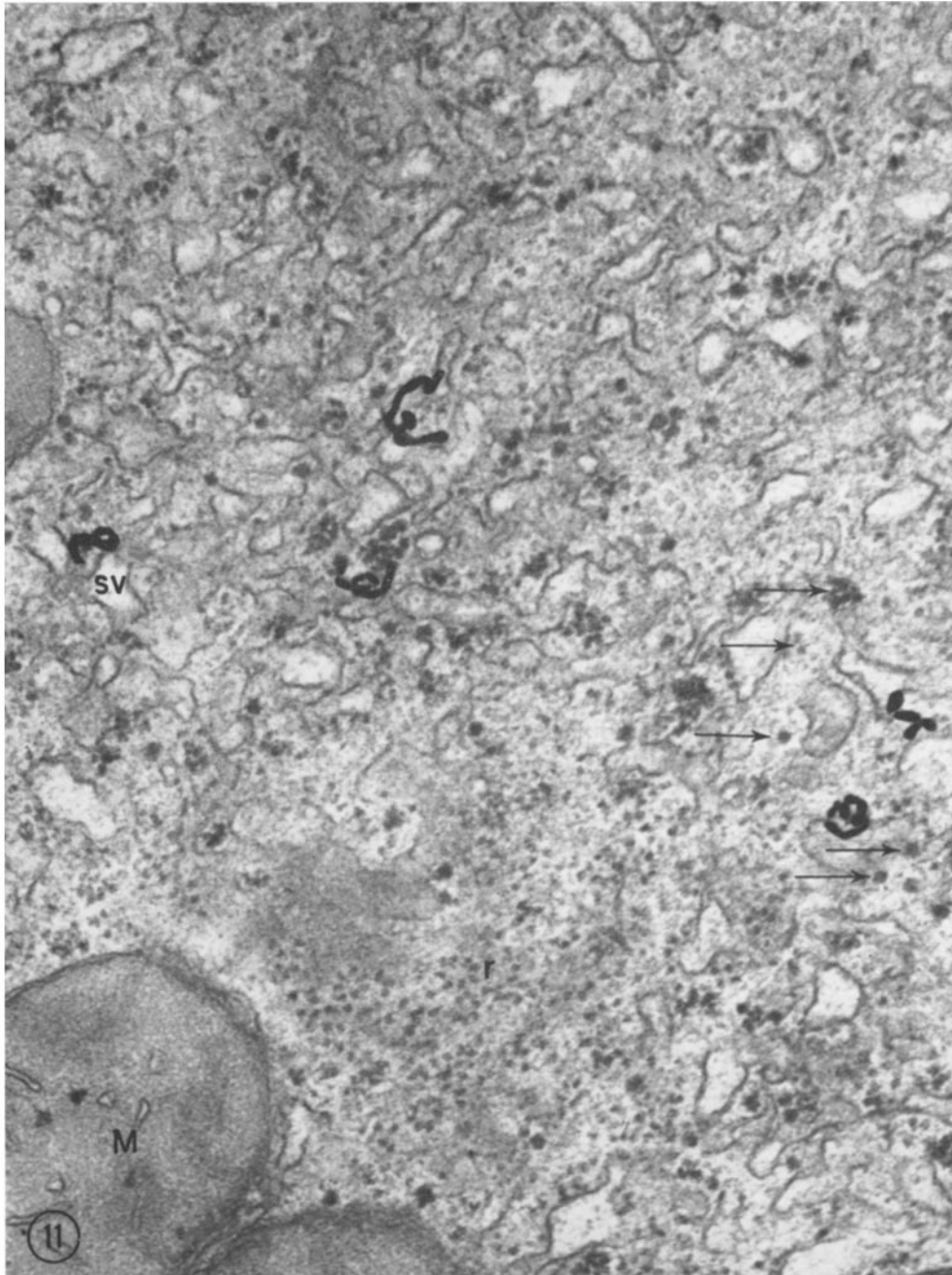
FIGURE 11   Electron microscope radioautograph of liver slice incubated with glucose H³ for 15 min.

The upper half of the picture consists of a glycogen area, whereas most of the lower part seems to be a transitional zone, containing numerous free ribosomes (r).

In the glycogen area, the cytoplasm separating the smooth vesicles (sv) contains few rosettes and many individual glycogen granules. Of the three silver grains over this area, the one at the left touches two smooth vesicles and its midportion is over a glycogen granule; the other two cross smooth vesicle walls, with the two extremities of the lower one being over glycogen and the upper one not far from a glycogen granule.

As for the two grains in the transitional zone (right), they are over the walls of smooth vesicles, but only the lower one is less than 0.2 μ from the nearby glycogen granules indicated by arrows. (Small dots nearby are taken to be ribosomes.) × 70,000.

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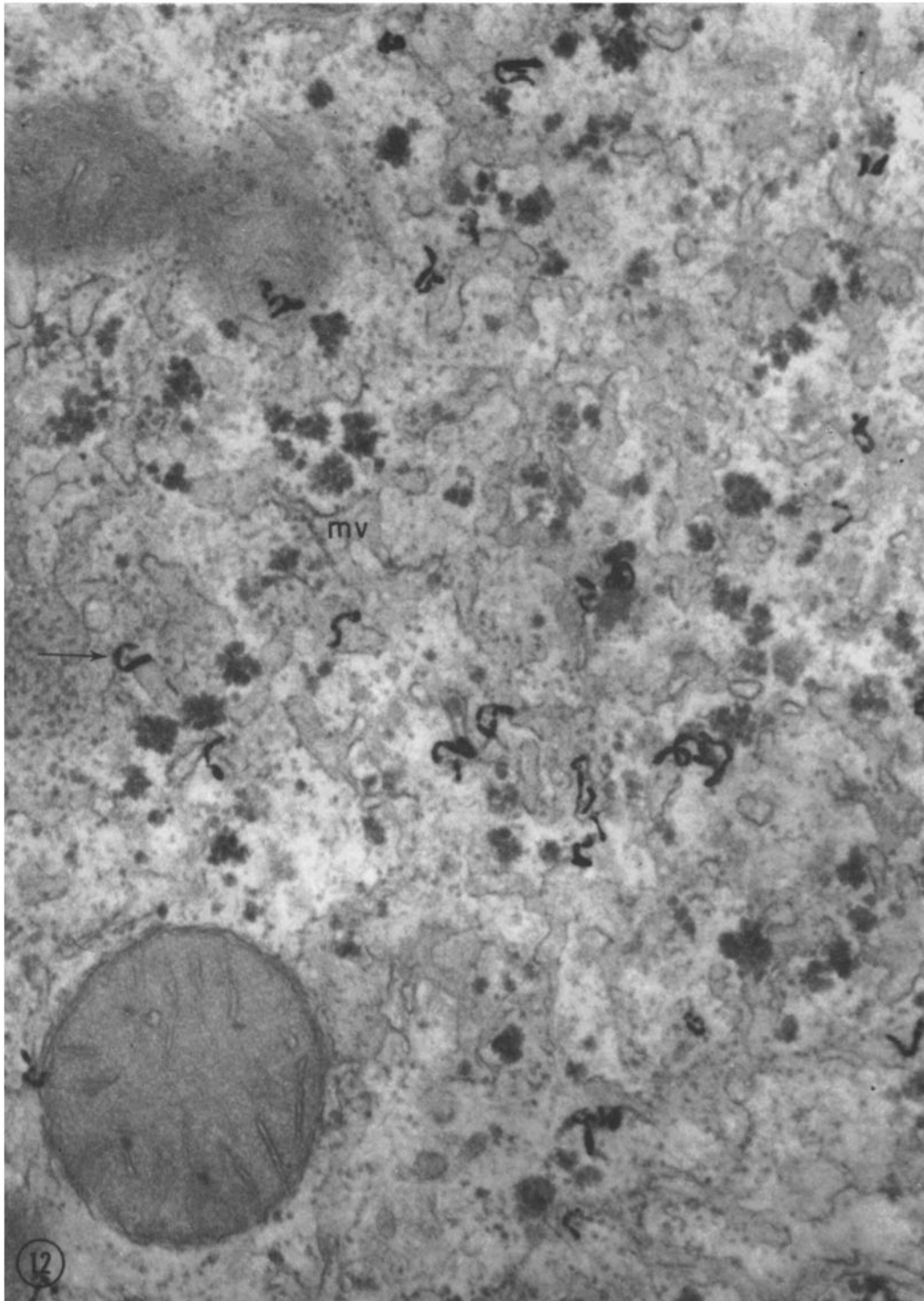
FIGURE 12 Electron microscope radioautograph of liver slice incubated with glucose H³ for 1 hr.

Most of the field appears to be a transitional zone. The smooth vesicles are arranged into an electron-opaque network, with some glycogen rosettes and granules in the hyaloplasm. The dotlike structures seen throughout most of the field are ribosomes.

After 1 hr' incubation, the number of silver grains is much increased over that at 15 min. Eighteen of the grains make contact with the walls of smooth vesicles, 13 are over or close to glycogen granules, 5 are at some distance though not more than 0.2 μ from glycogen granules. × 55,000.

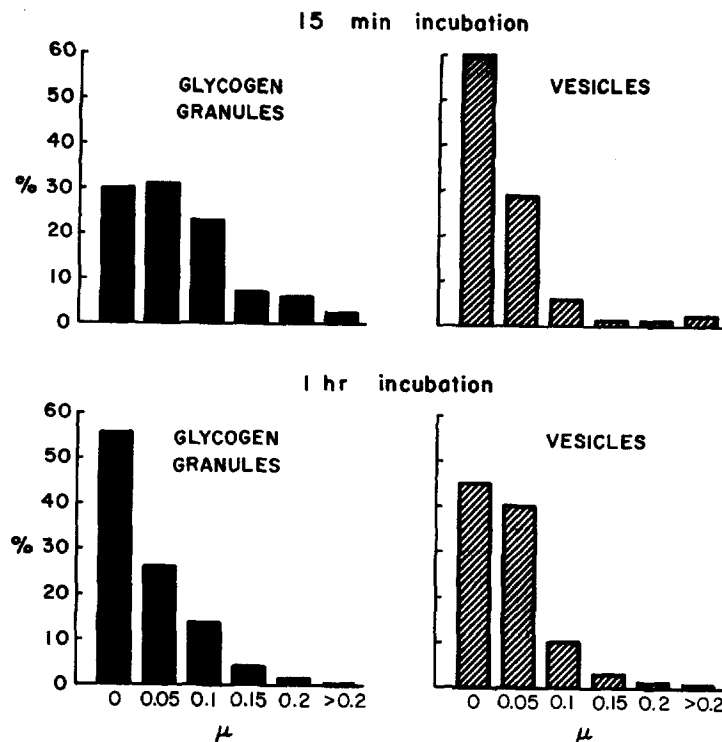FIGURE 13 In vitro experiments. Diagram giving the percentage of *silver grains* found at various distances (in μ) from the nearest glycogen granule and the nearest smooth (or mixed) vesicle. (Radioautographs of liver slices obtained after 15 min' or 1 hr' incubation in vitro in a medium containing glucose-H^3.) Most grains are close to both glycogen granules and vesicles.

and of rosettes and granules. In fact, the patches are "glycogen areas" rich in rosettes and granules (Figs. 16 and 17).

Effects of Glucose Administration on the Liver of Fasted Rats

Liver glycogen was drastically reduced by an 18 to 24 hr fast (Fig. 14), but was partly restored by ingestion of glucose (Fig. 23). Liver slices from fasted rats incubated in a glucose-rich medium also accumulated glycogen (Fig. 12). Whether added in vivo or in vitro, the new rosettes and granules of glycogen were located not only in glycogen areas, but also in zones transitional between these areas and the ergastoplasm.

After glucose administration, the glycogen areas appeared large, especially in vivo, and rich in smooth vesicles (Fig. 20); the transitional zones also appeared large, especially in vitro, but they mainly contained "mixed" vesicles, partly covered with ribosomes (Fig. 7). It seemed that glucose administration caused the rough-walled cisternae sur-

rounding a glycogen area to shed some of their ribosomes. As a result, the mixed vesicles increased in number, so that transitional zones appeared large. In some locations the presence of a number of free ribosomes next to smooth vesicles suggested that the shedding had been completed and the transitional zone became indistinguishable from the associated glycogen area, which then appeared to be larger and to contain more reticulum than in controls. Previous authors have suggested that proliferation of smooth endoplasmic reticulum alone accounted for the observations made in fasted refed rats (25) (and also for the abundant reticulum observed after various intoxications; references 12, 19, 31). However, this interpretation fits our observations less well than a transformation of rough into smooth reticulum at the periphery of glycogen areas.

Glycogen Synthesis

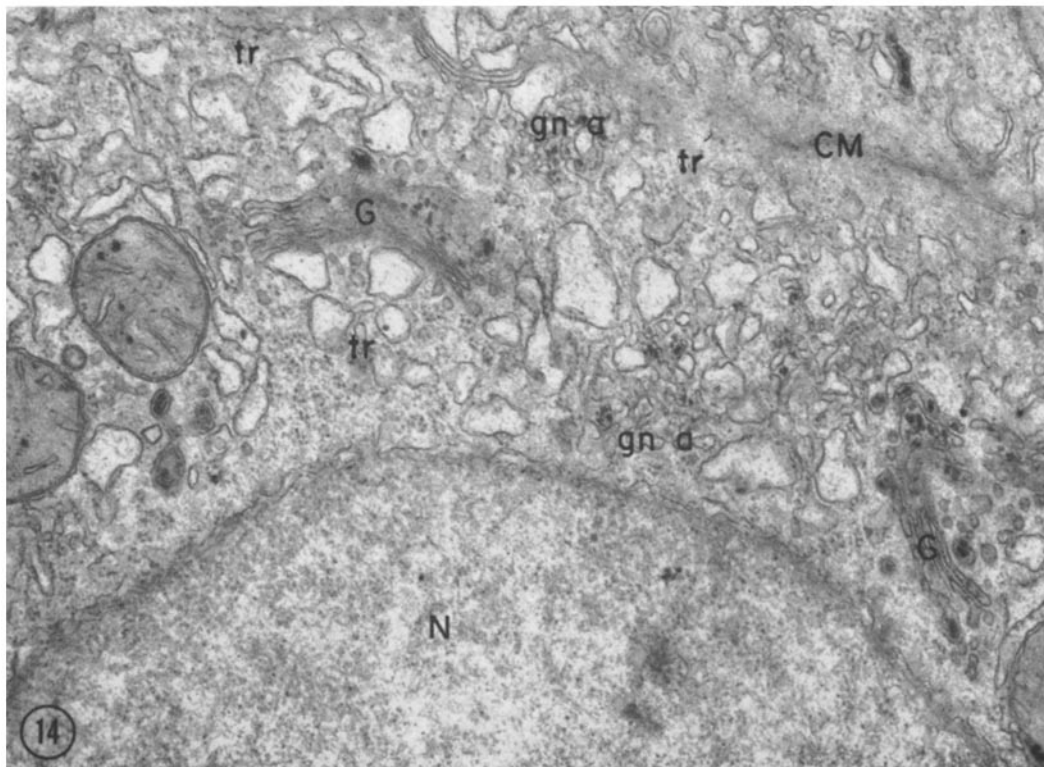Since alpha amylase treatment extracted most of the radioactivity appearing in the liver of fasted

FIGURE 14 Liver of 100 g rat fasted for 18 hr.

The glycogen areas (gn a) contain smooth vesicles with few glycogen granules. Much of the cytoplasm is occupied by smooth and mixed vesicles without glycogen granules, constituting transitional zones (tr). Two Golgi complexes have dense material within the saccules (G, lower right). × 35,000.

rats given glucose-H^3 in vivo or in liver slices incubated with glucose-H^3 in vitro (Tables I to IV; Figs. 8 to 10), this radioactivity must consist of glycogen. The labeled glucose administered must have been synthesized into glycogen.

The mode of administration of labeled glucose influenced the amount of glycogen synthesized. Thus, in vivo, when glucose-H^3 was given with added carrier glucose, nearly all liver radioactivity was removed by alpha amylase (Tables III and IV), but when glucose-H^3 was given without carrier, only three quarters of the radioactivity were extracted by the enzyme (Table II). Presumably, carrier glucose enhanced glycogen synthesis. The phenomenon was even more clear-cut in vitro, since the addition of excess glucose, 1%, was indispensable for any glycogen synthesis to take place.

Even under favorable conditions in vivo or in vitro, about 2 to 6% of the liver cell radioactivity was not removable by alpha amylase (Tables I and IV). The persisting radioactivity was attributed to the synthesis from glucose of substances other than glycogen, particularly glycoproteins and acid mucopolysaccharides (37) which are synthesized in the Golgi region of many cells (29), and perhaps even simple proteins which may be synthesized from glucose breakdown fragments in the ergastoplasm (6); but only 2 to 6% of the silver grains found in radioautographs would be due to such substances. The rest of the silver grains, that is, nearly all of them, would be due to newly-formed glycogen. The location of these grains might indicate the sites of glycogen synthesis.

Radioautographic Localization of Label

IN VITRO: Radioautography of liver slices incubated for 15 min in a medium containing 1% labeled glucose showed the presence of radioactivity over the PA-Schiff–staining glycogen present
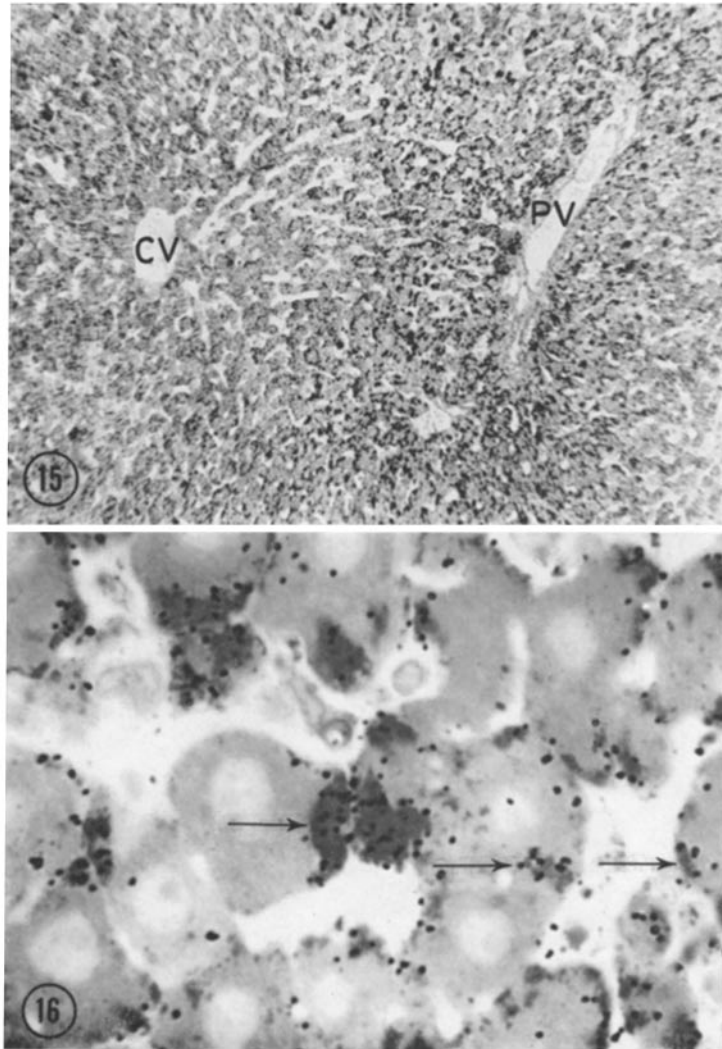
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FIGURE 15 Radioautograph of liver section from rat given intragastrically 5000 μc glucose-H³ with 120 mg of additional unlabeled glucose and sacrificed 1 hr later (experiment in vivo, third series). Fixation in Rossman fluid. Paraffin section. PA-Schiff-hematoxylin.

The dark material seen in liver cells corresponds to both PA-Schiff-stained material and silver grains covering it. There is slightly more radioactivity in the cells located near the portal space (PV, portal vein) than in those near the center of a hepatic lobule (CV, central vein). × 116.

FIGURE 16 Radioautograph of liver from the same experimental animal, but fixed in glutaraldehyde and postfixed in OsO₄, embedded in Epon, and stained with PA-Schiff (2 μ section).

Silver grains are located over PA-Schiff-stained material which corresponds to the "glycogen areas" described in the electron microscope. The three arrows from left to right point to large, medium-sized, and small glycogen areas, respectively. × 1300.

in cells (Fig. 8; Table I). The electron microscope recorded silver grains over glycogen areas and transitional zones. In both, the grains were usually close to glycogen granules and to vesicles (Figs. 11 and 12). This conclusion was confirmed by measurement of the distances from each silver grain to the nearest glycogen granule and to the nearest smooth or mixed vesicle at 15 min and 1 hr (Fig.
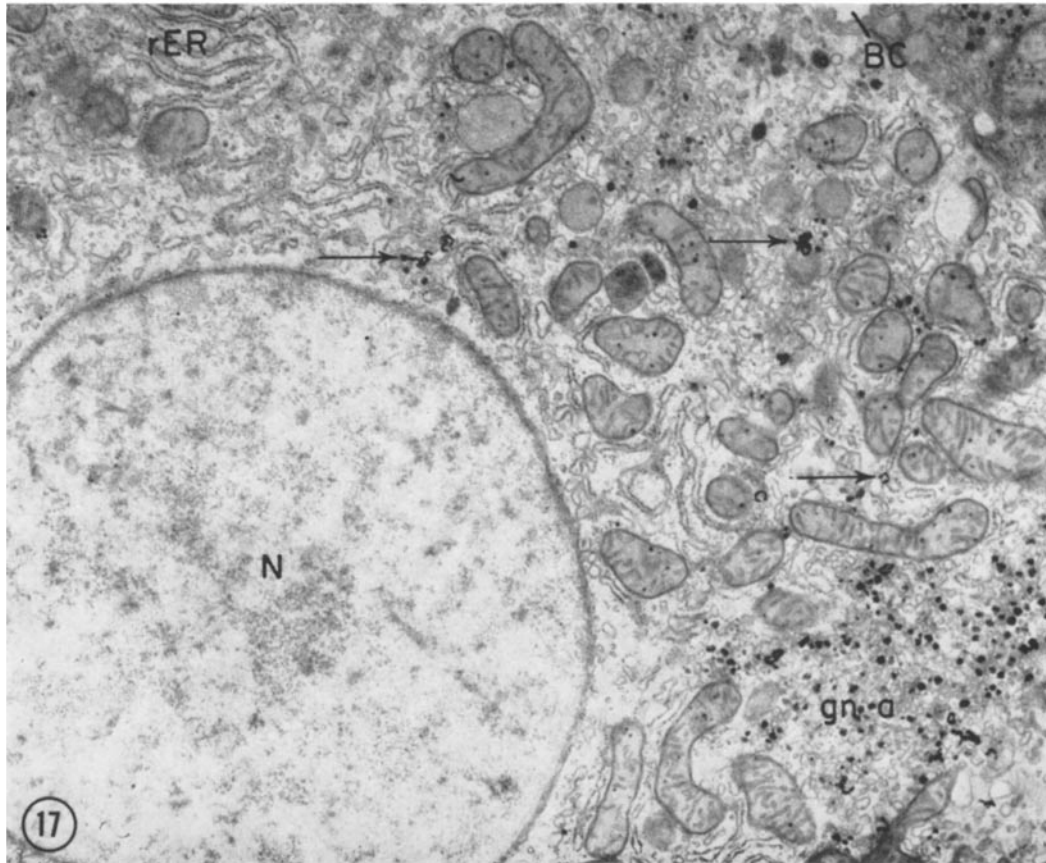
FIGURE 17   Electron microscope radioautograph of liver of fasted 100 g rat 1 hr after administration of glucose-H³.

Large portion of a liver cell with nucleus (N), a small stack of rough cisternae at upper left (rER), and a medium-sized glycogen area at lower right (gn a), in which rosettes are seen in the midst of a network of smooth vesicles, and a dozen silver grains show as tiny coils. Such a glycogen area corresponds to the medium-sized PA-Schiff–stained patch of Fig. 16.

Three arrows point to silver grains seen outside the main glycogen area; the arrow at left indicates two grains overlying a very small glycogen area; the grains shown by the other arrows are over mixed vesicles. Silver grains are lacking over the stack of rough endoplasmic reticulum (rER). × 15,000.

13); nearly all grains were found to be less than 0.2 μ from either. Furthermore, the distribution of these distances fitted well enough with theoretical diagrams (2, 8) to suggest that either glycogen granules or vesicles could be the source of the radioactivity eliciting the grains.

IN VIVO: Radioautographs of liver sections after administration of glucose-H³ to fasted animals showed a spotty distribution of radioactivity, with silver grains over glycogen patches (Fig. 16). In the electron microscope, most silver grains overlay glycogen areas (Fig. 17) and a few were

over transitional zones, being again close to glycogen granules and smooth vesicles (Fig. 20) or mixed vesicles (Fig. 19). Measurement of the distances (Fig. 24) again revealed that most silver grains were within 0.2 μ of a vesicle, but a fair number were farther than 0.2 μ from a glycogen granule: 15% at the 15 min interval. Yet, the over-all pattern was again consistent with theoretical diagrams (2, 8), so that either glycogen granules or vesicles could be the source of the radioactivity eliciting the majority of the grains.
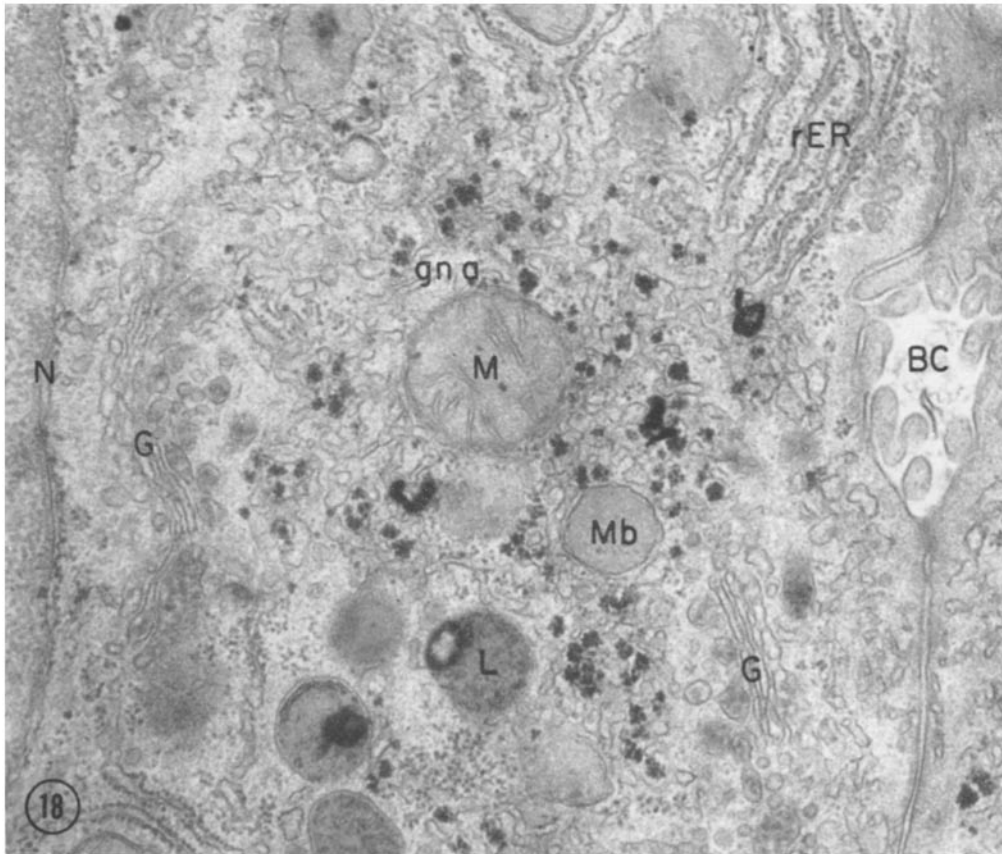
Figures 18 and 19   Radioautographs of liver of fasted 100 g rat 15 min after administration of glucose-H³.

Figure 18   A glycogen area with silver grains overlying both glycogen and vesicles. The grain at right is over a smooth vesicle connected to a nearby rough-walled cisterna. × 40,000.

Figure 19   Transitional zone with Golgi complex (G) near cell membrane (CM). Most of the vesicles in the field are partly coated with ribosomes (mv); smooth vesicles are seen at lower left (sv) and near the Golgi complex at upper right; ribosomes are numerous (r) and glycogen is scarce (gn). Of those silver grains located next to the walls of smooth or mixed vesicles, five are too far from glycogen rosettes to have been elicited by them. × 70,000.
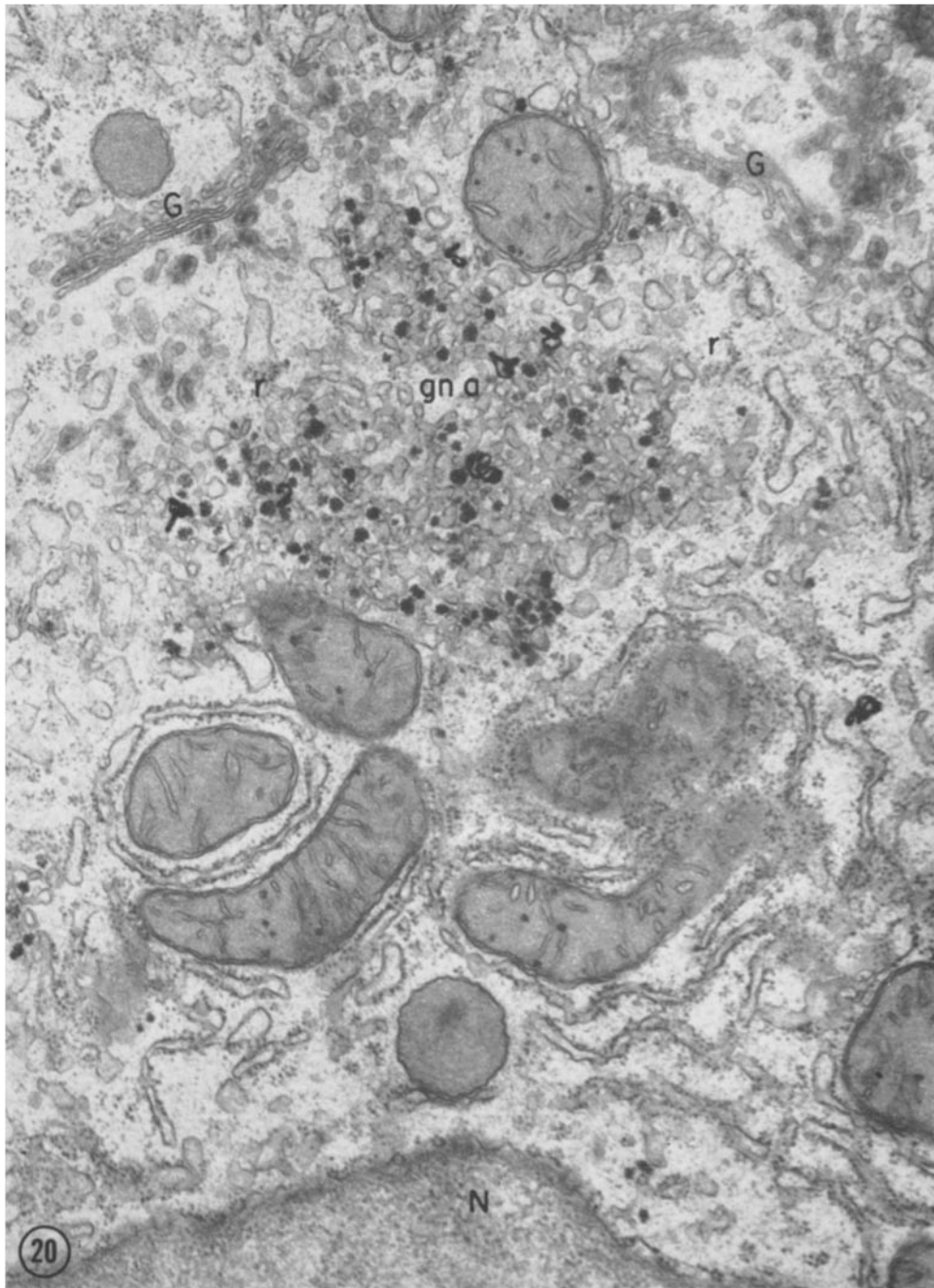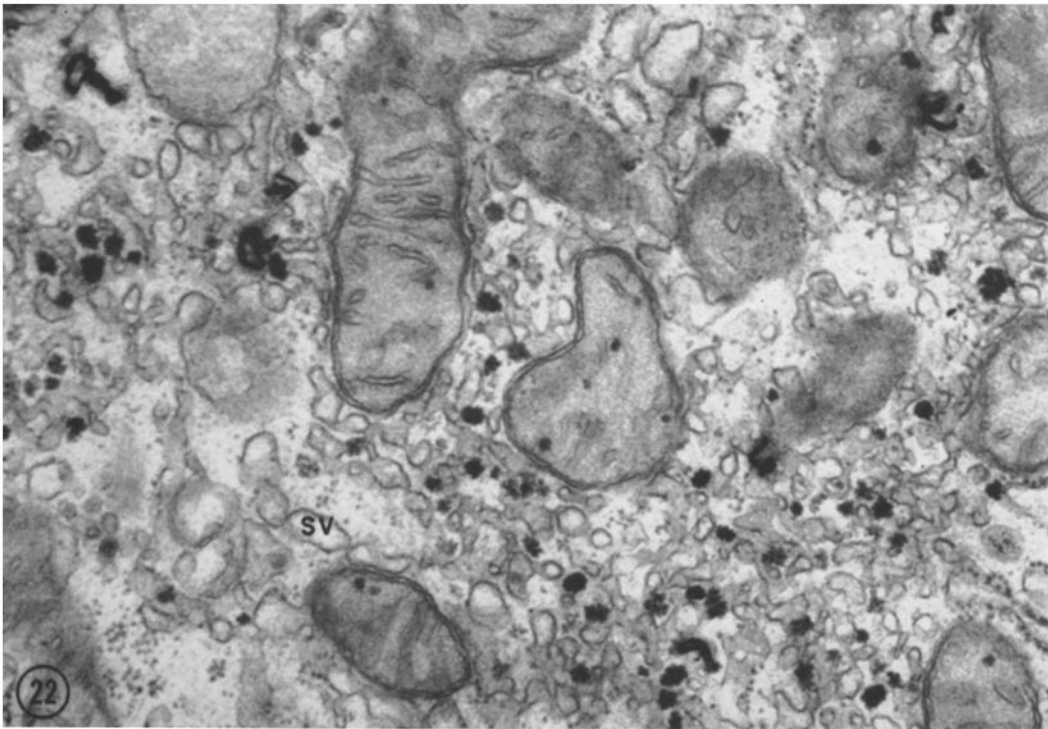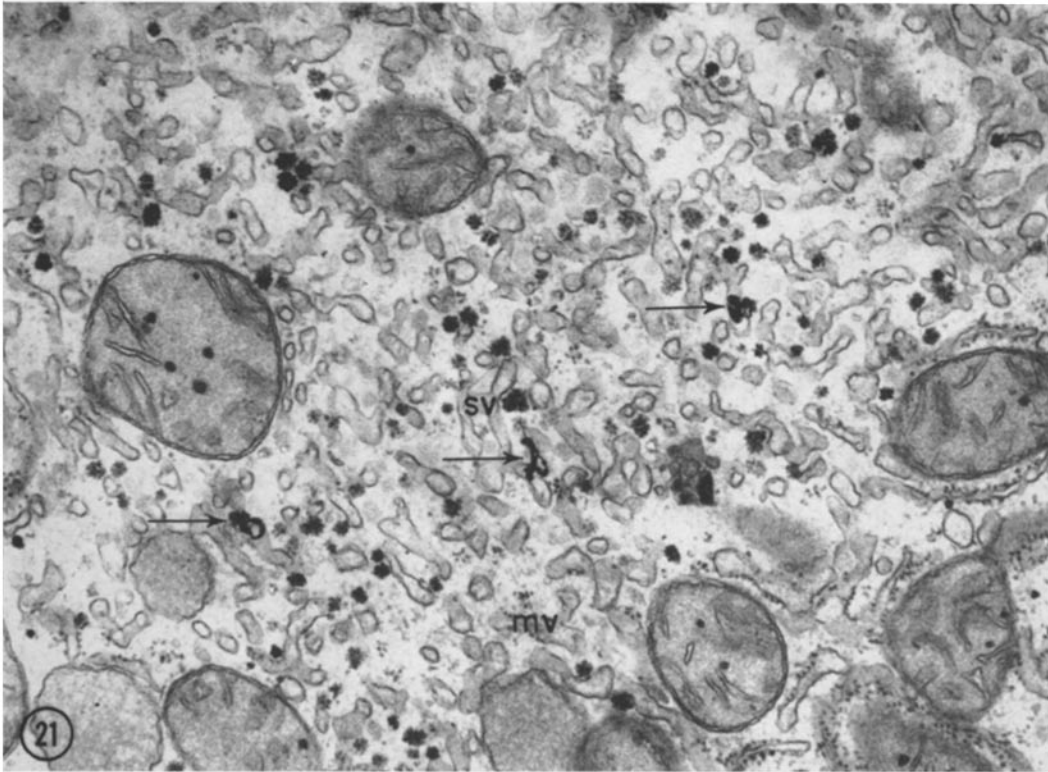
FIGURE 20 Radioautograph of liver of fasted 100 g rat 1 hr after administration of glucose-H^3. At upper center, a well limited glycogen area (*gn a*) contains smooth vesicles arranged into a network. Ribosomes are absent except at the periphery of the area (*r*). Of the seven grains within the glycogen area, all are fairly close to rosettes and all but one are placed over smooth vesicles. The grain found outside the area (center right) is over the smooth extremity of a rough-walled cisterna. × 31,500.

FIGURES 21 and 22   Radioautographs of liver of fasted 100 g rat 1 hr after administration of glucose-H³. × 49,000.

FIGURE 21   The field is occupied by a glycogen area in which many of the vesicles appear isolated, though interconnections are seen at right. Three silver grains (arrows) are over smooth vesicles and two of these are also over glycogen rosettes.

FIGURE 22   Glycogen area with seven grains over or not far from both rosettes and smooth vesicles.
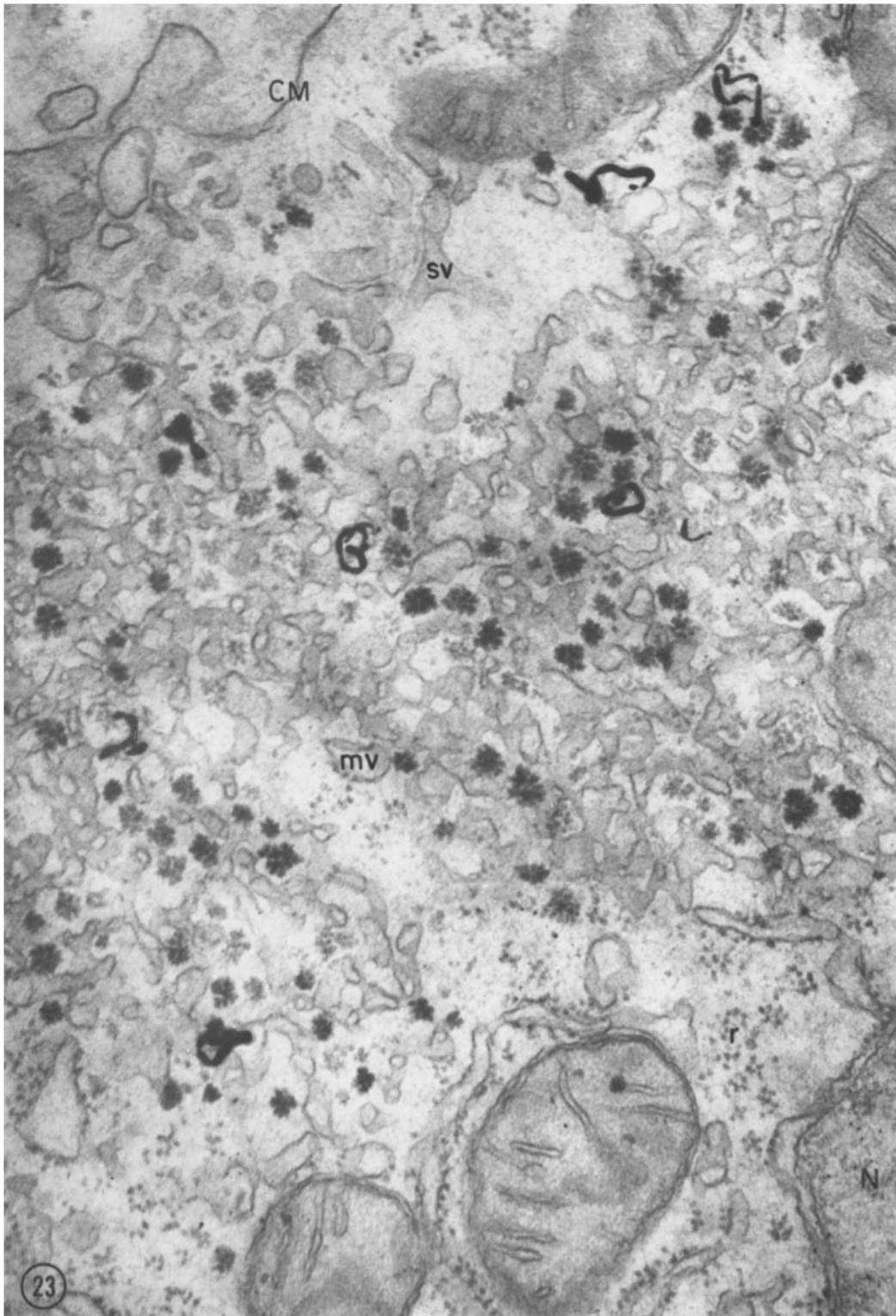
FIGURE 23 Radioautograph of liver of fasted 100 g rat 1 hr after administration of glucose-H³. The upper center region is occupied by a glycogen area and the lowest third by a transitional zone (with numerous free ribosomes and a few mixed vesicles). Nearly all grains are close to rosettes and vesicles. × 63,000.
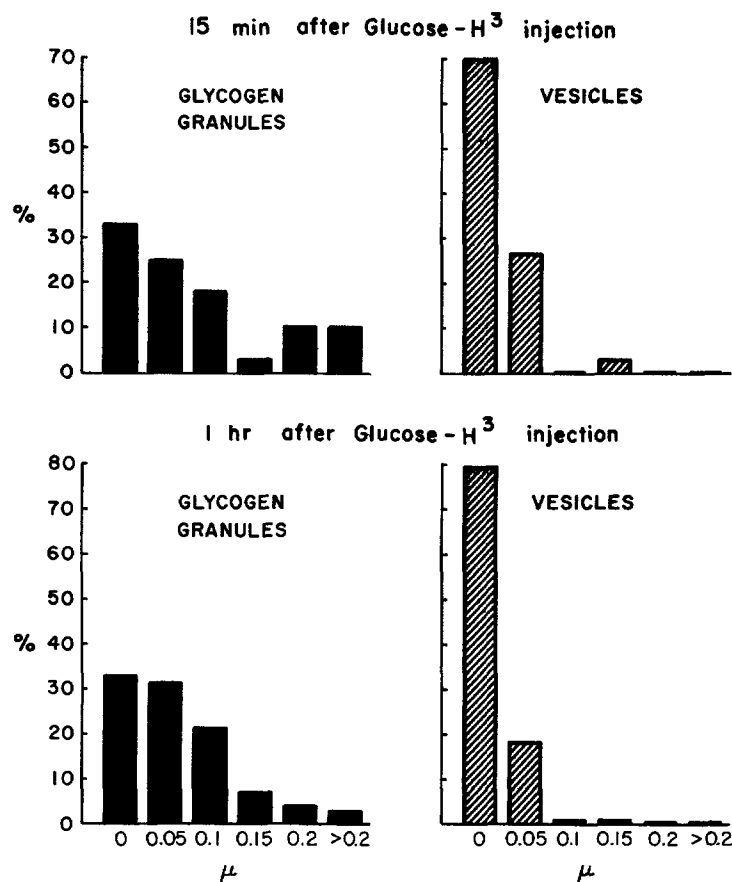
Figure 24 In vivo experiments. Diagram giving the percentage of *silver grains* found at various distances (in μ) from the nearest glycogen granule and the nearest smooth (or mixed) vesicle. (Radioautographs of liver sections obtained from in vivo experiments 15 min and 1 hr after glucose-H^3 injection.) Most grains are close to both glycogen granules and smooth vesicles. However, at 15 min and to a lesser extent at 1 hr, a fair number of grains is located 0.2 μ or farther from the nearest glycogen granule.

Site of Glucose Uptake into Glycogen

Since silver grains were near both glycogen granules and vesicles, one may wonder whether points placed at random on glycogen areas would also be near both. A test was actually carried out using twenty photographs of glycogen areas from the animals sacrificed 1 hr after injection. By drawing a grid over the pictures, 518 random points were determined; the distance of each was measured to the nearest glycogen granule and vesicle; and the distribution of these distances was plotted (Fig. 25). When the two distributions were compared to those of the distances from silver grains to nearest glycogen granule and vesicle as observed in vitro (Fig. 13) and in vivo (Fig. 24), the dia-

grams were quite comparable. The only significant difference seemed to be in the 15 min in vivo experiment, in which the number of silver grains located 2 μ or farther from glycogen granules exceeded the number of random points at these distances. With this exception, the over-all pattern of the experimental diagrams may be attributed to random effects. Hence, for most silver grains, it was not possible to decide whether they were due to radioactivity emanating from a glycogen granule or from a vesicle. Some conclusions may nevertheless be derived as will be shown below.

Let us first recall that, for glycogen synthesis, glucose must combine with uridine diphosphate and, in this form, be transferred by the enzyme
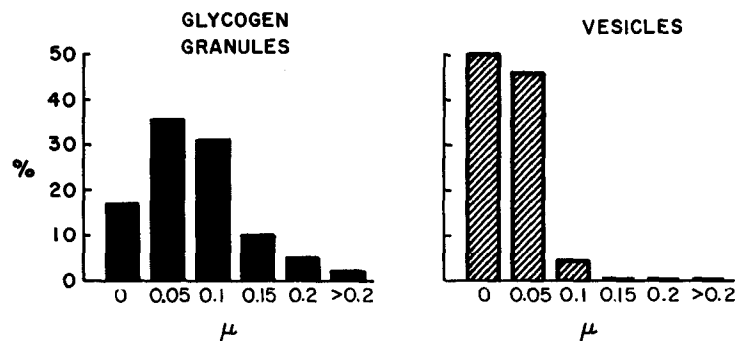
FIGURE 25 Percentage of *random points* found at various distances (in μ) from the nearest glycogen granule and the nearest smooth (or mixed) vesicle. (The points were placed at random in glycogen areas.)

The pattern is somewhat similar to that given by the percentages of silver grains at various distances from the nearest glycogen granules and vesicles, as shown in Figs. 13 and 24. (The lower columns at the 0 interval in this diagram may be due to the distances being measured from a point rather than from the swelling of the silver grains, as in Figs. 14 and 24.)

glycogen synthetase to a polysaccharide chain known as "acceptor" (20, 21). The best acceptor is glycogen itself (20). Thus, glycogen granules, or at least those which have not reached maximal size, could act as acceptors of glucose. In our experiments, a small number of glycogen granules persisted through the fasting period; and these could become labeled by addition of radioactive glucose. In addition, since the number of glycogen granules increased during the 15 min and 1 hr intervals in vivo as well as in vitro, new granules must have been formed; and these should be labeled. It has been suggested that a branch extending out of an existing glycogen granule may be the starting point on which glucose would be deposited to build up a new granule (26). Another possibility was that small molecules such as maltotetraose, maltotriose and maltose (7) or other oligosaccharides (1) could act as acceptors, and, presumably, could serve as starting points for new granules. Whatever the starting point is, it would not be detectable in the electron microscope. Neither would the early stages of the granules arising from them. (It is known that granules exist whose size is below the limit of visibility in the electron microscope; references 4, 28). Briefly then, the newly synthesized glycogen causing radioautographic reactions could be either added onto visible glycogen granules or arise in an "invisible" form.

Since the visible glycogen granules acquired in the course of the experiments in vivo as well as in vitro should be labeled and since nearly all silver grains were within the 0.167 μ range of beta rays

that would emanate from a labeled glycogen granule, it was reasonable to assume that most of the radioactivity was present in visible glycogen granules.

However, in many photographs a few silver grains were 0.2 μ or farther from the nearest glycogen granule. Particularly at 15 min in vivo, about 15% of the grains were at or beyond this limit (Fig. 24). These silver grains were too numerous to be accounted for by the 2 to 6% of radioactivity which is amylase resistant and, therefore, they must be produced by "invisible" labeled glycogen. On the other hand, these silver grains were invariably close to a smooth vesicle or to the smooth-walled portion of a mixed vesicle. Did the "invisible" phase of granule formation occur in relation with smooth membranes?

Current opinion does not favor a role of smooth membranes in glycogen synthesis. First, there is evidence that glycogen synthetase, the enzyme which transfers glucose from UDP-glucose to glycogen, is not held onto smooth membranes. Luck, who separated liver glycogen from smooth membranes by centrifugation, found the enzyme in the glycogen fraction only (23). Secondly, glycogen granules have been synthesized in vitro by Mordoh et al. using crystalline phosphorylase b, purified branching enzyme, and, as substrate, a small quantity of glycogen granules from rat liver (26). Therefore, it appears possible to synthesize glycogen in the absence of smooth membranes.

Our observations reopen the problem. The in vitro synthesis of Mordoh et al. (26) made use of

phosphorylase, whereas glycogen synthetase is probably the effective enzyme in vivo. Even though this enzyme does not seem to be associated with smooth membranes (23), Hizukuri and Larner found that a smooth membrane–rich fraction of liver contained a factor that allows glycogen synthetase to become active in the absence of glucose-6-phosphate and, thus, may control the activity of this enzyme (13). As a matter of fact, glucose-6-phosphate itself has been detected in liver smooth membranes (35), from where it could directly activate glycogen synthetase. Our own observations merely suggest that smooth mem-

branes may intervene at early stages in the formation of glycogen granules. Whether this role is enzymatic or whether the membranes simply have a steric effect facilitating the arrangement of glucose residues required for the initiation of glycogen granules, it remains possible that smooth membranes play some role in glycogen synthesis.

REFERENCES

1. BROWN, D. H. *in* Ciba Foundation Symposium, Control of Glycogen Metabolism, (W. J. Whelan and Margaret P. Cameron, editors), London, J. & A. Churchill Ltd., 1964, 82, 85, 403.
2. CARO, L. G., High-resolution autoradiography. II., *J. Cell Biol.*, 1962, 15, 189.
3. DEUTSCH, W., An improvement of Warburg's method for cutting tissue slices for respiratory experiments, *J. Physiol.*, London, 1936, 87, 56P(abstract).
4. DROCHMANS, P., Mise en évidence du glycogène dans la cellule hépatique par microscopie électronique, *J. Biophysic. and Biochem. Cytol.*, 1960, 8, 553.
5. FAWCETT, D. W., Observations on the cytology and electron microscopy of hepatic cells, *J. Nat. Cancer Inst.*, 1955, 15, 1475.
6. GAITONDE, M., DAHL, D. R., and ELLIOTT, K. A. C., Entry of glucose carbon into amino acid of rat brain and liver in vivo after injection of uniformly ^{14}C-labelled glucose, *Biochem. J.*, 1965, 94, 345.
7. GOLDENBERG, S. H., Specificity of uridine diphosphate glucose–glycogen glucosyltransferase, *Biochim. et Biophysica Acta*, 1962, 56, 357.
8. GRANBOULAN, P., *in* The Use of Radioautography in Investigating Protein Synthesis, Symposia International Society for Cell Biology, (C. P. Leblond and Katherine B. Warren, editors), New York, Academic Press Inc., 1966, 4, 43.
9. GRAUMANN, W., Untersuchungen zum cytochemischen Glykogennachweis, *Histochemie*, 1958, 1, 97.
10. HALE, A. J., The histochemistry of polysaccharides, *Internat. Rev. Cytol.*, 1957, 6, 193.
11. HASTINGS, A. B., TENG, C. T., NESBETT, F. B., AND SINEX, F. B., Studies on carbohydrate metabolism in rat liver slices, *J. Biol. Chem.*, 1952, 194, 69.

12. HERDSON, P. B., GARVIN, P. J., and JENNINGS, R. B., Reversible biological and fine structural changes produced in red liver by a thiohydantoin compound. And: Fine structural changes in red liver induced by phenobarbital, *Lab. Inv.*, 1964, 13, 1014, 1032.
13. HIZUKURI, S., and LARNER, J., Studies on UDPG: α-1,4-glucan α-4-glucosyltransferase. VII. Conversion of the enzyme from glucose-6-phosphate–dependent to independent form in liver, *Biochemistry*, 1964, 3, 1783.
14. HOTCHKISS, R. D., A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations, *Arch. Biochem.*, 1948, 16, 131.
15. ITO, K., Regulation of carbohydrate metabolism in liver tissue in vitro, *Japan J. Exp. Med.*, 1962, 32, 599.
16. KARNOVSKY, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, 11, 729.
17. KOPRIWA, BEATRIX M., and LEBLOND, C. P., Improvements in the coating technique of radioautography, *J. Histochem. and Cytochem.*, 1962, 10, 269.
18. KUGLER, J. H., and WILKINSON, W. J. C., A histochemical study of the incorporation of glucose-C^{14} into glycogen by liver slices in vitro, *J. Histochem. and Cytochem.*, 1962, 10, 143.
19. LAFONTAINE, J. G., and ALLARD, C., A light and electron microscope study of the morphological changes induced in rat liver cells by the azo dye 2-ME-DAB, *J. Cell Biol.*, 1964, 22, 143.
20. LELOIR, L. F., *in* Ciba Foundation Symposium, Control of Glycogen Metabolism, (W. J. Whelan and Margaret P. Cameron, editors), London, J. & A. Churchill Ltd., 1964, 68.

21. Leloir, L. F., and Cardini, C. E., *in* The Enzymes, (P. D. Boyer, H. Lardy, and K. Myrbäck, editors), New York, Academic Press Inc., 1961, **6**, 317.

22. Lison, L., Histochimie et Cytochimie Animales, 3rd edition, Paris, Gauthier-Villars, 1960.

23. Luck, D. J. L., Glycogen synthesis from uridine diphosphate glucose, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 195.

24. Millonig, G., Advantages of a phosphate buffer for OsO_4 solutions in fixation, *J. Appl. Physics*, 1961, **32**, 1637.

25. Millonig, G., and Porter, K. R., *Proc. European Regional Conf. Electron Micr.*, Delft, 1960, 655.

26. Mordoh, J., Leloir, L. F., and Krisman, C. R., In vitro synthesis of particulate glycogen, *Proc. Nat. Acad. Sc.*, 1965, **53**, 86.

27. Moses, M. J., Application of autoradiography to electron microscopy, *J. Histochem. and Cytochem.*, 1964, **12**, 115.

28. Orrell, S. A., Jr., Bueding, E., and Reissig, M., *in* Ciba Foundation Symposium, Control of Glycogen Metabolism, (W. J. Whelan and Margaret P. Cameron, editors), London, J. & A. Churchill Ltd., 1964, 29.

29. Peterson, Marian, and Leblond, C. P., Synthesis of complex carbohydrates in the Golgi region, as shown by radioautography after injection of labeled glucose, *J. Cell Biol.*, 1964, **21**, 143.

30. Peyrot, A., Il metodo del congelamento-sostituzione nella ricerca istologica e istochimica., *Riv. Istochim. Norm. e Patol.*, 1956, **2**, 197.

31. Porter, K. R., and Bruni, C., An electron microscope study of the early effects of 3'-Me-DAB on rat liver cells, *Cancer Research*, 1959, **19**, 997.

32. Revel, J. P., Electron microscopy of glycogen, *J. Histochem. and Cytochem.*, 1964, **12**, 104.

33. Revel, J. P., and Hay, Elizabeth, D., Autoradiographic localization of DNA synthesis in a specific ultrastructural component of the interphase nucleus, *Exp. Cell Research*, 1961, **25**, 474.

34. Roberts, P. J. P., and Whelan, W. J., The mechanism of carbohydrase action, *Biochem. J.*, 1960, **76**, 246.

35. Rosen, S. I., Tubular endoplasmic reticulum and glucose-6-phosphatase activity in the neonatal liver, *J. Cell Biol.*, 1964, **23**, 78A(abstract).

36. Sabatini, D. D., Bensch, K. G., and Barrnett, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity, *J. Cell Biol.*, 1963, **17**, 19.

37. Sarcione, E. J., The initial subcellular site of incorporation of hexoses into liver protein, *J. Biol. Chem.*, 1964, **239**, 1686.

38. Steiner, D. F., and Williams, R. H., Some observations concerning hepatic glucose-6-phosphate content in normal and diabetic rats, *J. Biol. Chem.*, 1959, **234**, 1342.

39. Stetten, D., Jr., and Stetten, M. R., Glycogen metabolism, *Physiol. Rev.*, 1960, **40**, 505.

40. Trott, J. R., An evaluation of methods commonly used for the fixation and staining of glycogen, *J. Histochem. and Cytochem.*, 1961, **9**, 703.

41. Woods, P. S., and Pollister, A. W., An ice-solvent method of drying frozen tissue for plant cytology, *Stain Technol.*, 1955, **30**, 123.

42. Young, B. A., and Kopriwa, Beatrix M., The use of Gevaert NUC-307 nuclear emulsion for radioautography at the electron microscope level, *J. Histochem. and Cytochem.*, 1964, **12**, 438.