

SERGE, the Subcellular Site of Initial Hepatic Glycogen Deposition in the Rat: A Radioautographic and Cytochemical Study

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ABSTRACT Hormonal control of hepatic glycogen and blood glucose levels is one of the major homeostatic mechanisms in mammals: glycogen is synthesized when portal glucose concentration is sufficiently elevated and degraded when glucose levels are low. We have studied initial events of hepatic glycogen synthesis by injecting the synthetic glucocorticoid dexamethasone (DEX) into adrenalectomized rats fasted overnight. Hepatic glycogen levels are very low in adrenalectomized rats, and DEX causes rapid deposition of the complex carbohydrate. Investigation of the process of glycogen deposition was performed by light and electron microscopic (EM) radioautography using [³H]galactose as a glycogen precursor. Rats injected with DEX for 2–3 h and [³H]galactose one hour before being killed displayed an increasing number of intensely labeled hepatocytes. EM radioautography revealed silver grains over small ($\pm 1 \mu\text{m}$) ovoid or round areas of the cytosome that were rich in smooth endoplasmic reticulum (SER) and contained a high concentration of small dense particles. These distinct areas or foci of SER and presumptive glycogen (SERGE) were most numerous during initial periods of glycogen synthesis. After longer exposure to DEX (4–5 h) more typical deposits of cytoplasmic glycogen were evident in the SERGE regions. Several criteria indicated that the SERGE foci contained glycogen or presumptive glycogen: resemblance of the largest dense particles to β -glycogen particles in EM; association of ³H-carbohydrate with the foci; removal of particles and label with α -amylase; and positive reaction with periodic acid–chromic acid–silver methenamine. The concentration of SER in the small foci and the association of newly formed glycogen particles with elements of SER suggest a role for this organelle in the initial synthesis of glycogen.

Glycogen in hepatocytes represents an available reserve of glucose that is used to maintain appropriate blood glucose levels. The synthesis and breakdown of glycogen are precisely regulated by hormonal mechanisms and blood glucose levels (12, 13, 30). In general, glycogen synthesis occurs as the glucose levels in the portal vein increase and breakdown of the complex carbohydrate is stimulated by low blood glucose levels (12, 13).

Although much information is available on the biochemistry of glycogen synthesis (12, 29) very little is known about the cellular and subcellular mechanisms in the synthesis of this important storage form of carbohydrate. More important,

almost nothing is understood about the role of cellular organelles in the synthesis of glycogen. In this article we address these questions by studying a model system that allows description of the early morphological events in hepatic glycogen synthesis. Our data implicate the smooth endoplasmic reticulum (SER)¹ in this process.

Patterns of glycogen deposition throughout the liver lobule are not necessarily uniform, but they are related in general to

¹ *Abbreviations used in this paper.* ADX, adrenalectomized; DEX, dexamethasone; EM and LM, electron and light microscopic (microscopy), respectively; SER, smooth endoplasmic reticulum; SERGE, distinct foci that contain SER, glycogen, and enzymes.

the nutritional status of the animal (7). The synthesis of glycogen occurs rather predictably in rats that have been maintained on a controlled feeding schedule (1, 2). However, even under these controlled feeding conditions hepatic glycogen in fasted rats is not reduced to uniformly low levels, and the use of such rats to investigate early glycogen deposition is undesirable because residual glycogen cannot be distinguished morphologically from newly deposited carbohydrate. In the current study we used another model—the adrenalectomized (ADX) rat, fasted overnight, that has hepatic glycogen levels <0.1%. Injection of a glucocorticoid causes rapid and predictable hepatic glycogen synthesis in this model (3, 6, 9, 10), and the initial events of glycogen deposition are more clearly demonstrated.

In the present light and electron microscopic (LM and EM, respectively) radioautographic study we used [³H]galactose as the glycogen precursor. The synthetic steroid dexamethasone (DEX) was employed as a glucocorticoid to stimulate glycogen synthesis. About 2 h after ADX rats had received DEX and a 1-h exposure to [³H]galactose, their responding hepatocytes had silver grains aggregated over small cytoplasmic areas or foci that were comprised of profiles of SER and small electron-dense particles. We refer to these areas as SERGE foci because they contain SER, glycogen, and enzymes (SERGE). In this paper we describe these foci and comment on their importance in hepatic glycogen synthesis.

MATERIALS AND METHODS

Young male Wistar rats, ~100–150 g at the time of sacrifice, were adrenalectomized 7 to 10 d before each experiment. The rats had constant access to water containing 0.9% NaCl and were fed ad lib. until the night before experimentation, when they were housed singly in wire-bottom cages and fasted.

The animals were divided into three groups: 2 normal rats which were fasted overnight; 4 ADX rats fasted overnight; and 14 ADX rats which were fasted overnight and given a 0.5-ml intraperitoneal injection of DEX, 4 mg/ml. Rats in group 3 were killed after DEX administration as follows: two rats after 1 h of DEX; four after 2 h; four after 3 h; two after 4 h; and two after 5 h. 1 h before death each of the rats in all groups was injected intraperitoneally with 2 mCi [³H]galactose/100 g body wt and 10 mg/100 g body wt of unlabeled galactose as carrier.

At death the rats were anesthetized with Nembutal and the abdomen was opened to expose the liver. Part of the liver was surgically removed and immediately immersed in fixative consisting of 2% glutaraldehyde, 2% formaldehyde (derived from paraformaldehyde) in 0.1 M sodium cacodylate buffer, pH 7.3, containing 0.01% calcium chloride. The tissue was sliced into strips with a razor blade, diced into 1-mm cubes, and fixed at room temperature for at least 2 h. After it was washed in 0.1 M sodium cacodylate buffer for 1 h, the tissue was postfixated in sodium cacodylate buffered 1% osmium tetroxide for 2 h. Dehydration in ethanol and then propylene oxide preceded infiltration of a mixture of propylene oxide and Epon. The blocks of tissue were embedded in Epon 812 (19). After initial fixation in the aldehyde fixative some tissue was incubated in a 1% solution of α -amylase at 37°C for 2 h to remove glycogen. To observe sites in the tissue where glycogen or glycoproteins were present the periodic acid–chromic acid–silver methenamine technique (26, 27) was employed.

Semithick sections (0.5 μ m) were cut from the tissue blocks and stained with toluidine blue. Blocks were trimmed so that a portal triad and/or central vein was present in the section. Radioautographs for LM and EM were prepared using the methods of Kopriwa and Leblond (15), and Kopriwa (16). Kodak NTB-2 emulsion was used for LM radioautography. The emulsion was exposed to unstained 1- μ m-thick sections for 8 d before being developed for 2 min in Kodak D-19 diluted 1:2 then cleared in Kodak fixer. The sections were stained with toluidine blue. Excess stain was removed with a very dilute solution of acetic acid if necessary.

Ilford L-4 nuclear track emulsion (Ilford Ltd., Basildon, Essex, U.K.) was used for EM radioautography. Thin sections (silver interference color) adhering to glass slides coated with a 2% solution of nitrocellulose in amyl acetate were usually prestained for EM (below) before coating with a thin layer of carbon in a vacuum evaporator. The carbon coated slides were dipped into emulsion

diluted to yield a monolayer of silver halide crystals. The emulsion was exposed to the sections for 43 d, developed for 2 min in fresh Kodak D-19 diluted 1:10, and fixed in 30% sodium thiosulfate. Sections that were floated off the slide were placed on grids, stained in uranyl acetate (31) and lead citrate (28) if not prestained, and examined in a Philips EM-301 electron microscope.

To evaluate quantitatively the distribution of silver grains among organelles in hepatocytes, a circle was placed over each silver grain. The diameter of this circle, which had a 50% probability of including the radioactive source, was calculated according to isotope, section thickness, and developer (32). Radioautographs of two different magnifications were used and the 50% probability circle (half-distance, 140 nm) calculated accordingly ($\times 20,000$, 6-mm-diam circle; $\times 30,000$, 8-mm diam). The grains were categorized according to the organelles included within the circle. When more than one organelle occurred within the 50% probability circle, each organelle received the appropriate fraction of a grain.

RESULTS

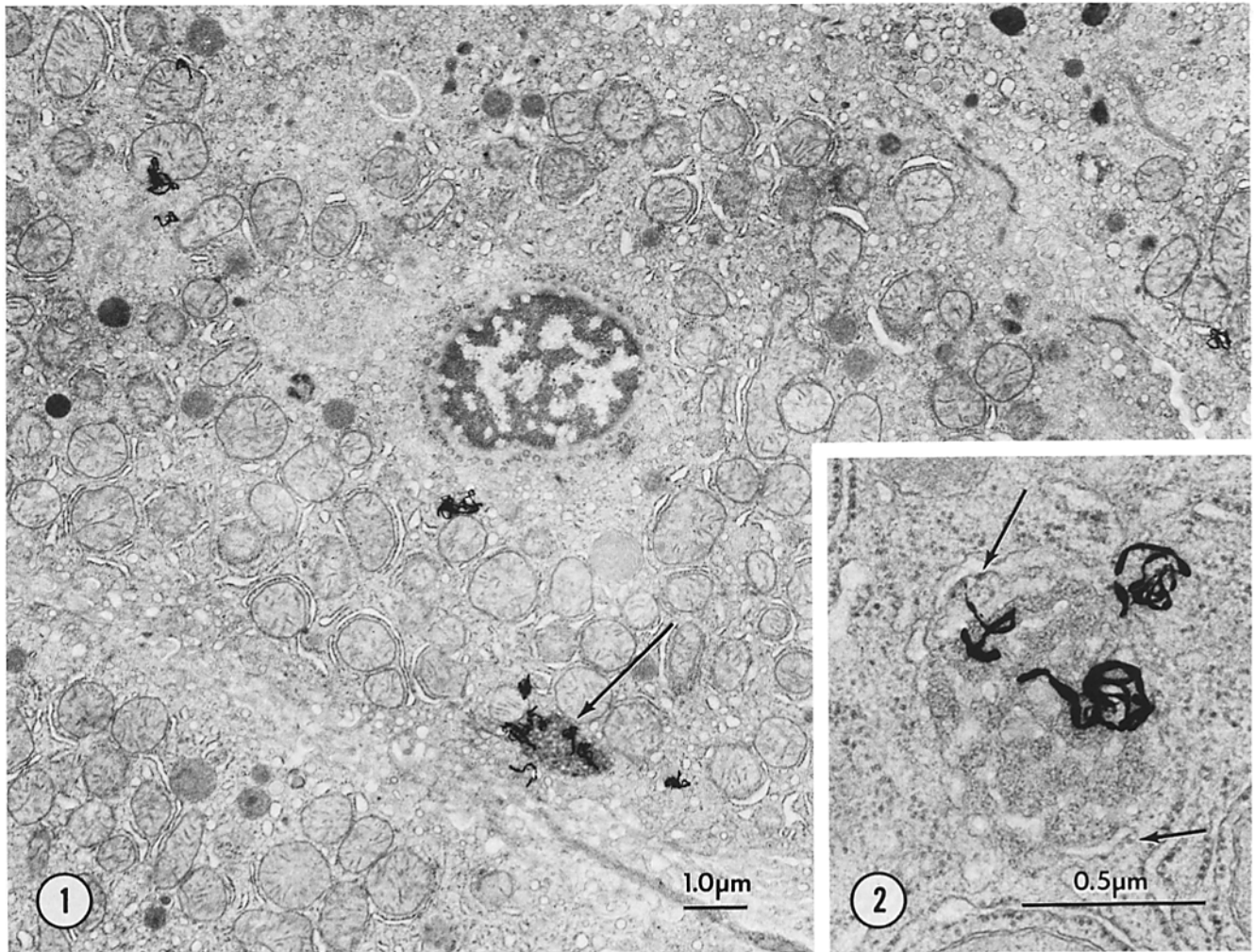
In the ADX rats that were fasted overnight and then injected with DEX and [³H]galactose 1 h before death most hepatocytes were lightly labeled, but an occasional cell showed intense labeling. Intensely labeled cells increased in number (10–20%) in the rats treated for 2 and 3 h with DEX and injected with [³H]galactose 1 h before death. In the LM radioautographs the silver grains over these hepatocytes were either dispersed or aggregated without regard to the intralobular location of the cells. A more detailed description and discussion of the LM radioautography was published recently by Michaels et al. (23).

The EM radioautographs of these cells defined in more detail the labeled intracellular regions. Silver grains indicating incorporation of labeled precursor into glycogen occurred principally over two regions of cytoplasm: small foci of SER and presumptive glycogen (SERGE) that did not contain typical glycogen particles (Fig. 1); and areas rich in SER and with sparse definite glycogen granules (not illustrated). Grains were found also over SER that had no obvious association with presumptive or definitive glycogen particles in that plane of the section. Occasionally silver grains were observed near mitochondria, rough endoplasmic reticulum, and Golgi complexes.

In sections of any labeled cell, usually from one to three SERGE foci were conspicuous because aggregated silver grains overlay them (Fig. 1). The foci were outlined by short profiles of endoplasmic reticulum (Fig. 2) that sometimes bore ribosomes, and consisted of elements of SER among a dense matrix of fine particulate matter that was variable in size but usually smaller than typical glycogen granules. Early or newly formed SERGE foci measured ~1 μ m in diameter.

In the radioautographs from rats treated for 3 h with DEX, silver grains were more numerous over the SERGE foci, and the dense particulate component was larger and resembled β -particles of glycogen. In periportal hepatocytes of rats treated with DEX for 4 and 5 h SERGE regions contained masses of glycogen particles, and these were associated with aggregated silver grains. Centrilobular cells displayed more dispersed glycogen over which silver grains were more spread. The regions of the cytosome in which glycogen deposits were present were more numerous and larger in area than those found earlier.

To identify the particulate component of the SERGE foci with more certainty, we performed two cytochemical procedures. Aldehyde-fixed tissue was washed and then incubated in 1% α -amylase, a glycogenolytic enzyme, before osmication and embedment. This treatment resulted in SERGE foci that lacked the dense granular component and were not labeled as



FIGURES 1 and 2 Fig. 1: This electron microscopic radioautograph shows hepatocytes from an ADX rat fasted overnight then injected with DEX for 2 h and with [^3H]galactose 1 h before being killed. Several silver grains are over an area (arrow) within the cytosome that has a higher electron density and more profiles of SER than does the surrounding cytoplasm. These specialized areas are referred to in the text as SERGE foci. A few silver grains are observed over or near rough endoplasmic reticulum (RER) and mitochondria throughout the cytosome. $\times 8,700$. Fig. 2: Hepatocyte from a rat that received the same treatment as in Fig. 1. At this magnification details of the morphological components of the SERGE region are evident. Profiles of SER outline the region and a few short cisterna of RER (arrows) are nearby. Within this unique area are many images of SER and fine electron dense particles. Three silver grains are associated with the SERGE region. $\times 51,000$.

evidenced by the lack of silver grains (Fig. 3).

Other tissue was stained by the periodic acid–chromic acid–silver methenamine techniques to show the presence of glycoproteins or glycogen. The SERGE foci displayed stained particles whose size ranged from very fine to that of β -glycogen particles. Some structures contained only fine particles (Fig. 4), others had a mixture of smaller and larger particles (Figs. 5 and 6), and still others contained primarily β -glycogen granules (Fig. 7).

To quantitate the relationship of silver grains to organelles, we scored the contact of the grains with several organelles as shown in Fig. 8. It is apparent that a close association of silver grains with SER and SERGE regions of the cell occurs especially after stimulation by DEX for 2 h or more. No other organelles appear significantly labeled, although mitochondria show an increased number of grains as DEX-stimulated glycogen synthesis progresses (2–5 h). This is expected because of the nearness of mitochondria to SERGE regions, the higher number of grains present, and the resolution of the EM radioautography procedure with ^3H (half-distance, 140 nm).

DISCUSSION

We have demonstrated in this study that unique areas within the cytosome of hepatocytes described for the first time as SERGE foci consisted of SER and particles of glycogen at various stages of formation. However, we should emphasize that SERGE foci containing presumptive glycogen were not found in every labeled hepatocyte, and the number of hepatocytes showing the labeled foci varied somewhat in different experiments. These observations probably reflect alterations in the morphology of the SERGE regions that depend on the metabolic state of the individual cell. Apparently the metabolic state of liver cells varies over a considerable range, especially during very early stages of glycogen synthesis.

The development of SERGE foci primarily occurred 2–4 h after DEX treatment. The concentration of silver grains over SERGE regions increased with the period of DEX treatment. In addition, there was an overall increase in the size of the dense particles during this interval so that by 4 h β -particles of glycogen were common and α -particles were present.

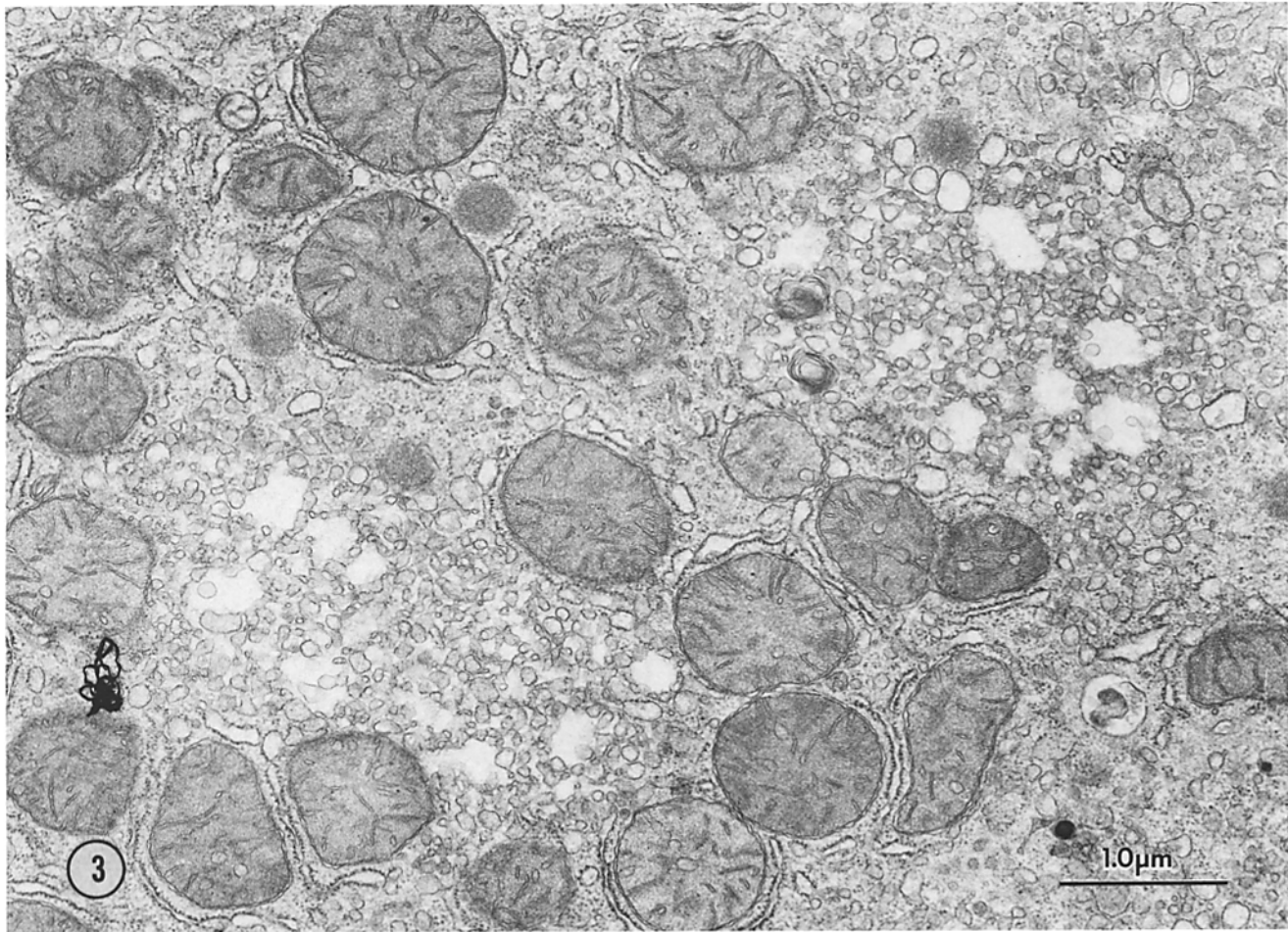


FIGURE 3 This electron microscopic radioautograph illustrates a portion of a hepatocyte from an ADX rat fasted overnight that had been injected with DEX for 3 h and had received [^3H]galactose 1 h before being killed. The tissue was incubated for 1 h in a 1% solution of α -amylase at 37°C before osmication, and most of the glycogen was removed. The micrograph shows a SERGE region from which glycogen particles and silver grains are absent and SER is conspicuous. $\times 22,600$.

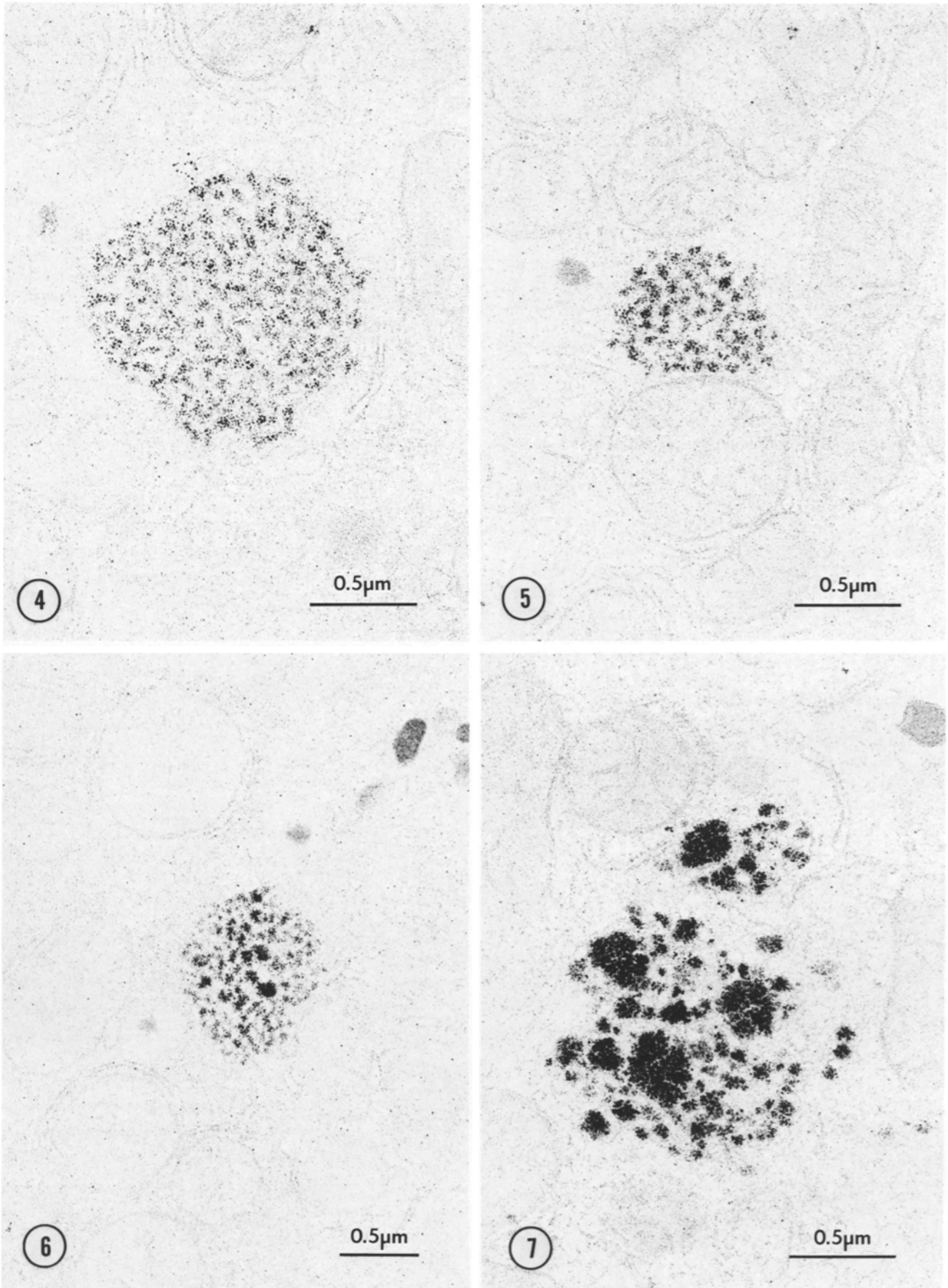
The particulate material located within the SERGE foci was identified as glycogen by several criteria: (a) in transmission electron microscopy the larger particles resembled β -glycogen granules; (b) silver grains derived from ^3H -carbohydrate were concentrated over SERGE areas; (c) α -amylase treatment of the tissue removed the particles and the radioactive label from SERGE foci; and (d) the periodic acid-silver methenamine technique used to identify glycogen and glycoproteins in sections for EM revealed that the particles within the SERGE foci contained a high concentration of carbohydrate. Moreover, it was evident by the latter technique that the size of particles ranged from ultrafine to that of granules that resembled β -glycogen granules. Such evidence clearly suggests glycogen formation and involvement of the SERGE foci in the initial stages of glycogen formation.

Once glycogen synthesis was well under way the SERGE regions were characterized by high accumulations of glycogen. After 4–5 h of DEX treatment glycogen was observed as clumps in periportal cells and dispersed in centrilobular cells, patterns that were less apparent in the earlier intervals of treatment. In the periportal cells profiles of SER continued to be associated with the periphery of the clumped glycogen, whereas SER profiles were dispersed throughout regions of centrilobular cells in which glycogen was concentrated.

Although several studies have investigated hepatic glycogen

deposition intensively (2, 6, 7, 14, 21) including by the use of EM radioautography (8, 9), the early SERGE foci with fine particulate presumptive glycogen were not described. This morphology probably appears mainly during the earliest stages of initiation of glycogen synthesis, and the smallness and relative sparsity make identification of these foci difficult without the overlying silver grains that result from radioautographic processing. For these reasons the foci without definitive glycogen were probably overlooked in earlier studies. The conditions of extremely low initial glycogen content followed by a potent stimulation of glycogen synthesis may be required for observation of early SERGE foci with reasonable frequency.

The reports that associate glycogen formation and SER are extensive, including early ultrastructural studies by Fawcett (11) and by Porter et al. (24, 25), radioautographic investigations (8, 9), and more recent studies by Cardell et al. (2, 7, 20). Although it appears that glycogen synthase is not contained within the membranes of SER (18), it has been suggested that the activating enzyme glycogen synthase phosphatase is present in this organelle (20). The presence of this enzyme in SER may be one of the primary reasons for the association of SER and newly synthesized glycogen. The intimate association of profiles of SER and glycogen or presumptive glycogen in the SERGE foci again suggests a role of



FIGURES 4-7 These electron micrographs are from ADX rats fasted overnight injected with DEX for 2 or 3 h. The sections were stained with periodic acid-chromic acid-silver methenamine, which demonstrates glycogen. Note that the glycogen occurs in restricted regions of the cytosome. The particles of glycogen vary from fine particles (Fig. 4) to a few larger granules (Figs. 5 and 6) and mostly large granules (Fig. 7). A sequence in the formation of glycogen within SERGE foci is suggested. Figs. 4, 5, and 7, $\times 38,250$; Fig. 6, $\times 28,000$.

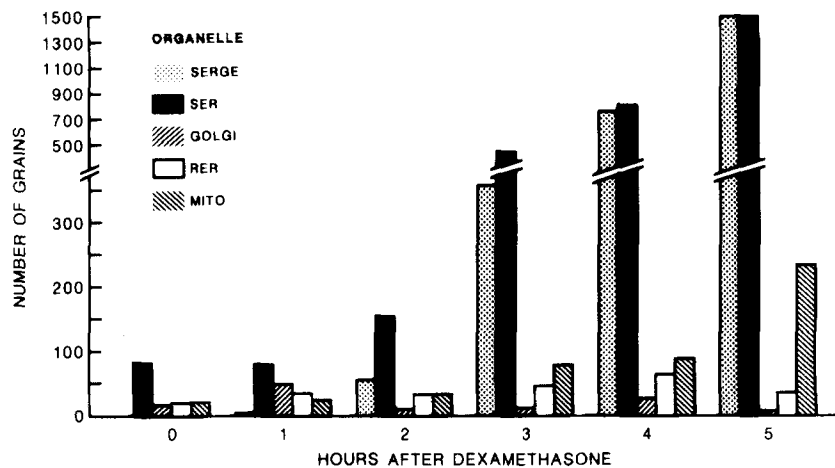


FIGURE 8 Hepatic grain distribution in ADX rats. This bar graph illustrates the distribution of silver grains associated with cellular organelles in electron microscopic radioautographs of rat hepatocytes. The animals were ADX, fasted overnight, injected with DEX for 0-5 h, and injected with 2 mCi [^3H]galactose 1 h before being killed. The association of silver grains with organelles was determined by scoring the structures included in a 50% probability circle (half-distance, 140 nm) placed over each grain. It is obvious that most of the grains are associated with SERGE and SER especially after 2 h of stimulation with DEX. Mito, mitochondria.

this membrane organelle in glycogen synthesis. We should point out that there probably are other factors or components, yet to be identified, that may be present in the SERGE foci and that are important in the process of glycogen deposition. For example, an appropriate stimulus (hormonal or otherwise) could cause an increase or decrease in the calcium concentration (4) in the SERGE regions, which could activate or inactivate enzymes involved in glycogen metabolism, thus resulting in glycogen deposition in restricted regions of the cell. Furthermore, glycogen deposition would be expected to occur in regions of the cytosome in which substrate levels are high. It is possible that SERGE foci can generate or retain substrates important for glycogen synthesis. Finally, several investigators (5, 17, 22) have suggested that a protein core is required for early glycogen synthesis. The SERGE regions may provide the cytoplasmic site for such proteins which in turn may cause the early deposition of glycogen in SERGE regions of the cell.

Yet to be revealed are the mechanisms involved in sequestering either ions, substrate, or proteins in SERGES, the specific limited areas within the cell where all essential factors required for the conversion of soluble sugars to glycogen come together.

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