The American Journal of PATHOLOGY

April 1971 • Volume 63, Number 1

Fine Structural Aspects of the Mobilization of Hepatic Glycogen

I. Acceleration of Glycogen Breakdown

Orthon B. Kotoulas, MD, PhD and Melville J. Phillips, MD

SINCE THE ORGINAL OBSERVATIONS by Porter and Bruni in 1959,[1] suggesting a relationship between the metabolism of glycogen and the smooth endoplasmic reticulum, the role of cellular organelles in the metabolism of glycogen has been the subject of extensive investigation and much controversy.[2-12] The endoplasmic reticulum-bound enzyme glucose-6-phosphatase was particularly implicated in the postnatal mobilization of glycogen.[5,13,14] A role of lysosomes in degrading glycogen was suggested from reported studies of glycogenesis type II (Pompe's disease). In this disease, the absence of the lysosomal enzyme, α-glucosidase, which is capable of degrading glycogen, leads to an accumulation of large amounts of undegraded polysaccharide inside the lysosomes.[15-17]

The postnatal period provides an excellent biologic model for studying the breakdown of hepatic glycogen, since during the first 12 hours after birth, enormous stores of glycogen are completely mobilized.[18,19]

In a previous ultrastructural study of postnatal rat liver,[10] it was found that a number of changes in cell organelles accompanied the breakdown of glycogen. It was not possible to decide conclusively from that study which, if any, of these changes were related to the breakdown of glycogen and which were developmental changes or other

From the Department of Pathology, McGill University, Montreal, Canada.
Supported by Grant-in-Aid MA-785 from the Medical Research Council of Canada.
Accepted for publication December 14, 1970.
Address for reprint requests: Dr. M. J. Phillips, Banting Institute, University of Toronto, 100 College Street, Toronto, Canada.

unrelated phenomena. In the present study, by accelerating the breakdown of glycogen by glucagon and adrenalin, it was hoped that the glycogen-associated organelle changes could be promoted and identified. Puromycin, which exerts a known glycogenolytic effect on liver,[55] and cyclic AMP, which plays an important role in glycogenolysis,[21] were also used. Biochemical estimations of glycogen in the liver and glucose in the blood were carried out for quantitative purposes. Since the lysosomes and the endoplasmic reticulum are the organelles most commonly implicated in the metabolism of glycogen, the activities of acid phosphatase and glucose-6-phosphatase were estimated on liver homogenates. The electron microscopic findings were quantitated, using morphometric analysis.

In a companion paper,[22] a study using the same experimental method deals with the effects of the inhibition of glycogen breakdown. Some of these observations have already been reported in abstract.[23]

Materials and Methods

Animals and Handling of Tissues

The newborn rats used were delivered naturally from Wistar pregnant females obtained from the Canadian Breeding Laboratories. The females were maintained at room temperature and fed Purina Chow until delivery. Twenty-five pregnant females were used; the average litter contained 10 newborns. The newborns were separated from their mothers within the first 5 minutes of birth, weighed and kept in an incubator at 35 C until they were sacrificed. Markedly cyanotic newborns as well as those outside the weight range of 5.5–6.5 g were excluded (mean weight of newborns, 6 g). The animals were sacrificed by decapitation at birth, or after 4, 6 or 12 hours unless otherwise specified. Immediately before sacrificing, blood was obtained from the cervical veins for determining blood glucose. After decapitation, the liver was excised immediately. A small piece was processed for electron microscopy. The rest of the liver was immersed in liquid nitrogen, then stored at approximately −35 C and used for biochemical determinations, which were completed usually within 10 days.

Chemicals

The following principal chemical and pharmacologic materials were used: glucagon, crystalline (lot 258-234 B-167-1, Eli Lilly & Co); epinephrine (adrenalin) bitartrate, 1:1000 solution (lot 77526, British Drug Houses (Canada) Ltd); puromycin dihydrochloride (control No. 8903, Nutritional Biochemical Corp); cyclic AMP (adenosine 3′,5′-cyclic monophosphoric acid) crystalline (lot 78B-7240); glucose-6-phosphate, barium salt heptohydrate (lot 56B-0420); β-glycerophosphate disodium salt pentahydrate (lot 68B-4130) and the reagents for deproteinizing blood and for determining glucose and inorganic phosphorus (Sigma Chemical Company). For electron microscopy, all reagents, except araldite, were obtained from Fisher Scientific Co. Araldite 502 was obtained from Ciba Co Ltd.

Biochemical Methods

All the colorimetric methods were carried out using a Bausch and Lomb Spectronic 20 colorimeter. Glycogen in the liver was estimated by the method of Carroll *et al.*[24] The results were expressed as mg glycogen/mg of liver protein. Protein was determined by the method of Lowry *et al.*[25] The enzyme assays were performed on homogenates of tissues in a 10% dilution with distilled water. The tissue was homogenized in a glass tissue grinder with Teflon pestle (from AH Thomas Co, Philadelphia, Pennsylvania). Usually 100 µl of homogenate were used for the assays. Glucose-6-phosphatase was determined by the method of Swanson.[26] Total acid phosphatase was determined as in DeDuve *et al.*[27] Inorganic phosphorus was measured according to the method of Fiske and Subbarow.[28] The enzymatic activities were expressed as µmole inorganic phosphorus liberated/mg protein/hr. Blood glucose was estimated by the method of Raabo and Terkildsen,[29] modified according to Sigma Tentative Technical Bulletin No. 510, 1967, using glucose oxidase, peroxidase and dianisidine. The blood was deproteinized by the method of Somogyi.[30]

Electron Microscopy

Blocks of liver tissue were fixed for 1 hour at 0 C in 1% osmium tetroxide buffered with 0.1 M phosphate buffer (pH 7.2). The blocks were dehydrated in graded series of ethanol solution, transferred to propylene oxide and then to a mixture of propylene oxide and resin.

The embedding medium was prepared essentially according to Mollenhauer.[31] Sections from the plastic-embedded tissue were cut with glass knives using Porter Blum or LKB microtomes. Sections 1.0 µ thick were stained with toluidine blue[32] and examined by light microscopy. Ultrathin sections of gray interference color were picked up on uncoated grids and stained at room temperature with a saturated aqueous solution of uranyl acetate for 10 minutes and Reynold's solution of lead citrate for another 10 minutes.[33,34] The sections were examined in Philips 300 and Hitachi 11B electron microscopes.

Morphometric Analysis

The ultrastructural alterations were assessed quantitatively according to the methods of quantitative stereology.[35–38] For this purpose, from each group 3 experimental animals and an equal number of control animals were used. From each liver, five blocks were used. From each block, sections of gray interference color were cut and mounted in 300-mesh grids. Two to four randomly taken micrographs from each block were used, except for the group II (see Experimental Design) where one or two micrographs/block were used.

Randomness of the sampling was ascertained by taking the first micrograph from the upper right, the second from the lower left corner of the hole of the grid, and so on.

The pictures were taken at a magnification of 5200 and enlarged 2.5 times to a final magnification of 13,000. Thus an area of 400 sq cm on the print represented an area of 0.00000237 sq cm (or 237 sq µ) of the tissue. Pictures containing less than 50% hepatic cell cytoplasm were not used for analysis. For the morphometric work, a 20 × 20 cm square lattice composed of lines drawn on a transparent material was superimposed on the prints. The lattice was made of 40 horizontal and 40 vertical lines at regular intervals (0.5 cm). The cross points of the lines of the lattice were used for planimetric work. According to the principles of stereology, the fractional value occupied by a component is equal to the fraction of the random points enclosed within the component's area in a random cross section of tissue. This fractional volume was expressed as a percentage of cytoplasmic volume. In

the cases of lyososomes and the lysosomal glycogen, the data of the micrograps from the same block were combined. Therefore, the means and standard errors in these cases were calculated from these combined data. The results were evaluated statistically by Student's t test.[39] Values of P less than 0.05 were considered significant.

Experimental Design, Doses, Routes and Mode of Administration

The animals were divided into groups according to the agent used. For the postnatal ages of 4, 6 and 12 hours, approximately equal numbers of experimental animals and their controls of the same age and from the same litters were sacrificed during the same experimental period.

The control animals were injected with carrier or they represented untreated normal animals, as in the case of group II.

Group I: Glucagon Administered. Glucagon was administered subcutaneously in 0.3 ml of 0.2 M glycine-NaOH buffer (pH 9.5).[40] Control animals were injected with the carrier only. The animals were injected at 0 and 3 hours after birth, each time with a dose of 15 mg/kg and sacrificed at 4, 6 and 12 hours.

Group II: Adrenalin Administered. Adrenalin was administered subcutaneously in 0.03 ml of a 1:10 dilution (with distilled water) of the original 1:1000 solution. Normal animals of the same age and from the same litters were used as controls. The adrenalin-treated animals were injected at 0 and 3 hours after birth (a dose of 0.5 mg/kg each time) and sacrificed at 6 hours.

Group III: Puromycin Administered. Puromycin was dissolved in 1.8% NaCl and the pH was brought to 7 with NaOH. The solution was diluted with distilled water to a final concentration of 0.9% NaCl.[41]

The newborns were injected intraperitoneally with 0.1 ml of the above solution at 0 and 3 hours after birth, each time with a dose of 20.8 mg/kg. Control animals were injected with carrier. All animals were sacrificed at 6 and 12 hours.

Group IV: Cyclic AMP Administered. Cyclic AMP was administered intraperitoneally in 0.2 ml of an 0.3% solution of the compound in 0.9% NaCl. The treated animals were injected at 0 and 3 hours after birth (a dose of 100 mg/kg each time). Control animals were injected with 0.9% NaCl.

Results

Biochemical Results

Twelve animals from the same dam were used in a preliminary experiment in order to determine the hyperglycemic response of the

Table 1. Blood Glucose Values in Glucagon-Treated and Control Rats

	Blood glucose value (mg%)					
	Time after first injection			Time after second injection		
Treatment	2 min	1 hr	2 hr	2 min	1 hr	2 hr
Control	53.0	53.0	40.0	40.0	56.5	50.0
Glucagon*†	60.0	56.5	46.5	80.0	80.0	100.0

* Two subcutaneous injections of glucagon, 15 mg/kg each, were given. The first injection was given at birth and the second at 3 hours of age.

† Note the hyperglycemic response after two injections of glucagon.

Table 2. Effects, 4-12 Hours after Birth, of Administering Glucagon to Newborn Rats

	Glycogen (mg/mg of protein)*			
	Time of sacrifice after birth (hours)			
Treatment	0	4	6	12
Control	0.855 ± 0.260 (4)	0.367 ± 0.116 (6)	0.207 ± 0.110 (13)	<0.020 (4)
Glucagon		0.193 ± 0.053 (5)	0.127 ± 0.064 (10)	<0.020 (4)
P		<0.02	<0.05	

* The results are the means ± standard deviations. Numbers in parentheses represent number of observations included in results.

newborn rat to glucagon administered subcutaneously at doses of 15 mg/kg. Six of the animals were injected at birth with carrier and 6 with glucagon. One animal from each group was sacrificed at 2 minutes after the injection and hourly thereafter. At 3 hours after birth, a second injection was given to the remaining newborns from each group and the animals continued to be sacrificed in the same pattern. The hyperglycemic response was negligible after the first injection but appreciable after the second (Table 1). The response to two injections of glucagon was studied in more definitive experiments. The results are depicted in Tables 2 and 3.

Table 3. Effects, 4 and 6 Hours after Birth, of Administering Glucagon to Newborn Rats*

Treatment	Blood glucose (mg/100 ml blood)	Glucose-6-phosphatase (μM P/mg protein/hr)	Acid phosphatase (μM P/mg protein/hr)
	4 HOURS AFTER BIRTH		
Control	53.7 ± 14.6 (5)	3.68 ± 0.386 (5)	1.91 ± 0.28 (5)
Glucagon	73.5 ± 16.1 (5)	3.47, 2.83	1.78, 1.51
P	<0.1		
	6 HOURS AFTER BIRTH		
Control	61.8 ± 15.2 (7)	5.16 ± 0.92 (12)	1.68 ± 0.39 (12)
Glucagon	94.6 ± 23.3 (7)	5.05 ± 1.23 (9)	1.97 ± 0.67 (9)
P	<0.02	<0.5	<0.3

* Results are means ± standard deviations. Numbers in parentheses represent number of observations included in results. Values without standard deviation represent single observations.

Glucagon administration elevated glucose in the blood ($P < 0.02$ at 6 hours).

At birth, the liver contains a large amount of glycogen, which is mobilized postnatally. At 4 hours after birth, mobilization of glycogen by glucagon was enhanced appreciably, the treated animals having significantly lower amounts of glycogen in the liver than the controls. A difference in the glycogen content of the liver between glucagon-treated and control animals was observed also at 6 hours. At 12 hours, both treated animals and controls showed a negligible amount of glycogen in the liver. The activities of glucose-6-phosphatase or acid phosphatase were not increased significantly by glucagon.

The concentration of glycogen in the liver was significantly lower in the adrenalin-treated animals than in untreated control animals (Table 4).

Administering puromycin resulted in hyperglycemia and depletion of hepatic glycogen. No significant changes were noted in the activities of glucose-6-phosphatase or acid phosphatase (Table 5).

The level of blood glucose was elevated in 4 animals treated with cyclic AMP, with a mean 172.8 mg glucose/100 ml blood with a standard deviation ±27.2. Five control animals had a mean level of 83.2 mg glucose/100 ml blood with a standard deviation of ±18.0. The difference was statistically significant ($P < 0.01$).

Morphologic Results

Both qualitative and quantitative changes of the various cytoplasmic components were studied. In the description of the morphologic and morphometric results as well as in the tables of morphometric analysis, the term *lysosomes* includes lysosomes and related particles, from autophagic vacuoles to residual bodies,[42,43] unless otherwise specified. Structures described as *ribosome-coated and sparsely coated vesicles of the rough endoplasmic reticulum* largely include the "mixed" vesicles

Table 4. Effects, 6 Hours after Birth, on Hepatic Glycogen after Administering Adrenalin to Newborn Rats

Treatment	Glycogen (mg/mg protein)*
Control	0.214 ± 0.081 (3)
Adrenalin	0.075 ± 0.009 (3)
P	<0.05

* Results are means ± standard deviations. Numbers in parentheses are number of observations included in results.

Table 5. Effects, 6 and 12 Hours after Birth, of Administering Puromycin to Newborn Rats*

Treatment	Blood glucose (mg/100 mg blood)	Glycogen (mg/mg protein)	Glucose-6-phosphatase (μM P/mg protein/hr)	Acid phosphatase (μM P/mg protein/hr)
		6 HOURS AFTER BIRTH		
Control	50.8 ± 4.3 (5)	0.125 ± 0.035 (5)	6.37 ± 1.75 (5)	2.74 ± 0.38 (5)
Puromycin	139.0 ± 53.1 (5)	0.057 ± 0.018 (4)	4.85 ± 1.17 (5)	2.70 ± 0.53 (5)
P	<0.02	<0.02	<0.2	>0.5
		12 HOURS AFTER BIRTH		
Control		<0.020 (4)		
Puromycin		<0.020 (3)		

* The results are means ± standard deviations. Numbers in parentheses represent number of observations included in results.

of previous investigators.[10,44] The term *glycogen* in the tables of morphometric analysis refers to hyaloplasmic glycogen unless otherwise specified.

Ultrastructure of the Hepatocyte at Birth

A detailed description of the normal fine structure of the rat hepatocytes during the first postnatal hours has been reported by Phillips *et al.*[10] It is worth emphasizing certain points here. At birth, the cytoplasm is divided into a large glycogen-containing area and a glycogen-free area into which the remaining organelles are crowded. (Fig 1). Lysosomes and autophagic vacuoles are small and rarely seen. The Golgi apparatus is near the margins of the glycogen areas. The endoplasmic reticulum appears mainly in the form of cisternae. Some cisternae show bulbous terminations. Ribosome-coated vesicles are not frequent.

Morphometric analysis of the various cell components is shown in Table 6. The hyaloplasmic glycogen occupied 50.0% of the cytoplasmic volume, mitochondria 11.5%, peroxisomes 0.58%, lysosomes and related particles (including autophagic vacuoles) 0.33% and glycogen in autophagic vacuoles 0.16%. The last column of Table 6 depicts the fraction of the volume of lysosomes which is occupied by glycogen. This fraction was 0.48.

Table 6. Volume Fractions of Cytoplasmic Components of Rat Hepatic Cell at Birth*

Percent of cytoplasmic volume†					
Glycogen	Mitochondria	Peroxisomes	Lysosomes	Glycogen in autophagic vacuoles	Fraction of glycogen in lysosomes‡
50.0 ± 3.3	11.5 ± 0.6	0.58 ± 0.06	0.33 ± 0.08	0.16 ± 0.03	0.48

* Results computed from a total of 30 micrographs and an area of 5250 sq μ.
† Results means ± stqndard errors.
‡ Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.

Group I: Glucagon Administered

In control animals at 4 and 6 hours after birth (Fig 2 and 3) the areas of glycogen varied in size but generally were reduced compared to those seen at birth. At 6 hours, lysosomal activity increased noticeably and numerous autophagic vacuoles appeared. At both 4 and 6 hours, the lysosomes and autophagic vacuoles were not distributed randomly throughout the cell but were usually found near the junction of glycogen areas with glycogen-free areas. They often were related closely to the Golgi apparatus. They usually occurred in groups. The autophagic vacuoles were limited by a single or double membrane and contained glycogen and other cytoplasmic components as well as amorphous electron-dense material. Most of the glycogen within the autophagic vacuoles was of the monoparticulate type. Also noticeable in the cytoplasm were ribosome-coated or sparsely coated vesicles.

In glucagon-treated animals at both 4 and 6 hours, hyaloplasmic depletion of glycogen was advanced and in many cells complete (Fig 4). Lysosomes and autophagic vacuoles were numerous and again often were related closely to Golgi apparatus (Fig 5). In those cells containing residual hyaloplasmic glycogen, the lysosomes and autophagic vacuoles occurred at the margins of the stores of glycogen, as noted in the control animals. The autophagic vacuoles were predominantly depleted of glycogen; in this respect they differed from those of control animals. The cisternae of the rough endoplasmic reticulum were more elongated than those in normal cells and numerous focal areas of dilatation were observed along their course. The membranes in these dilatations were usually sparsely coated or completely devoid of ribosomes (Fig 4).

Morphometric analysis (Tables 7 and 8) reveals a significant reduction in the volume of glycogen in the glucagon-treated animals. The volume of lysosomes increased in the glucagon-treated animals at the

Table 7. Effects, 4 Hours after Birth, on Hepatic Cells of Administering Glucagon to Newborn Rats

	Percent of cytoplasmic volume*			
Treatment	Glycogen	Mitochondria	Peroxisomes	Lysosomes
Control†	15.0 ± 2.2	12.9 ± 0.5	0.67 ± 0.05	0.56 ± 0.09
Glucagon‡	9.0 ± 1.4	13.1 ± 1.4	0.82 ± 0.07	0.86 ± 0.12
P	<0.05	>0.5	<0.1	<0.05

* Results are means ± standard errors.
† Results computed from a total of 30 micrographs and an area of 4900 sq μ.
‡ Results computed from a total of 40 micrographs and an area of 7600 sq μ.

age of 4 hours (Table 7). It is estimated that at least 85% of the lysosomal volume in both control and treated animals (at 4 and 6 hours) is occupied by lysosomes of the autophagic vacuole type. A smaller fraction of the lysosomal volume is occupied by glycogen in the glucagon-treated animals as compared with the controls (last column of Table 8). No statistically significant change of the other organelles was noted (Table 7).

Group II: Adrenalin Administered

The control animals at the age of 6 hours were observed to have the same fine structural features described for the control animals of Group I.

Treating the animals with adrenalin resulted in accelerated depletion of glycogen. The autophagic vacuoles were predominantly depleted of glycogen. Other changes in organelles were not conspicuous. The results of morphometric analysis are shown in Table 9.

Table 8. Effects, 6 Hours after Birth, on Hepatic Cells of Administering Glucagon to Newborn Rats

	Percent of cytoplasmic volume*			
Treatment	Glycogen	Lysosomes	Glycogen in autophagic vacuoles	Fraction of glycogen in lysosomes†
Control‡	12.0 ± 2.7	1.37 ± 0.24	0.23 ± 0.05	0.17
Glucagon§	5.2 ± 1.2	1.30 ± 0.12	0.12 ± 0.03	0.09
P	<0.02	>0.5	<0.05	

* Results are means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 30 micrographs and an area of 5400 sq μ.
§ Results computed from a total of 30 micrographs and an area of 5150 sq μ.

Table 9. Effects, 6 Hours after Birth, on Hepatic Cells of Administering Adrenalin to Newborn Rats

	Percent of cytoplasmic volume*			
Treatment	Glycogen	Lysosomes	Glycogen in autophagic vaculoes	Fraction of glycogen in lysosomes†
Control‡	13.0 ± 2.0	1.40 ± 0.50	0.24 ± 0.10	0.17
Adrenalin§	7.0 ± 3.0	1.20 ± 0.50	0.11 ± 0.05	0.09

* Results are means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 18 micrographs and an area of 2900 sq μ.
§ Results computed from a total of 18 micrographs and an area of 2600 sq μ.

Group II: Puromycin Administered

Control animals were observed to have the same fine structural features as the control animals of the other groups. Advanced hyaloplasmic depletion of glycogen was observed by 6 hours in animals treated with puromycin.

Lysosomes and autophagic vacuoles were small. The latter sometimes contained small amounts of glycogen. The results of the morphometric analysis are shown in Table 10. In the puromycin-treated animals, the volume of hyaloplasmic glycogen was reduced. Lysosomes were reduced markedly in their volume. It is estimated that at least 85% of the volume of these organelles in both control and treated animals was occupied by lysosomes of the autophagic vacuole type. No statistically significant changes were noted in the volumes of mitochondria and peroxisomes.

Table 10. Effects, 6 Hours after Birth, on Hepatic Cells after Administering Puromycin to Newborn Rats

	Percent of cytoplasmic volume*				
Treatment	Glycogen	Mitochondria	Peroxisomes	Lysosomes	Glycogen in autophagic vacuoles†
Control‡	11.3 ± 2.1	18.5 ± 0.8	0.68 ± 0.05	1.65 ± 0.20	0.29 ± 0.06
Puromycin§	3.4 ± 0.7	16.5 ± 0.7	0.67 ± 0.03	0.39 ± 0.05	<0.07
P	<0.01	<0.1	>0.5	<0.01	<0.01

* Results refer to means ± standard errors.
† Volume of lysosomes (including autophagic vacuoles) occupied by glycogen.
‡ Results computed from a total of 38 micrographs and an area of 6100 sq μ.
§ Results computed from a total of 52 micrographs and an area of 8500 sq μ.

Discussion

From the biochemical data presented in this study, it is apparent that the rate of the postnatal mobilization of glycogen in rat hepatocytes can be accelerated by the use of various agents including the two hormones, glucagon and adrenalin, which have been considered the main physiologic stimuli for postnatal hepatic glycogenolysis. The glycogenolytic and hyperglycemic effects of these hormones observed in our experiments can be explained on the basis of an activation of the enzyme phosphorylase.[19,45,46] The hyperglycemia observed after cyclic AMP is administered may also be explained on the same basis.[47,48]

The fact that the activity of the enzyme glucose-6-phosphatase remains unchanged despite a significantly accelerated breakdown of glycogen by glucagon supports conclusions by others [19,45,46] that postnatal mobilization of glycogen is not directly related to an increase in activity of this enzyme.

The changes in the amount of liver glycogen, as determined chemically, are reflected in changes in the volume of the glycogen in the hepatocytes, as observed in the electron microscope. Accelerated mobilization of liver glycogen results in a decrease in the volumes of both hyaloplasmic and lysosomal glycogen. This positive correlation between the volumes of hyaloplasmic glycogen and lysosomal glycogen is evident from the morphologic study and is confirmed by the morphometric analysis. (The fraction of lysosomal volume occupied by glycogen is shown in the last column of Tables 8 and 9).

It is also noted that during the postnatal period studied, formation of autophagic vacuoles was not random but occurred predominantly at the margins of the areas of glycogen.

In addition to these changes in the lysosomal glycogen, changes in the volume of lysosomes *per se* are noted. Thus, glucagon, which accelerates breakdown of glycogen, is shown to increase the lysosomal volume in these cells. Since the lysosomes observed are predominantly of the autophagic vacuole type, this increase undoubtedly reflects the induction of cellular autophagy, a well-known effect of the hormone.[43,49,50] These findings suggest that catabolism of lysosomal glycogen is accelerated under these experimental conditions.

The fact that the volume of the lysosomal glycogen is relatively small compared with the volume of the hyaloplasmic glycogen does not necessarily indicate that the lysosomal breakdown of glycogen is of small physiologic importance. Indeed, the relative importance of the two kinds of breakdown (lysosomal as opposed to hyaloplasmic) should depend mainly on the relative velocities of the two processes.

Quantitation of lysosomal glucosidase is required in order to assess the degree of involvement of lysosomes in this process; this is presently under study and will be the subject of a subsequent report.

At the present time, the mechanisms by which the lysosomal pathway is controlled are obscure.[21] However, it is of interest to note that administering adrenalin to rabbits for several days results in increased activity of lysosomal acid glucosidase.[51]

The observation that formation of autophagic vacuoles in these cells is not random but occurs near areas of glycogen and the findings that the volume of lysosomes and autophagic vacuoles is increased and the glycogen content of the latter particles decreased by those agents that enhance the breakdown of hyaloplasmic glycogen, lead us to the conclusion that formation of autophagic vacuoles in these cells is related to the breakdown of glycogen. Further, on the basis of this data, we formulate the hypothesis that those agents—*ie*, glucagon, that regulate hyaloplasmic breakdown of glycogen (phosphorylation) also influence lysosomal breakdown of glycogen (hydrolysis) in these hepatocytes.

In a companion paper,[22] it is shown that measures that inhibit hyaloplasmic breakdown of glycogen also inhibit lysosomal breakdown of glycogen. Further, Ho *et al*[52] have reported that inhibition of the hepatic breakdown of glycogen, induced by administering glucose, results in inhibition of the formation of lysosomes in these cells. These above findings further support our hypothesis.

Under certain pathologic conditions, in particular Pompe's disease, a different situation exists since it has been shown that administering hormones depletes hyaloplasmic glycogen but not lysosomal glycogen.[17] However, this disease is a special instance, since lysosomal acid glucosidase is absent.[15,16]

Rosenfeld[51] suggested that the hydrolytic (lysosomal) pathway may be important in cases where there is a demand for the rapid liberation of free glucose. It may be that the physiologic participation of lysosomes in the breakdown of glycogen is a reflection of this requirement.

The experiment with puromycin provides further insight into the regulation of lysosomal formation. This agent exerts a glycogenolytic effect on liver through activation of phosphorylase.[53–55] Lysosomal development is inhibited by puromycin. Most of the lysosomes in both control and treated animals belong to the autophagic type. It has been shown by previous investigators that formation of autophagic vacuoles does not depend on protein synthesis but on energy.[43] Accordingly, the inhibition of lysosomal formation by puromycin may be explained on the basis that the antibiotic interferes with the energy metabolism of these cells. A possible interference by puromycin with the metabolism

of adenine and its derivatives has been suggested by Hofert and Boutwell.[55]

It is of interest that the changes in lysosomal volume are not associated with changes in the total acid phosphatase activity, under the conditions of these experiments. Such a phenomenon has been observed previously by Arstila and Trump in glucagon-treated animals.[43] According to these investigators, the hydrolases within autophagic vacuoles were provided by Golgi-derived vesicles—primary lysosomes—which fused with the newly formed autophagic vacuoles. No synthesis of lysosomal hydrolases seemed to take place during the induction of cellular autophagy, under the experimental conditions studied. Our data also supports this view. Moreover, the occurrence of autophagic vacuoles at the edge of the glycogen areas and near the Golgi complexes would facilitate the transfer of the Golgi vesicles to vacuoles. This particular disposition of the autophagic vacuoles may also be related to the formation of these structures from the endoplasmic reticulum in the Golgi zone.[20]

The closely meshed network of tubular smooth endoplasmic reticulum, which is prominent in adult animals and has been implicated by others in the breakdown of glycogen [2-6] or release of glucose,[8,12] is absent in postnatal rat hepatocytes and shows no change after the administration of various agents that produce breakdown of glycogen and hyperglycemia in this study. Accordingly, it is concluded that this organelle plays no role in the mobilization of glycogen or release of glucose in these animals.

The ribosome-coated and sparsely coated vesicles of the rough endoplasmic reticulum that were implicated by previous investigators in the metabolism of glycogen [10,44] did not change markedly during the mobilization of glycogen by various glycogenolytic agents under the conditions of these experiments. The other organelles studied, such as mitochondria and peroxisomes, did not change after the acceleration of the breakdown of glycogen in postnatal rat hepatocytes.

Summary

A study was made of relationships between glycogen and organelles in postnatal rat hepatocytes during the phase of rapid mobilization of glycogen and under experimental conditions in which breakdown of glycogen was accelerated. The electron microscopic findings were quantitated by morphometric analysis. Biochemical estimations of hepatic glycogen, glucose-6-phosphatase, total acid phosphatase and blood glucose were performed also.

Lysosomes appeared to play a definite role in the breakdown of

glycogen in these cells. Formation of autophagic vacuoles was not random but occurred particularly at the margins of the glycogen areas. A positive correlation between lysosomal glycogen and hyaloplasmic glycogen was noted and confirmed by morphometric quantitation. The hypothesis was formulated that those factors—*ie*, glucagon, that regulate hyaloplasmic glycogen breakdown, also influence the catabolism of lysosomal glycogen.

Puromycin, which mobilized the hyaloplasmic glycogen, inhibited the development of lysosomes. The significance of the latter findings is discussed.

The smooth endoplasmic reticulum played no role in the breakdown of glycogen or release of glucose in these cells.

Glucose-6-phosphatase was not a key enzyme in these cells for mobilizing glycogen after glucagon and puromycin were administered.

No changes in the mitochondrial and peroxisomal volumes were observed during the acceleration of the breakdown of glycogen after glucagon and puromycin were administered.

References

1. Porter KR, Bruni C: An electron microscope study of the early effects of 3-Me-DAB on rat liver cells. Cancer Res 19:997–1009, 1959
2. Millonig G, Porter KR: Structural elements of rat liver cells involved in glycogen metabolism. Proceedings of European Regional Conference on Electron Microscopy, Delft, Holland, 1960, Vol 2, p 655
3. Porter KR: The ground substance: observations from electron microscopy, The Cell. Vol 2. Edited by J Brachet, AE Mirsky. New York, Academic Press, Inc, 1961, p 621
4. Peters VB, Dembitzer HM, Kelly GW, Baruch E: Ergastoplasmic changes associated with glycogenolysis. Proceedings of the Fifth International Congress on Electron Microscopy, Philadelphia, 1962, Vol 2, pp TT-7
5. Peters VB, Kelly GW, Dembitzer HM: Cytologic changes in foetal and neonatal hepatic cells of the mouse. Ann NY Acad Sci 111:87–103, 1963
6. Jones AL, Fawcet DW: Hypertrophy of the agranular endoplasmic reticulum in hamster liver induced by phenobarbital (with a review of the functions of this organelle in liver). J Histochem Cytochem 14:215–232, 1966
7. Steiner JW, Baglio CM: Electron microscopy of the cytoplasm of parenchymal liver cells in alpha-naphthyl isothiocyanate-induced cirrhosis. Lab Invest 12:765–790, 1963
8. Jézéquel A, Arakawa K, Steiner JW: The fine structure of the normal, neonatal mouse liver. Lab Invest 14:1894–1930, 1965
9. De Man JCH, Block APR: Relationship between glycogen and agranular endoplasmic reticulum in rat hepatic cells. J Histochem Cytochem 14:135–146, 1966
10. Phillips MJ, Unakar NJ, Doornewaard G, Steiner JW: Glycogen depletion in the newborn rat liver: an electron microscopic and electron histochemical study. J Ultrastruct Res 18:142–165, 1967

11. Revel JP, Napolitano L, Fawcett DW: Identification of glycogen in electron micrographs of thin tissue sections. J Biophys Biochem Cytol 8:575–589, 1960
12. Biava C: Identification and structural forms of human particulate glycogen. Lab Invest 12:1179–1197, 1963
13. Nemeth AM: Glucose-6-phosphatase in the liver of the foetal guinea pig. J Biol Chem 208:773–776, 1954
14. Rosen SI, Kelly GW, Peters VB: Glucose-6-phosphatase in tubular endoplasmic reticulum of hepatocytes. Science 152:352–354, 1966
15. Hers HG: Alpha-glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). Biochem J 86:11–16, 1963
16. Baudhuin P, Hers HG, Loeb H: An electron microscopic and biochemical study of type II glycogenosis. Lab Invest 13:1139–1152, 1964
17. Hug G, Garancis JC, Schubert WK, Kaplan S: Glycogen storage disease, Types II, III, VIII and IX: a biochemical and electron microscopic analysis. Amer J Dis Child 111:457–474, 1966
18. Shelley HJ: Glycogen reserves and their changes at birth and in anoxia. Brit Med Bull 17:137–143, 1961
19. Dawkins MJ: Glycogen synthesis and breakdown in fetal and newborn rat liver. Ann NY Acad Sci 111:203–211, 1963
20. Novikoff AB, Shin WY: The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. J Microscopie 3:187–206, 1964
21. Smith EE, Taylor PM, Whelan WJ: Carbohydrate metabolism and its disorders. Vol 1. Edited by F Dickens, PJ Randle, WJ Whelan. New York, Academic Press, Inc, 1968
22. Kotoulas OB, Ho J, Adachi F, Weigensberg B, Phillips MJ: Fine structural aspects of the mobilization of hepatic glycogen. II. Inhibition of glycogen breakdown. Amer J Path 63:23–34, 1971
23. Kotoulas OB, Phillips MJ: Observations on glycogen-containing cytolysomes in postnatal rat hepatocytes. Amer J Path 59:85a, 1970, abstr
24. Carroll NV, Longley RW, Roe JH: The determination of glycogen in liver and muscle by use of anthrone reagent. J Biol Chem 220:583–593, 1956
25. Lowry OH, Rosebrough NJ, Farr AL, Randall, RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951
26. Swanson MA: Glucose-6-phosphatase from liver, Methods in Enzymology. Vol 2. New York, Academic Press, Inc, 1955, p 541
27. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F: Tissue fractionation studies. Biochem J 60:604–617, 1955
28. Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. J Biol Chem 66:375–400, 1925
29. Raabo E, Terkildsen TC: On the enzymatic determination of blood glucose. Scand J Clin Lab Invest 12:402–407, 1960
30. Somogyi M: Determinations of blood sugar. J Biol Chem 160:69–73, 1945
31. Mollenhauer HH: Plastic embedding mixtures for use in electron microscopy. Stain Techn 39:111–114, 1964
32. Trump BF, Smuckler EA, Benditt EP: A method for staining epoxy sections for light microscopy. J Ultrastruct Res 5:343–348, 1961
33. Glauert AM: Techniques for Electron Microscopy. Second edition. Edited by DH Kay. Oxford, Blackwell Scientific Publications, 1965, p 254

34. Pease DC: Histological techniques for electron microscopy. New York, Academic Press, Inc, 1964, p 216
35. Weibel ER: Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 26:235–302, 1969
36. Weibel ER, Kistler GS, Scherle, WF: Practical stereological methods for morphometric cytology. J Cell Biol 30:23–38, 1966
37. Loud AV: A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. J Cell Biol 37:27–46, 1968
38. Wiener J, Loud AV, Kimberg DV, Spiro D: A quantitative description of cortisone-induced alterations in the ultrastructure of rat liver parenchyma cells. J Cell Biol 37:47–61, 1968
39. Hill AB: Principles of Medical Statistics. Eighth edition. Oxford, Oxford University Press, 1966
40. Deter RL, De Duve C: Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. J Cell Biol 33:437–449, 1967
41. Dallner G, Siekevitz P, Palade GE: Biogenesis of endoplasmic reticulum membrances. II. Synthesis of constitutive microsomal enzymes in developing rat hepatocyte. J Cell Biol 30:97–117, 1966
42. De Duve C, Wattiaux R: Functions of lysosomes. Ann Rev Physiol 28:435–492, 1966
43. Arstila AU, Trump BF: Studies on cellular autophagocytosis. Amer J Path 53:687–733, 1968
44. Coimbra A, Leblond CP: Sites of glycogen synthesis in rat liver cells as shown by electron microscope radioautography after administration of glucose-H^3. J Cell Biol 30:151–175, 1966
45. Dawkins MJ: Biochemical aspects of developing function in newborn mammalian liver. Brit Med Bull 22:27–33, 1966
46. Dawes GS, Shelley HJ: Carbohydrate metabolism and its disorders. Vol 2. Edited by F Dickens, PJ Randle, WJ Whelan. New York, Academic Press, Inc, 1968, p 87
47. Sutherland EW, Rall TW: The relation of adenosine-3′,5′-phosphate and phosphorylase to the actions of catecholamines and other hormones. Pharmacol Rev 12:265–299, 1960
48. Henion WF, Sutherland EW, Posternak T: Effects of derivatives of adenosine 3′,5′ phosphate on liver slices and intact animals. Biochem Biophys Acta 148:106–113, 1967
49. Ashford TP, Porter KR: Cytoplasmic components in hepatic cell lysosomes. J Cell Biol 12:198–202, 1962
50. Deter RL, Baudhuin P, De Duve C: Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. J Cell Biol 35:C11–C16, 1967
51. Rosenfeld EL: Control of Glycogen Metabolism: Ciba Foundation Symposium. Edited by WJ Whelan, MP Cameron, London, J & A Churchill Ltd, 1964, p 176
52. Ho J, Adachi F, Kotoulas O, Weigensberg B, Phillips MJ: Ultrastructural observations on hepatic glycogen in newborn rats given parenteral glucose. Fed Proc 28:365, 1969
53. Hofert JF, Boutwell RK: Effect of pyromycin on hepatic glycogen phosphorylase activity. Proc Soc Exp Biol Med 121:532–536, 1966

54. Appleman MM, Kemp RG: Puromycin: a potent metabolic effect independent of protein synthesis. Biochem Biophys Res Comm 24:564–568, 1966
55. Hofert JF, Boutwell RK: Puromycin-induced glycogenolysis as an event independent from inhibited protein synthesis in mouse liver: effects of puromycin analogs. Arch Biochem 103:338–344, 1963

The authors are grateful to Dr. B. I. Weigensberg for valuable advice and help throughout the project and for critical review of parts of this work. Appreciation is expressed to Miss Kathleen Tasnadi, Mr. Gerald Doornewaard and Mme. Raymond LeMorvan for excellent technical assistance. The crystalline glucagon was kindly donated by Dr. J. M. McGuire, Eli Lilly and Co., Indianapolis, Indiana.

[*Illustrations follow*]

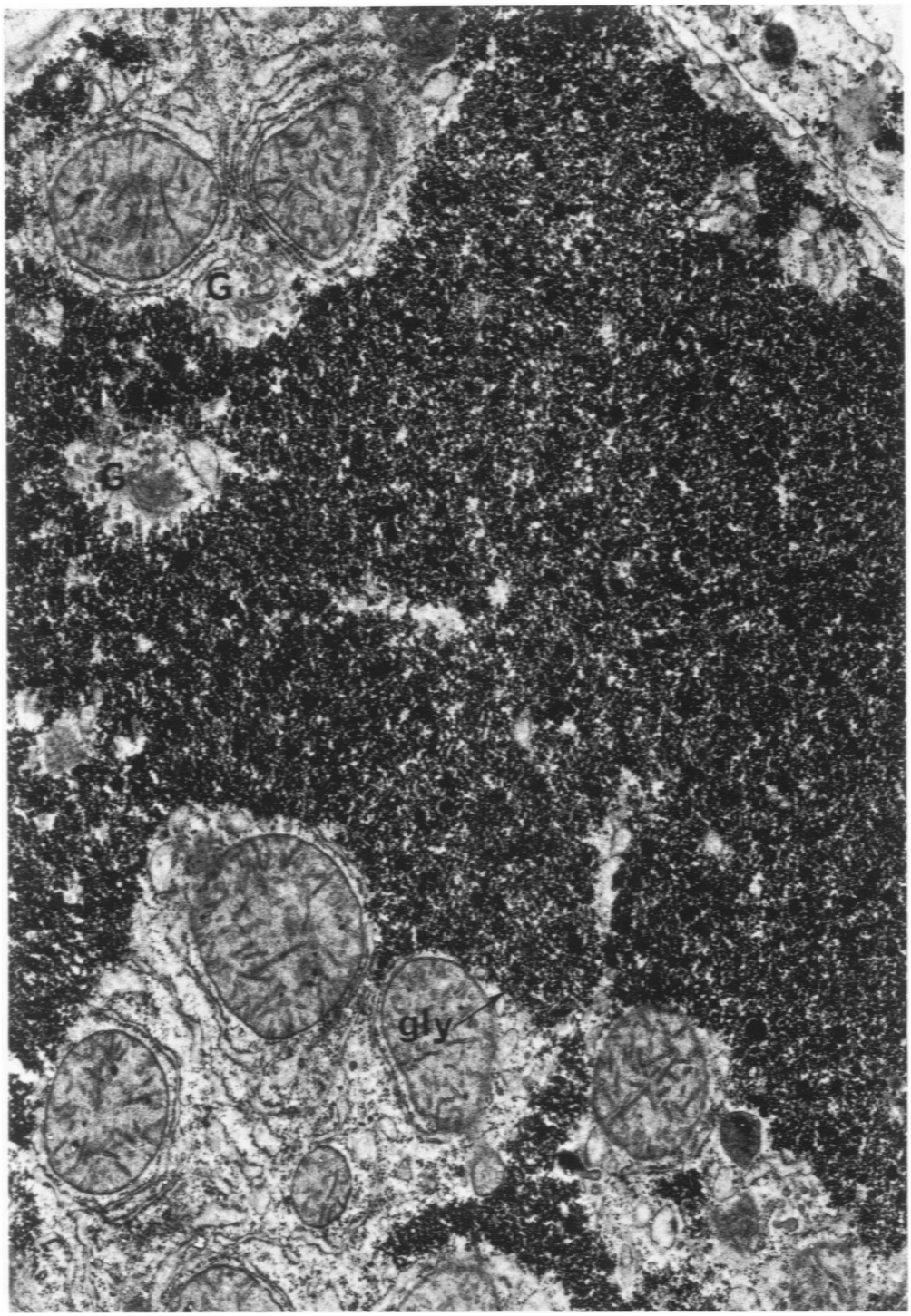
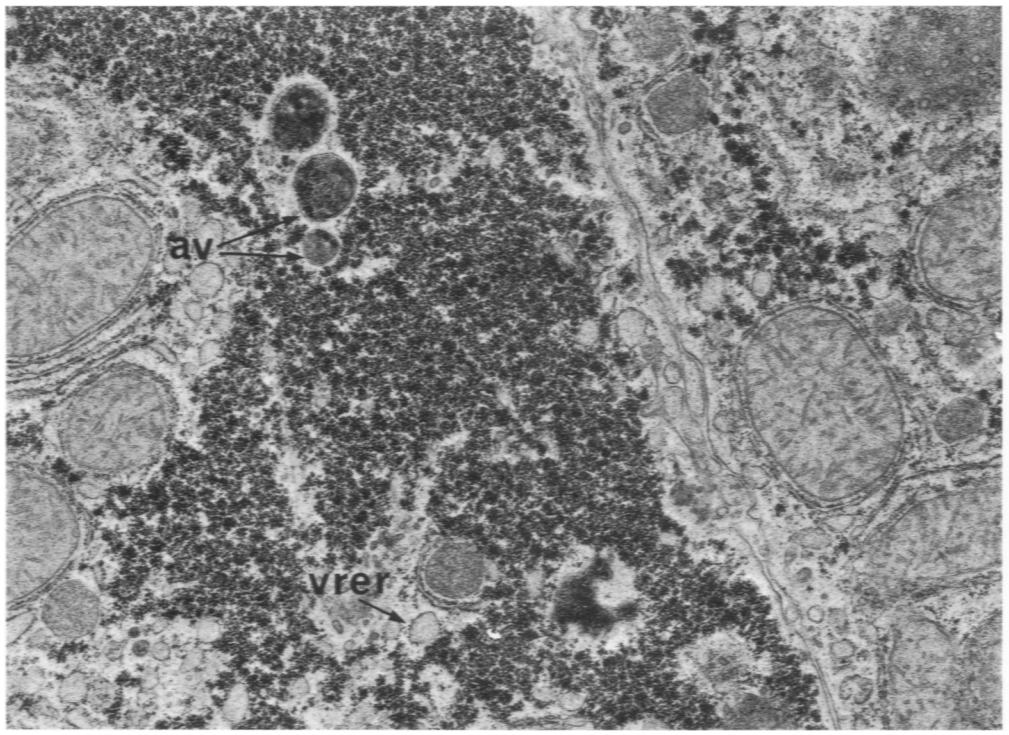


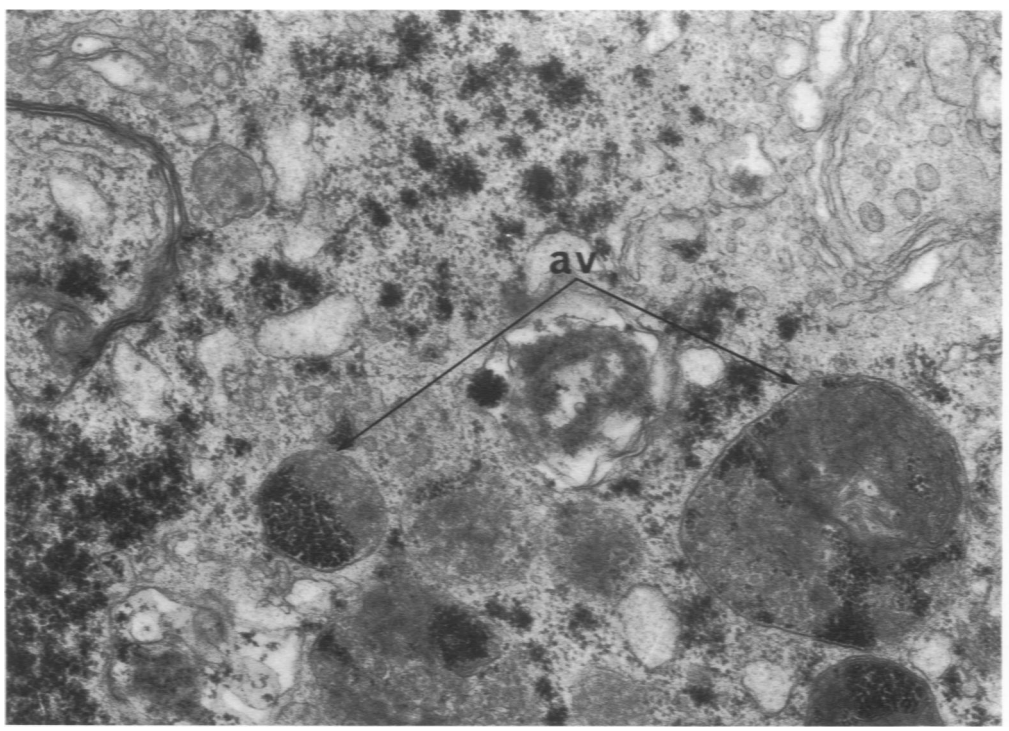
Fig 1.—Portion of normal rat hepatocyte at birth. Note large glycogen area (*gly*) and exclusion of other organelles to glycogen-free parts of cytoplasm. Small Golgi zones (*G*) can be seen at margin of glycogen area. Endoplasmic reticulum is mainly in form of rough-surfaced cisternae (uranyl acetate–Reynold's lead citrate, × 21,600).

Fig 2.—Portions of two hepatocytes of normal rat at 4 hours. Stores of glycogen are partly mobilized in cell on right. Note presence of autophagic vacuoles (*av*) containing particles of glycogen. Vacuoles are located at margin of glycogen area. Several vesicles of rough endoplasmic reticulum are present (*vrer*) (uranyl acetate–Reynold's lead citrate, × 13,000).

Fig 3.—Portion of normal rat hepatocyte at 6 hours. Note presence of autophagic vacuoles (*av*) containing discrete particles of glycogen. Golgi zone can be seen near autophagic vacuoles (uranyl acetate–Reynold's lead citrate, × 31,500).

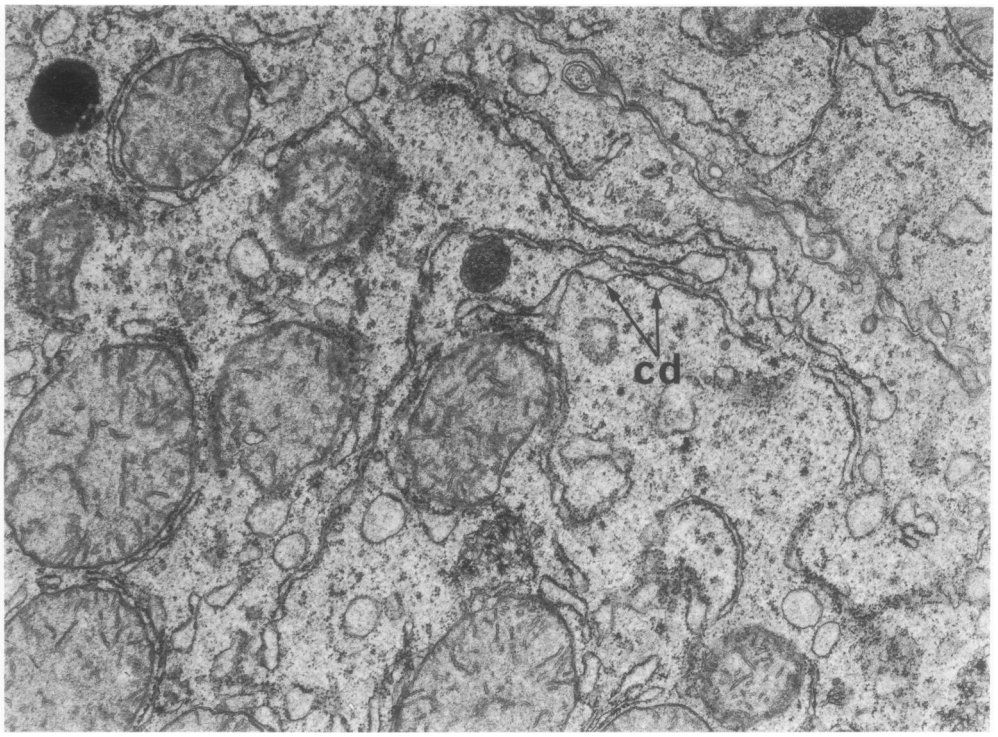


2



3

4



5

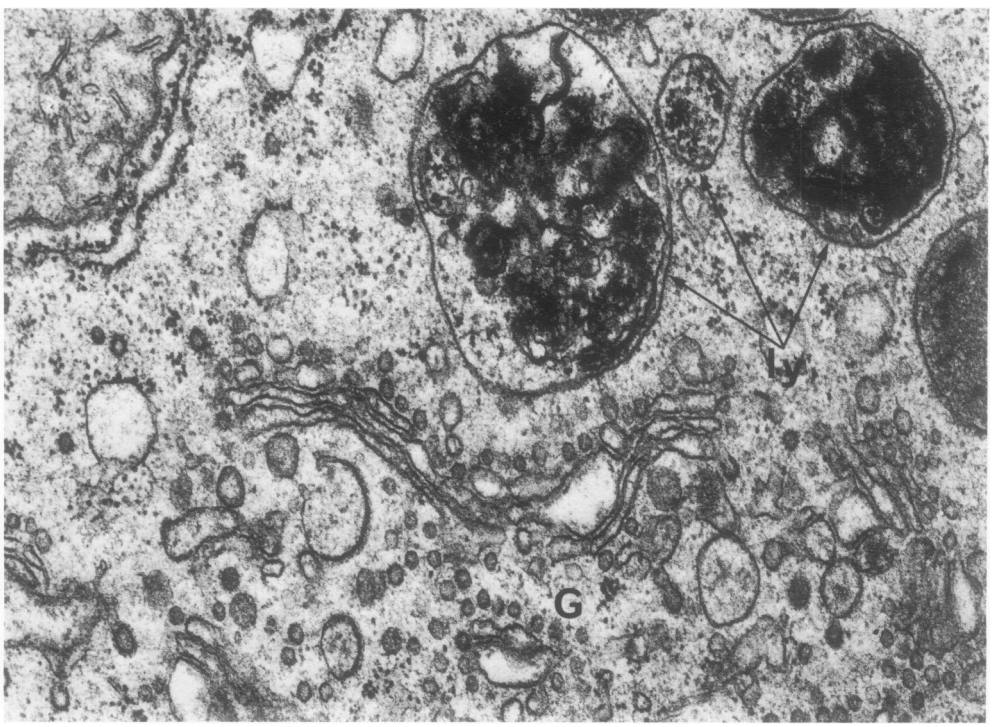


Fig 4.—Portions of two hepatocytes of glucagon-treated rat at 6 hours. Cisternae of rough endoplasmic reticulum are elongated and show, focally, areas of dilatation (*cd*). Glycogen is absent (uranyl acetate–Reynold's lead citrate, × 15,000).

Fig 5.—Portion of hepatocyte of glucagon-treated rat at 6 hours. Large Golgi zone (*G*) is seen at lower half of micrograph. Nearby are several lysosomes (*ly*). Glycogen is absent (uranyl acetate–Reynold's lead citrate, × 24,000).