

# AUTOPHAGIC DEGRADATION OF GLYCOGEN IN SKELETAL MUSCLES OF THE NEWBORN RAT

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## ABSTRACT

Large amounts of glycogen accumulate in rat skeletal muscle fibers during the late fetal stages and are mobilized in the first postnatal days. This glycogen depletion is relatively slow in the immature leg muscles, in which extensive deposits are still found 24 hr after birth and, to some extent, persist until the 3rd day. In the more differentiated psoas muscle and especially in the diaphragm, the glycogen stores are completely mobilized already during the early hours. Section of the sciatic nerve 3 days before birth or within the first 2 hr after delivery does not affect glycogen depletion in the leg muscles. Neonatal glycogenolysis in rat muscle fibers takes place largely by segregation and digestion of glycogen particles in autophagic vacuoles. These vacuoles: (a) are not seen in fetal muscle fibers or at later postnatal stages, but appear concomitantly with the process of glycogen depletion and disappear shortly afterwards; (b) are prematurely formed in skeletal muscles of fetuses at term treated with glucagon; (c) contain almost exclusively glycogen particles and no other recognizable cell constituents; (d) have a double or, more often, single limiting membrane and originate apparently from flattened sacs sequestering glycogen masses; (e) are generally found to contain reaction product in preparations incubated from demonstration of acid phosphatase activity. The findings emphasize the role of the lysosomal system in the physiological process of postnatal glycogen mobilization and appear relevant in the interpretation of type II glycogen storage disease.

## INTRODUCTION

Glycogen accumulates in liver and skeletal muscle in different mammalian species during the late fetal stages and is rapidly mobilized at birth (3). The glycogen reserves are thought to represent an important source of energy for the newborn mammal in the first crucial period of extra-uterine life.

The mechanisms responsible for perinatal glycogen changes in the liver have been the object of several investigations. Biochemical studies have been mainly concerned with the identification of hormonal factors regulating fetal and neonatal glycogen metabolism and especially with the

enzymic mechanisms directly implicated in glycogenolysis: this process has been generally associated with an activation of liver phosphorylase, induced by glucagon or, possibly also, by catecholamines through the mediation of cyclic AMP (see 3, 4, 10, 34, 35). On the other hand, morphological observations on the appearance of autophagic vacuoles loaded with glycogen in the newborn mouse and rat liver suggested that the lysosomal system takes part in glycogen breakdown at birth (15, 27).

Perinatal glycogen changes in skeletal muscle have received less attention. The rate of glycogen

depletion in muscle after birth has been reported to be slower than in liver (see 3), but most data refer to whole muscle groups without taking into account separate muscles with different functions and degrees of maturity. In a morphological study, Heuson-Stiennon and Drochmans (12) described the presence of glycogen-containing vacuoles and of "glycogen bodies" in the newborn rat muscle, but the relative importance of these two types of structures and their role in glycogen mobilization was not ascertained.

We have reinvestigated perinatal glycogen changes, and especially the process of glycogen depletion, in rat skeletal muscles, making use of cytochemical techniques for the selective staining of glycogen and for the demonstration of acid phosphatase activity. Our observations were performed at precisely timed pre- and postnatal stages on skeletal muscles which differ in function and degree of maturity at birth. The effects of denervation and of glucagon administration on perinatal glycogen changes were also investigated.

#### MATERIALS AND METHODS

Wistar rats at late fetal and early postnatal stages of development were used. Fetal age was determined by rump-to-crown length (33). Some litters were delivered by Cesarean section and kept for varying periods, at 35°–37°C or at room temperature, separated from their mothers. Section of the sciatic nerve was performed in fetuses 3 days before birth or in newborn rats within the first 2 hr after delivery as previously described (28, 31). Fetuses at term (day 21 or 22 of gestation) were treated *in utero* with glucagon essentially as described by Greengard and Dewey

(9), but the injections were given subcutaneously in order to avoid possible inactivation of the hormone in the liver following intraperitoneal injection. Fetuses within the same mother received either glucagon (0.05 mg in 0.1 ml of saline) or saline, and were left *in situ* for 3–6 hr.

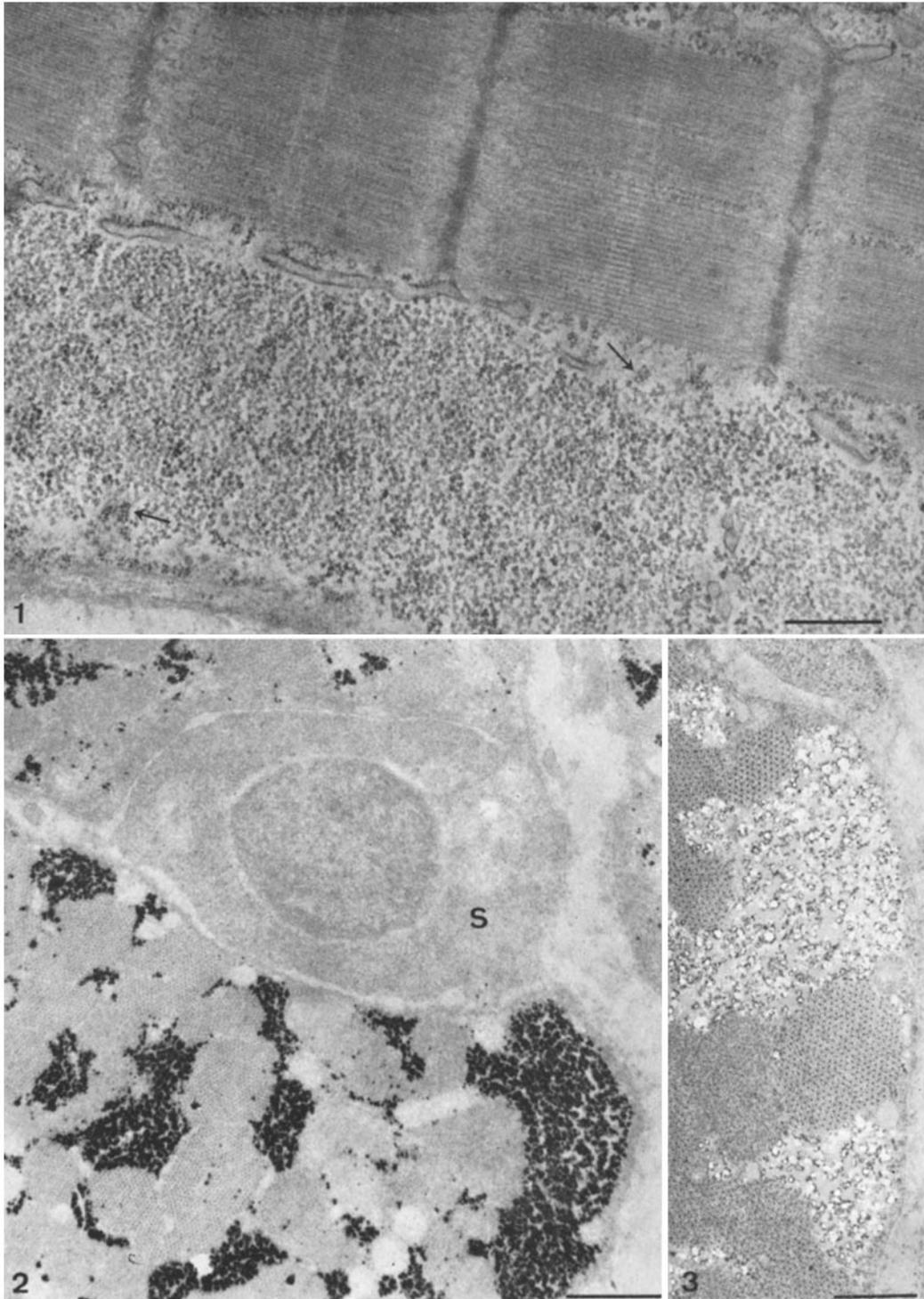
The diaphragm, psoas, gastrocnemius, soleus, and extensor digitorum longus muscles, generally from the same animal or from animals of the same litter, were studied. The muscles were fixed *in situ* in 2.5 or 4% glutaraldehyde buffered with 0.1 M sodium cacodylate. During subsequent washing in buffer containing 0.2 M sucrose, thin bundles of fibers were dissected from the superficial layers of the muscles. Some of these pieces were then incubated for cytochemical demonstration of acid phosphatase activity according to the method of Gomori as specified by Miller and Palade (22); substrate-free medium was used as enzyme control. All specimens were postfixed in 1% osmium tetroxide in phosphate or cacodylate buffer, dehydrated in alcohol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate or with lead hydroxide (16). In addition, sections collected on gold or nickel grids were treated according to the periodic acid-thiosemicarbazide-osmium tetroxide (PATO) procedure devised by Seligman et al. (32) for the demonstration of polysaccharides. With the reagent concentration and the incubation times used (1% periodic acid for 10–15 min and 1% thiosemicarbazide for 30–45 min), only glycogen was stained by this method in our material. Enzymatic extraction of glycogen was also performed on sections collected on gold or nickel grids, following the procedure described by Monneron and Bernhard (23). After periodate oxidation (10% periodic acid for 20 min), the sections were incubated for 1–4 hr at 37°C in 0.5%  $\alpha$ -amylase in 0.05 M phosphate buffer,

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FIGURE 1 18-day old rat fetus; longitudinal section through a muscle fiber from the extensor digitorum longus muscle. The figure illustrates a large glycogen deposit in the subsarcolemmal space. Glycogen particles vary in size and intensity of staining, and are often clumped in small aggregates. Elongated profiles of sarcoplasmic reticulum, partially encrusted with ribosomes, extend between the glycogen area and adjacent myofibrils, where glycogen particles are characteristically absent. Free ribosomes and polyribosomes are in close proximity to and at some places intermingled with the glycogen particles (arrows). Lead hydroxide. Scale mark, 0.5  $\mu$ .  $\times$  28,000.

FIGURE 2 Soleus muscle, 24 hr after birth; transverse section. Abundant glycogen deposits, selectively stained with the PATO procedure, are present in the muscle fibers. PATO-positive granules are not seen in an undifferentiated satellite cell (S), which is filled with barely visible, free ribosomes. No counterstain. Scale mark, 0.5  $\mu$ .  $\times$  28,000.

FIGURE 3 Gastrocnemius muscle, 24 hr after birth; transverse section. The glycogen deposits have been largely extracted by  $\alpha$ -amylase, leaving empty spaces and holes of varying size. Treatment with 0.5%  $\alpha$ -amylase for 1 hr, followed by the PATO procedure; slight uranyl acetate and lead citrate counterstain. Scale mark, 0.5  $\mu$ .  $\times$  24,000.



pH 6.9, and subsequently processed by the PATO method. Specimens were examined in a Siemens Elmiskop 1 A electron microscope.

## RESULTS

### *Changes in Quantity and Distribution of Muscle Glycogen during the Perinatal Period*

Large glycogen deposits in muscle cells were observed from the 18th day of the gestation period in all investigated rat muscles (Fig. 1). Glycogen particles were selectively stained by the PATO method (Fig. 2), specificity of which was confirmed by amyolytic digestion (Fig. 3). The PATO method permitted a clear-cut distinction between glycogen particles and ribosomes, which are abundant in developing muscle fibers, and thus made possible a ready evaluation of glycogen content and distribution. Glycogen particles in fetal muscle fibers were 150-300 Å in diameter, cor-

responding to the  $\beta$  particles of Drochmans (6), but were often clumped together in larger aggregates of variable complexity (see also reference 12). Glycogen deposits were especially conspicuous in the central, paranuclear areas of myotubes and in subsarcolemmal spaces of both myotubes and fibers. As shown in Fig. 1, the glycogen masses had initially no relation to the myofibrils, but at later stages rows of glycogen particles assumed the characteristic intermyofibrillar disposition: Fig. 2 represents an intermediate stage in this transition. Glycogen accumulated also in satellite cells displaying differentiated features (presence of myofilaments), but was almost absent at any age from undifferentiated satellite cells as well as from interstitial cells (Fig. 2).

The size of the glycogen deposits further increased until the end of the gestation period. In the leg muscles, such as the gastrocnemius and the soleus, huge glycogen masses, not infrequently

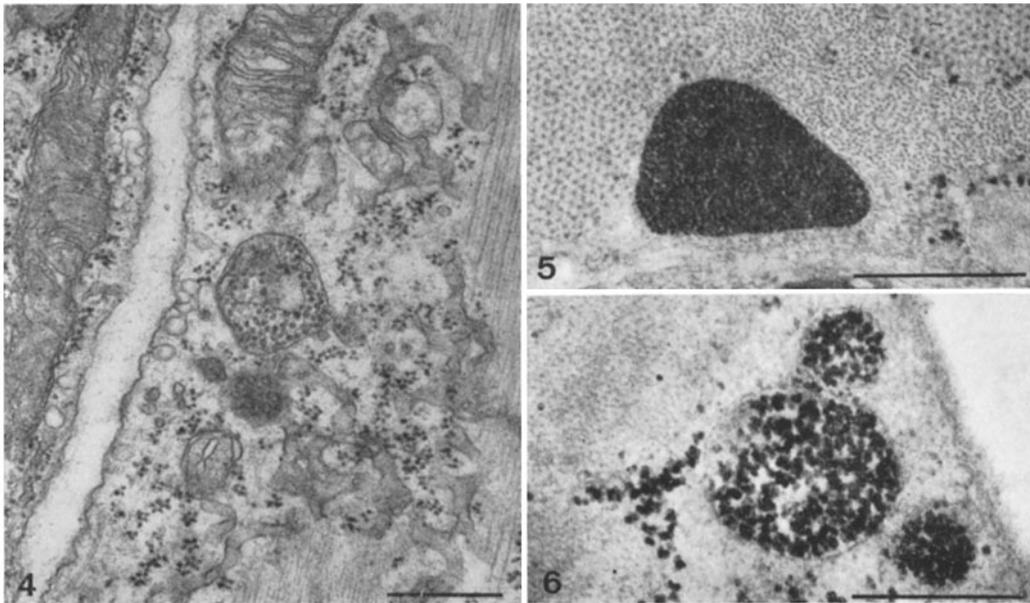
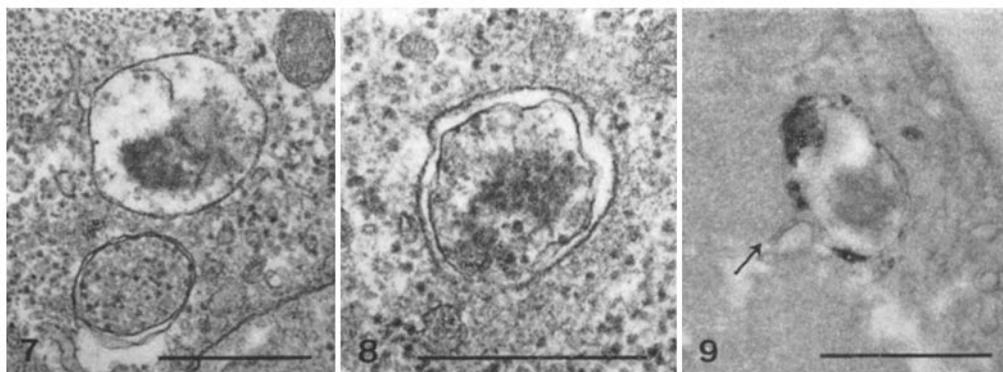


FIGURE 4 Psoas muscle, about 24 hr after birth; longitudinal section. The figure shows a vacuole bounded by a single membrane and containing, predominantly, glycogen particles. The granules dispersed in the surrounding cytoplasm are almost exclusively ribosomes. Glycogen deposits are no longer seen in psoas muscle fibers at this time. Lead citrate. Scale mark,  $0.5 \mu$ .  $\times 30,000$ .

FIGURE 5 Same material as in Fig. 4; transverse section. The figure illustrates a large vacuole completely filled with PATO-positive granules. PATO staining with uranyl acetate counterstain. Scale mark,  $0.5 \mu$ .  $\times 45,000$ .

FIGURE 6 Rat diaphragm, 3 hr after birth. A cluster of glycogen vacuoles is seen in the subsarcolemmal space of a cross-sectioned muscle fiber. PATO staining without counterstain. Scale mark,  $0.5 \mu$ .  $\times 45,000$ .



FIGURES 7 and 8 Rat diaphragm, 3 hr after birth. The figures show different glycogen-containing vacuoles bounded by a double or single membrane. The membranes of the double-walled vacuole in Fig. 7 appear to fuse, at sites, into a single, thick layer. Lead hydroxide. Scale marks,  $0.5 \mu$ . Fig. 7,  $\times 40,000$ ; Fig. 8,  $\times 60,000$ .

FIGURES 9–12 Rat diaphragm, 3 hr after birth, incubated for demonstration of acid phosphatase activity. FIGURE 9 Most of the lead phosphate precipitate is confined to the space between the two limiting membranes of a vacuole. Glycogen is not seen in this unstained section, but most probably it corresponds to the white translucent areas within the vacuole and outside in the cytoplasm. Reaction product is also present in a small vesicle close to the vacuole. Note, by contrast, the absence of precipitate in a junctional cisterna of the sarcoplasmic reticulum in diadic contact with an enlarged T tubule (arrow). Scale mark,  $0.5 \mu$ .  $\times 45,000$ .

occupying more than 40% of the cross-sectional area of the fiber, were still found 24 hr after birth (Figs. 2 and 3). In these muscles, glycogen depletion occurred gradually in the first 3–5 days postpartum. However, in the psoas muscle, which at birth is more differentiated<sup>1</sup> than the leg muscles, postnatal glycogen mobilization proceeds more rapidly. Large glycogen deposits were present in the majority of the psoas fibers 1 hr after delivery, but were considerably reduced in size by 12 hr and had totally disappeared by 24 hr after birth. In the even more differentiated muscle fibers of the diaphragm, glycogen stores were almost completely mobilized in the first 6 hr of life. At this time, the subsarcolemmal spaces formerly occupied by glycogen deposits were invaded by long cisternae encrusted with ribosomes, mitochondria, and abundant free ribosomes (Fig. 14).

<sup>1</sup>The following morphological criteria of muscle differentiation were especially considered: position of nuclei, frequency and structure of satellite cells (1, 17), and organization of the sarcotubular system (30). Fiber type differences as seen in adult muscles (29) appear later in postnatal development.

#### *Autophagic Processes Accompanying Glycogen Mobilization*

Concomitantly with the disappearance of the glycogen stores, numerous autophagic vacuoles containing glycogen particles were consistently encountered in muscle fibers of newborn animals (Figs. 4–14). Most vacuoles contained exclusively glycogen particles, which were intensely stained after the PATO treatment (Figs. 5 and 6). In addition, some vacuoles were found to contain also granular and amorphous material, which was often PATO-positive as well and might represent, in part, a product of glycogen degradation (Figs. 7, 8, and 13). Small vesicles and membranous fragments were also occasionally present within autophagic vacuoles (Figs. 4 and 13). However, identifiable cytoplasmic components, such as mitochondria, elements of sarcoplasmic reticulum, or myofilaments, were never seen inside autophagic vacuoles in muscle fibers of newborn animals. Glycogen-containing vacuoles varied with respect to size, ranging from  $0.1$  to  $1 \mu$ , and degree of packing of the glycogen particles. They were lined by a single or, less frequently, double membrane. In the latter case, the two membranes were at some

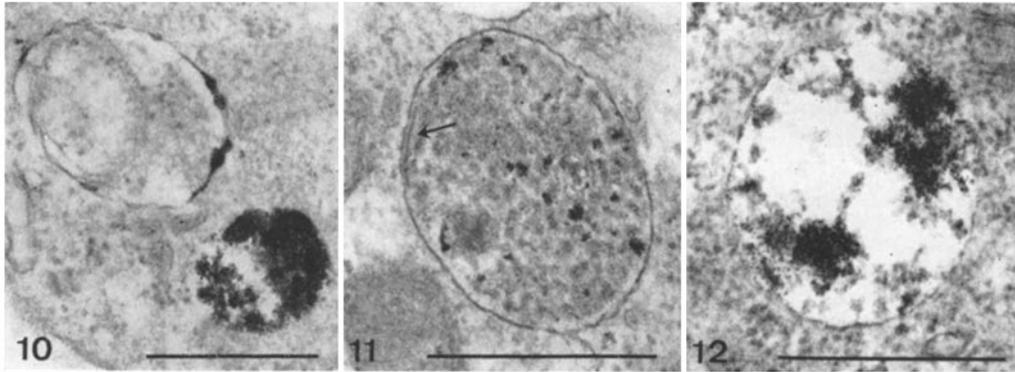


FIGURE 10 Acid phosphatase activity is abundant in the lysosome at right, and is more sparse, confined to peripheral spots closely adherent to the limiting membrane, in the glycogen-containing vacuole at left. Lead hydroxide. Scale mark,  $0.5 \mu$ .  $\times 45,000$ .

FIGURE 11 Sparse precipitates are seen in a glycogen-laden vacuole. The arrow indicates what may be residues of the inner limiting membrane. Lead hydroxide. Scale mark,  $0.5 \mu$ .  $\times 60,000$ .

FIGURE 12 The reaction product is more abundant in this vacuole, and the glycogen particles are more sparse in comparison with the vacuole shown in the previous figure. Lead hydroxide. Scale mark,  $0.5 \mu$ .  $\times 60,000$ .

places apparently fused (Fig. 7). Flattened sacs incompletely surrounding areas of cytoplasm filled with glycogen particles were also observed close to glycogen vacuoles (Fig. 13). In preparations incubated for acid phosphatase, reaction product was frequently but not consistently found in glycogen-containing vacuoles (Figs. 9–12). In vacuoles with a double wall, the precipitate was generally present in the space between the two limiting membranes (Fig. 9). The PATO treatment caused extraction of lead phosphate precipitates, so that a simultaneous demonstration of acid phosphatase and PATO staining on the same section was not possible. Acid phosphatase activity was also observed in Golgi cisternae and vesicles.

The timing of the appearance and persistence of the glycogen-containing vacuoles in different muscles was in direct relation to the time-course of glycogen depletion. Thus, glycogen vacuoles were only sparsely distributed in fibers from leg muscles during the first 3 days after birth, while in the psoas muscle they were especially found on the first day. In the diaphragm muscle, glycogen vacuoles were particularly frequent 3 hr after birth, often grouped in clusters (Figs. 6 and 13), and more rarely seen at 6 hr (Fig. 14). It must be emphasized that similar structures were hardly ever seen in fetal muscles and that their number decreased 3–10 days after birth, being virtually absent at later

stages. Premature formation of glycogen vacuoles in muscles of fetuses at term could be induced by administration of glucagon. The vacuoles were sparse in muscle fibers from glucagon-treated animals 3 hr after the injection and appeared to increase in frequency at 6 hr. Glycogen deposits were, however, still present at that time.

#### *Effect of Denervation; "Glycogen Bodies"*

Postnatal glycogen depletion occurred practically unchanged in muscles denervated before birth or within the first hours of life. In agreement with biochemical data (20), the time-course of glycogen mobilization in the leg muscles appeared to be accelerated. During the early postnatal days, glycogen-containing vacuoles were seen in denervated muscle fibers (Fig. 15). In addition, "glycogen bodies," i.e. concentric arrays of smooth membranes with interposed rows of glycogen particles, were also found (Fig. 16). Similar glycogen-membrane complexes were very rarely encountered in the normal muscles (Fig. 17).

#### DISCUSSION

The results of the present study are in agreement with previous observations on newborn mouse and rat liver (15, 27) and support the view that autophagic processes are implicated in the breakdown

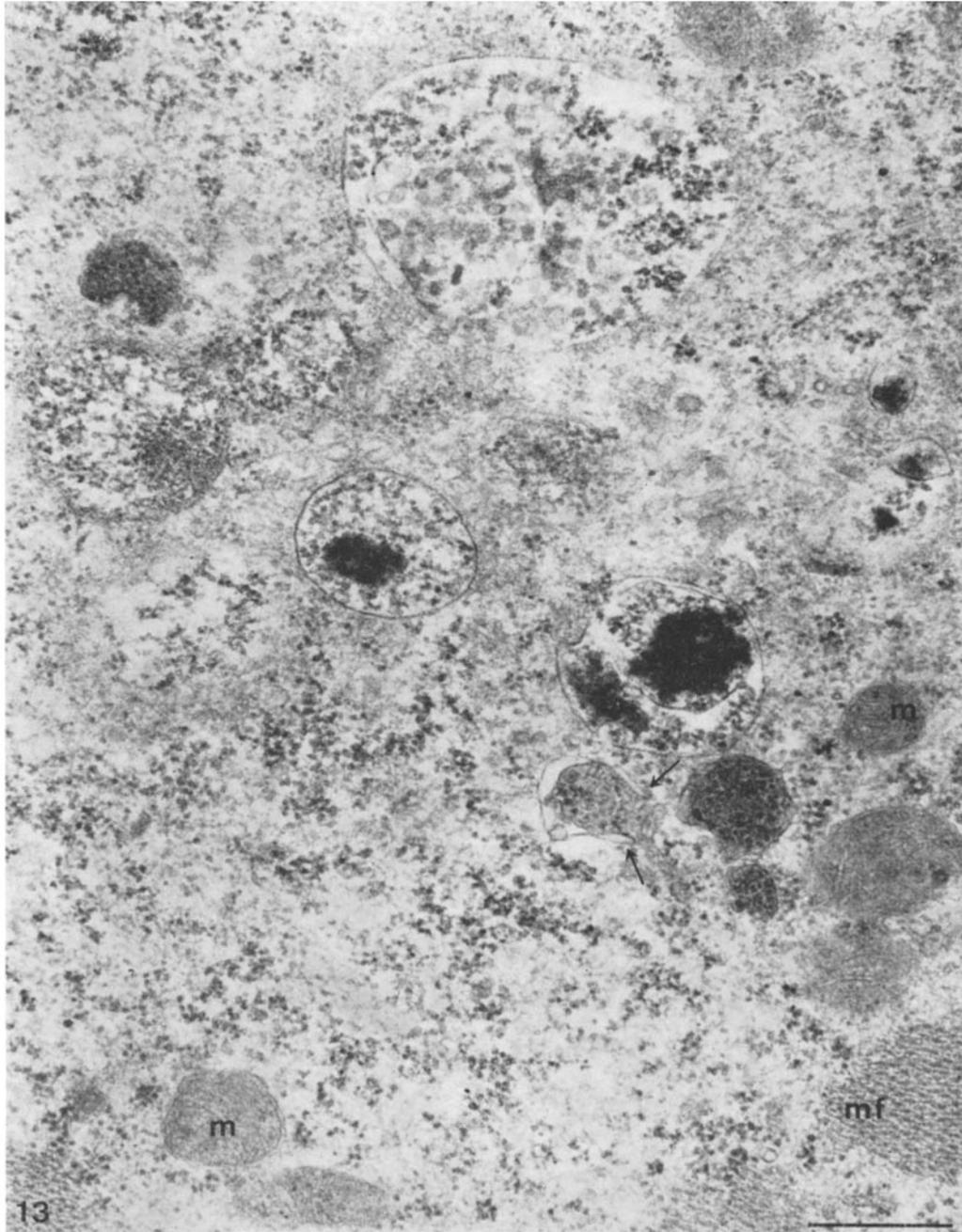


FIGURE 13 Rat diaphragm, 3 hr after birth; transverse section. Numerous vacuoles of variable size are seen in a peripheral, glycogen-rich area. They are filled with glycogen particles which, at some places, appear to be clumped in homogeneous dark masses. Profiles of small vesicles are also seen in the large vacuole in the upper part of the figure. The arrows point to cup-shaped sacs partially encircling a portion of cytoplasm filled with glycogen particles. *m*, mitochondria; *mf*, myofibril. Lead hydroxide. Scale mark,  $0.5 \mu. \times 40,000$ .

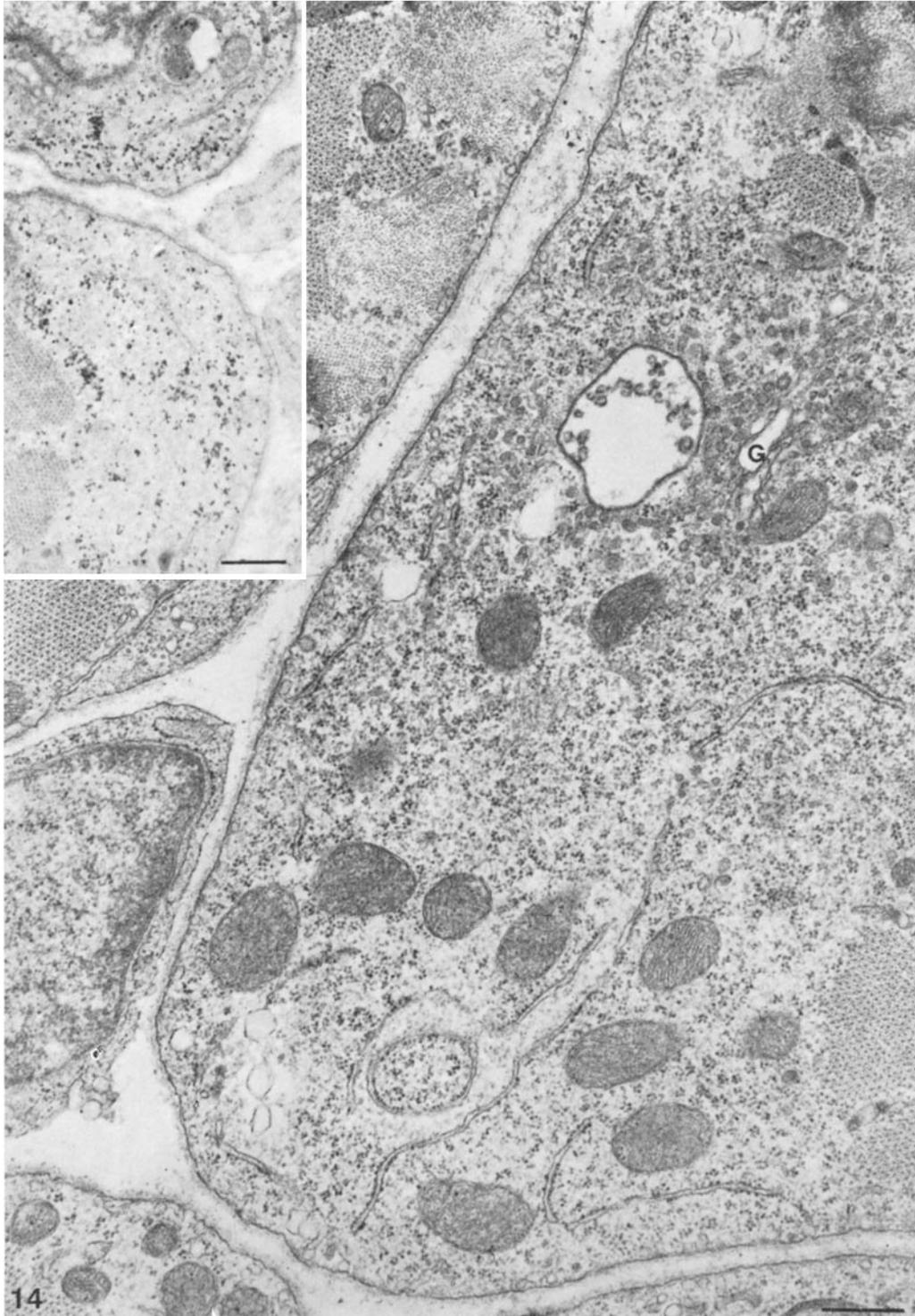


FIGURE 14 Rat diaphragm, 6 hr after birth. Transverse section illustrating a large subsarcolemmal area predominantly occupied by free ribosomes and polyribosomes, long cisternae partially studded with ribosomes and mitochondria. Glycogen particles recognizable by lighter staining are sparsely scattered in this area. A Golgi complex (*G*), surrounded by numerous small dense vesicles, and a large, thick-walled multivesicular body are also seen. Lead citrate staining. The paucity of glycogen in the subsarcolemmal spaces at this stage is also evident with the specific PATO staining (*inset*). Note the apparent fusion of a glycogen-containing vacuole with a small, dense lysosome in the upper part of the *inset*. No counter-stain. Scale marks, 0.5  $\mu$ .  $\times$  24,000, *Inset*,  $\times$  18,000.

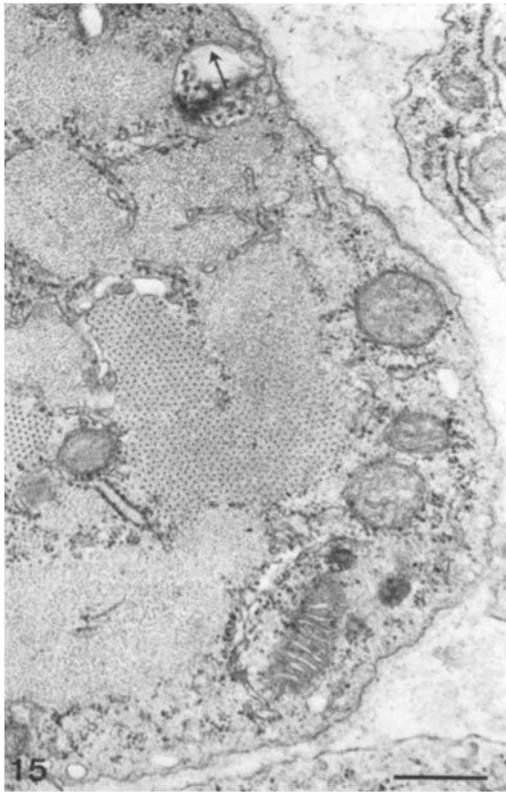


FIGURE 15 Extensor digitorum longus of 3-day old rat denervated 3 days before birth; transverse section. Subsarcolemmal glycogen deposits have largely disappeared. A vacuole containing glycogen particles can be seen in the upper part of the field. The wall of the vacuole is made up by a single membrane, except for a short segment with a double membrane (arrow). Lead citrate. Scale mark,  $0.5 \mu$ .  $\times 24,000$ .

of the glycogen deposits which accumulate in different mammalian tissues at the end of the fetal life. The role of glycogen bodies in this process appears to be of minor importance. Glycogen bodies are present in some rat muscles also at later stages of development (8) and are seen in a variety of other tissues under physiological and pathological conditions (see reference 19). Their significance in glycogen metabolism has not yet been defined.

Our observations suggest that the glycogen-containing autophagic vacuoles in newborn muscle arise from smooth-surfaced sacs which enclose areas of cytoplasm filled with glycogen particles. These sacs may derive from Golgi cisternae or Golgi-associated endoplasmic reticulum (autophagosome formation occurs frequently in the

Golgi zone), or from elements of the sarcoplasmic reticulum proper. The double-walled vacuoles thus formed are presumably transformed into vacuoles lined by a single membrane through fragmentation and dissolution of the inner membrane or by a process of "compaction" (see 7 and 26) of the two enveloping membranes. Acid hydrolases may be supplied to the autophagosomes by Golgi vesicles which are occasionally seen in close proximity to glycogen vacuoles. Alternatively, acid hydrolases could be present already in the enveloping sacs before vacuole formation (see reference 25): indeed, acid phosphatase was frequently found in Golgi cisternae and associated endoplasmic reticulum. Our observations do not provide unambiguous evidence in favor of one or the other interpretation, although the not infrequent finding of autophagosomes lacking acid phosphatase activity seems to be in contrast with the latter possibility.

The most interesting aspect of the autophagic processes related to muscle glycogen depletion at birth concerns their unusually selective character. Most autophagosomes in neonatal muscle fibers do, in fact, contain exclusively glycogen particles, at variance with autophagic vacuoles seen in denervated muscle fibers at later stages (28). The mechanism of such a selective segregation of glycogen is at present not clear, but at least two possibilities may be discussed. The first is that some changes may occur in the perinatal period in the glycogen particles, involving either the glycogen molecules proper or associated proteins (see reference 21), and this modification might induce a directed segregation process. The changes in the association properties of the glycogen particles during development (12) may be of relevance in this respect. An alternative possibility is that the segregation process is primarily induced at birth and that its apparently discriminating character is only due to the "topographical disposition of the membranous systems involved in it" (5). The latter hypothesis, however, seems unlikely, as one would expect that also other cell components would be trapped, at least occasionally, by the sequestering membranes.

The physiological factors which induce glycogen breakdown in muscles of newborn animals remain at present unknown, but the finding that the process is basically independent of neural influences would suggest that hormonal factors are operative. Glucagon has been recently implicated in postnatal glycogenolysis in the rat liver (10, 18). This hormone does not promote glycogenolysis in adult

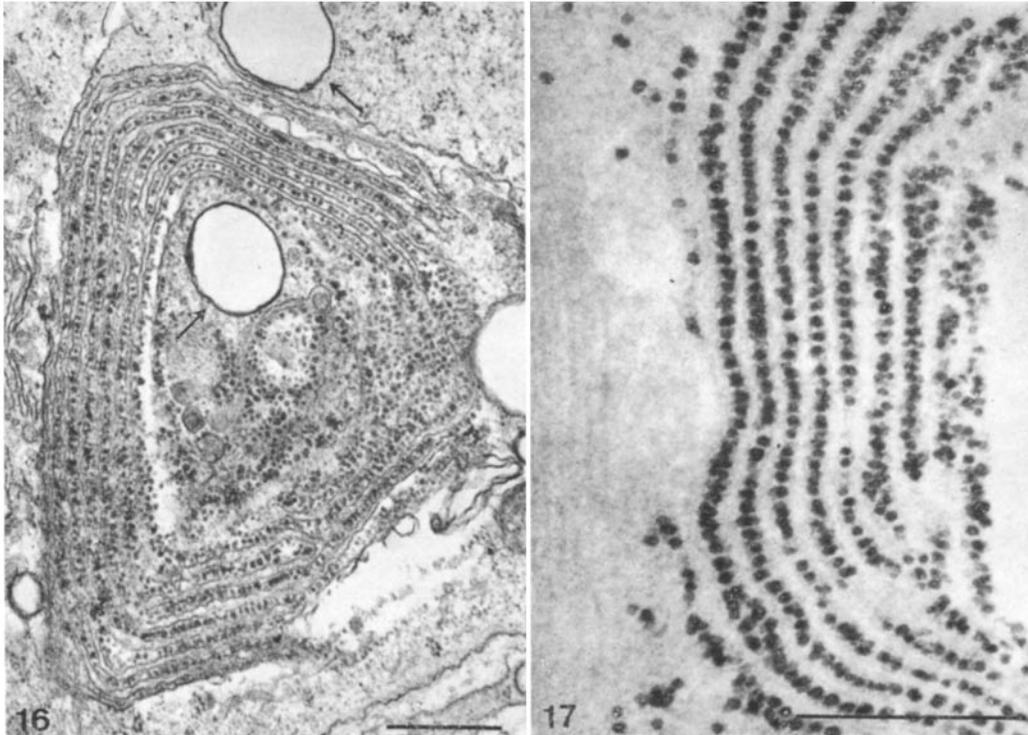


FIGURE 16 Same material as in Fig. 15. The figure illustrates a typical "glycogen body," formed by a concentric array of membranes with intervening rows of glycogen particles. Lipid droplets are also present (arrows). Lead citrate. Scale mark,  $0.5 \mu$ .  $\times 30,000$ .

FIGURE 17 Rat psoas muscle, about 24 hr after birth; longitudinal section. Portion of a "glycogen body" as seen after PATO staining without counterstain. The rows of glycogen particles are intensely stained whereas the intervening membranes are hardly visible. Scale mark,  $0.5 \mu$ .  $\times 60,000$ .

skeletal muscle (see reference 24), but its effect on fetal and neonatal muscle has not been studied. Our experiments indicate that glucagon can induce the formation of glycogen-containing vacuoles in fetal muscle. This process may have some relation to glucagon's well-known property of inducing cellular autophagy in the adult liver (2). Glycogen depletion, however, was rather limited in muscles of glucagon-treated fetuses, and it seems likely that additional factors are involved in this process. Peripheral factors, such as the degree of maturation and active function of the different muscles during the immediate postnatal period, apparently set the timing of the glycogenolytic process. The diaphragm and the gastrocnemius represent two extreme situations in this respect. Denervation experiments indicate that active function is not a necessary prerequisite for glycogenolysis in muscles of newborn animals, although it may accelerate glycogen mobilization.

The participation of the lysosomal system in glycogen mobilization in liver and skeletal muscle of the newborn mammal may be relevant to an understanding of the human "inborn lysosomal disease," type II glycogenosis or Pompe's disease. This condition, which is characterized by the abnormal accumulation of glycogen within autophagic vacuoles in muscles, liver, and other tissues, is associated with absence of lysosomal  $\alpha$ -glucosidase (11, 13).<sup>2</sup> It is possible that the disease is initiated in the autophagic processes which occur physiologically at birth, but which cannot be completed owing to the lack of acid  $\alpha$ -glucosidase. A

<sup>2</sup> This enzyme has been shown to have both  $\alpha$ -1,4- and  $\alpha$ -1,6-glucosidase activity and can thus completely break down glycogen to free glucose (14). The possibility may thus be raised that muscle glycogen makes a direct contribution to circulating glucose during the early postnatal period.

progressive accumulation and enlargement of the glycogen-laden vacuoles would then follow. This interpretation would also be consistent with the fact that the disease becomes manifest generally during the first months of life.

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