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# Type III A Glycogenosis

## A Biochemical and Ultrastructural Study*

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### *Typ III A Glykogenose*

*Eine biochemische und ultrastrukturelle Untersuchung*

*Zusammenfassung.* Die Beobachtung einer Glykogenose (Typus III A) bei einem 11 Jahre alten Jungen wurde biochemisch, elektronenmikroskopisch, cytotopochemisch und durch eine Reihe sog. Negativfärbungen durchgearbeitet. In Leber, Muskulatur und Leukocyten wurden starke Glykogenanhäufungen nachgewiesen. Das Leberglykogen war, sowohl in Schnittpräparaten als auch bei isolierter Untersuchung, von dem normalen Glykogen des gesunden Menschen deutlich verschieden. Sogenannte typische Rosetten wurden nur ganz ausnahmsweise gefunden. Dagegen fanden sich sog. kleine Rosetten, jeweils zusammengesetzt aus einigen wenigen Partikeln, vor allem aber monopartikuläre Granula. In der Muskulatur jedoch trat das Glykogen, wie auch beim gesunden Menschen, monopartikulär und granuliert auf. *Biochemisch* konnte die Diagnose einer Glykogenose des Typus III A eindeutig gestellt werden. Es haben sich keine Anzeichen dafür nachweisen lassen, daß ein sog. Fermentdefekt, etwa wie bei einer Glykogenose des Typus II, zugrunde lag.

*Summary.* A combined biochemical and ultrastructural study, including cytochemical and negative staining techniques, has been made of three tissues, liver, muscle and leucocytes, in a case of type III A glycogenosis.

The electron microscopic studies revealed an increased accumulation of glycogen in the liver, in the skeletal muscle and in the leucocytes.

The hepatic glycogen, either isolated or within the hepatocytes, is mostly represented in the form of monoparticulate granules and of rosettes with fewer component units, whereas the typical rosettes are rare. The isolated and intracellular glycogen of muscle cells appears composed of monoparticulate granules, as in normal muscle cells.

The biochemical studies confirm the diagnosis of type III A glycogenosis and seem to exclude the simultaneous occurrence of the enzymatic defect responsible for type II glycogenosis.

Type III glycogenosis (CORI, 1957) or limit dextrinosis is a glycogen storage disease characterized by the congenital absence of the debranching enzyme, amylo-1,6-glucosidase (ILLINGWORTH *et al.*, 1956). In its most frequent type, limit dextrinosis A, a complete lack of the enzyme occurs in both liver and muscle (HERS, *et al.*, 1964). Amylo-1,6-glucosidase is absent also in leucocytes of patients affected by such disease (WILLIAMS *et al.*, 1963). Biochemical studies (ILLINGWORTH and BROWN, 1964) revealed that in type III glycogenosis the polysaccharide has shorter outer chains and a higher end-group percentage than normal

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glycogen. Previous studies (SALOMON, *et al.*, 1961; HUG *et al.*, 1966) of patients affected by type III glycogenosis are mainly concerned with the ultrastructural changes in the liver and in the muscle.

This report deals not only with the ultrastructure of liver, skeletal muscle and leucocytes in a patient affected by type III A glycogenosis, but also includes cytochemistry, and negative staining of the glycogen particles. Glycogen, amylo-1,6-glucosidase and acid and neutral maltase activities were evaluated in homogenates of liver and muscle. The acid and neutral maltase activities were studied according to ILLINGWORTH (1961) observation of the possible simultaneous occurrence of type II and type III glycogenosis.

The fine structure of intracellular and isolated glycogen particles was studied in order to investigate a possible relationship between the biochemical and morphologic changes.

## Materials and Methods

### *Clinical Data*

In the patient, an 11-years-old-boy, the first manifestations of the disease date back to the age of 1 year and 4 months when muscular weakness, hypoglycemic shocks and hepatomegaly became evident. During his last admission in November 1966 surgical biopsies of the liver and of the muscle rectus abdominis were performed under total anesthesia. The tissues obtained were employed for the present study.

### *Morphologic Techniques*

Fragments of tissues were partly fixed in 2% osmium tetroxide for 90′ and partly fixed in 2.5% glutaraldehyde for 2 hours with and without osmium postfixation for 30′. Both fixatives were buffered at pH 7.2 according to MILLONIG (1961). The tissue blocks were embedded in Araldite (Durcupan ACM). Heparinated blood samples were centrifuged at 200 r.p.m. for 10′. The supernatant, containing leucocytes and platelets, was partly fixed in osmium tetroxide for 30′ and partly in glutaraldehyde for 60′ with osmium postfixation for 15′ and embedded in Araldite. Thick sections (0.5—1 μ) were stained with toluidine blue (TRUMP *et al.*, 1961) and observed in the light microscope. Thin sections were stained with lead hydroxide (KARNOVSKY, 1961), with uranyl acetate and lead hydroxide, and with 10% phosphotungstic acid (PTA) (WATSON, 1958).

Other fragments of liver and muscle were immediately frozen in a carbon dioxide bath, kept at —20° and then processed according to ORREL and BUEDING (1958) for the glycogen extraction. For "negative staining" (DROCHMANS, 1962) a distilled water suspension of isolated glycogen was mixed with an equal volume of 2% PTA adjusted to pH 7.2 with 1 N KOH, and then collected on carbon coated grids.

### *Biochemical Techniques*

For determination of the *glycogen content* in fragments of liver and muscle, the tissues were immediately frozen after biopsy and then weighed and thawed. Samples of 100 mg were minced with scissors and homogenized in a mortar with distilled water, the final concentration being 5% w/v. The glycogen content of the homogenates was measured with the anthrone method (SEIFTER *et al.*, 1950) as modified by HERS (1964). The values of glycogen concentration were expressed as per cent of the weight of the frozen tissue. The glycogen of the leucocytes was extracted and measured according to WILLIAMS and FIELD (1961) and expressed as μg per $10^7$ cells. The *amylo-1,6-glucosidase activity* was tested on the 5% homogenates according to HERS (1959). The acid and neutral maltase activities were assayed on samples of tissue immediately frozen after biopsy and stored at —20°. The *maltase activities* were determined by measuring the rate of formation of glucose from maltose by a modification of the HERS (1963) method previously described (AURICCHIO and BRUNI, 1967).

Samples of human livers obtained at open biopsy from patients without liver diseases were processed in the same way for testing the maltase activities. Activities were expressed as μmoles of substrate utilized per minute per gram of frozen tissue.

## Results

### *Liver*

### Morphologic Data

*Light microscopy.* The examination of the thick sections stained with toluidine blue shows that the architecture of the hepatic lobules is changed for the presence of septa of connective tissue arising from the portal spaces. The hepatocytes

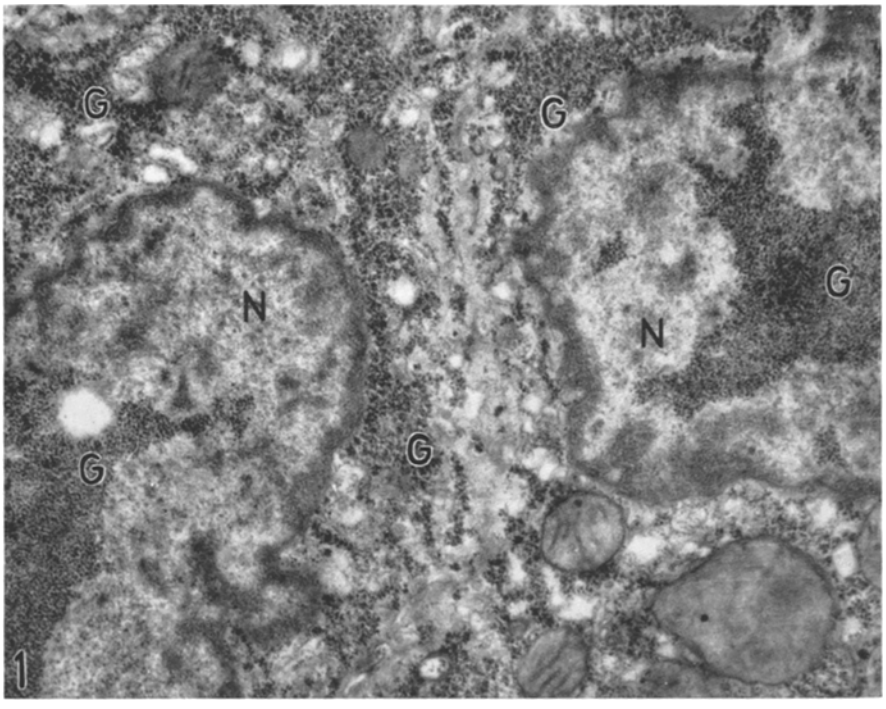



Fig. 1. The micrograph illustrates that the nuclei (*N*) of the hepatocytes have irregular margins and contain glycogen deposits (*G*). In the cytoplasm, large glycogen deposits are visible. Osmium fixation, lead hydroxide staining, × 19,000

appear swollen, their shape is irregular, their cytoplasm is clear. The nuclei are eccentric and some of them are vacuolated. The number and size of the sinusoidal spaces are reduced and the number of the limiting endothelial and Kupffer cells is decreased. The lipid droplets, often visible within the hepatic cells, are metachromatically stained by toluidine blue.

*Electron microscopy.* The observation of thin sections stained with lead hydroxide or with uranyl acetate and lead hydroxide shows that the nuclei of hepatocytes have irregular margins (Fig. 1). Some of the nuclei contain glycogen deposits in the form of small granules, 200 Å in diameter, (β particles) (Drochmans, 1962), and rosettes about 130 mμ in diameter (α particles) (Drochmans, 1962) (Fig. 2). The mitochondria are mainly located at the periphery of the cells.

Some mitochondria show fibrillar and vacuolar degeneration (Fig. 3). Smooth and rough endoplasmic reticulum and free ribosomes are scarce. On the vascular pole of the hepatocytes the plasma membrane has a straight course and appears devoid of microvilli. The cytoplasm is almost completely filled with large deposits of glycogen (Figs. 1, 4) in which some lipid vacuoles are visible. The deposits do not appear surrounded by a membrane and are composed of $\beta$ particles (250 Å) and of rare rosettes (100 mμ), (Fig. 4).

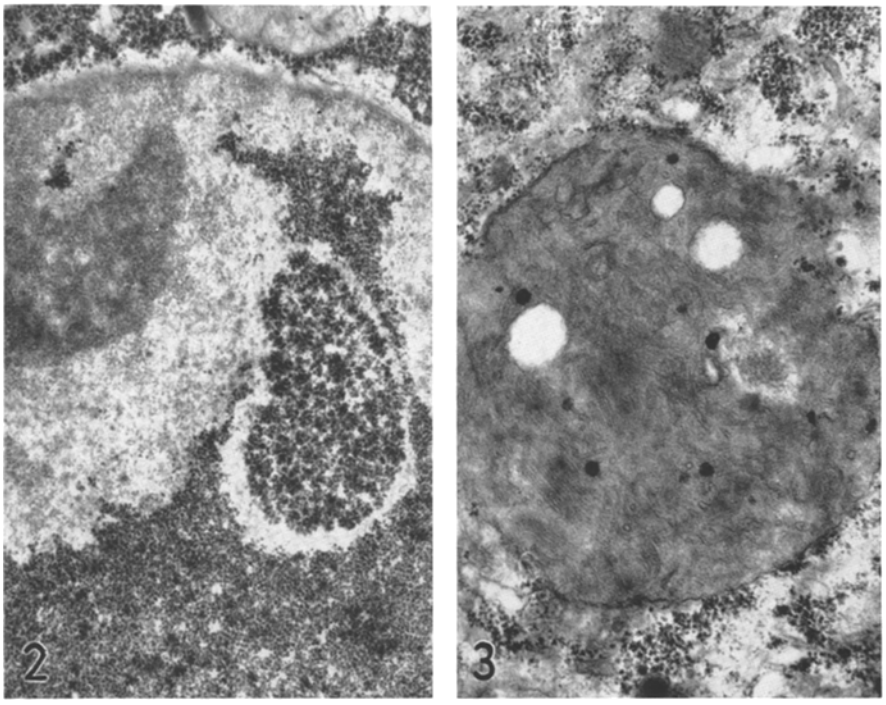



Fig. 2. Part of an hepatocyte. The intranuclear deposits of glycogen are composed of some $\alpha$ particles but mostly of $\beta$ particles. Osmium fixation, lead hydroxide staining, × 18,000

Fig. 3. In a giant mitochondrion of an hepatocyte fibrillar inclusions and "vacuolar degeneration" are present. Osmium fixation, lead hydroxide staining, × 21,600

In sections embedded in Araldite and treated with PTA only collagen fibers and glycogen particles are specifically stained. The appearance of glycogen particles in both nuclei and cytoplasm is similar to that observed in sections stained with lead hydroxide or with uranyl acetate and lead hydroxide (Fig. 5).

After "negative staining" the isolated glycogen appears mostly in the form of $\beta$ particles (250 Å). The $\alpha$ particles (150 mμ) are scarce, whereas numerous rosettes with fewer component units (40—80 mμ) are present (Fig. 6).

## Biochemical data

Amylo-1,6-glucosidase activity is not detectable in the liver tissue. The glycogen content is 11.80 per cent. The acid and neutral maltase activities are 0.16 and 0.025 μmoles/min/g respectively; the values in the normal subjects in the same experimental conditions are 0.6 and 0.15 μmoles/min/g.

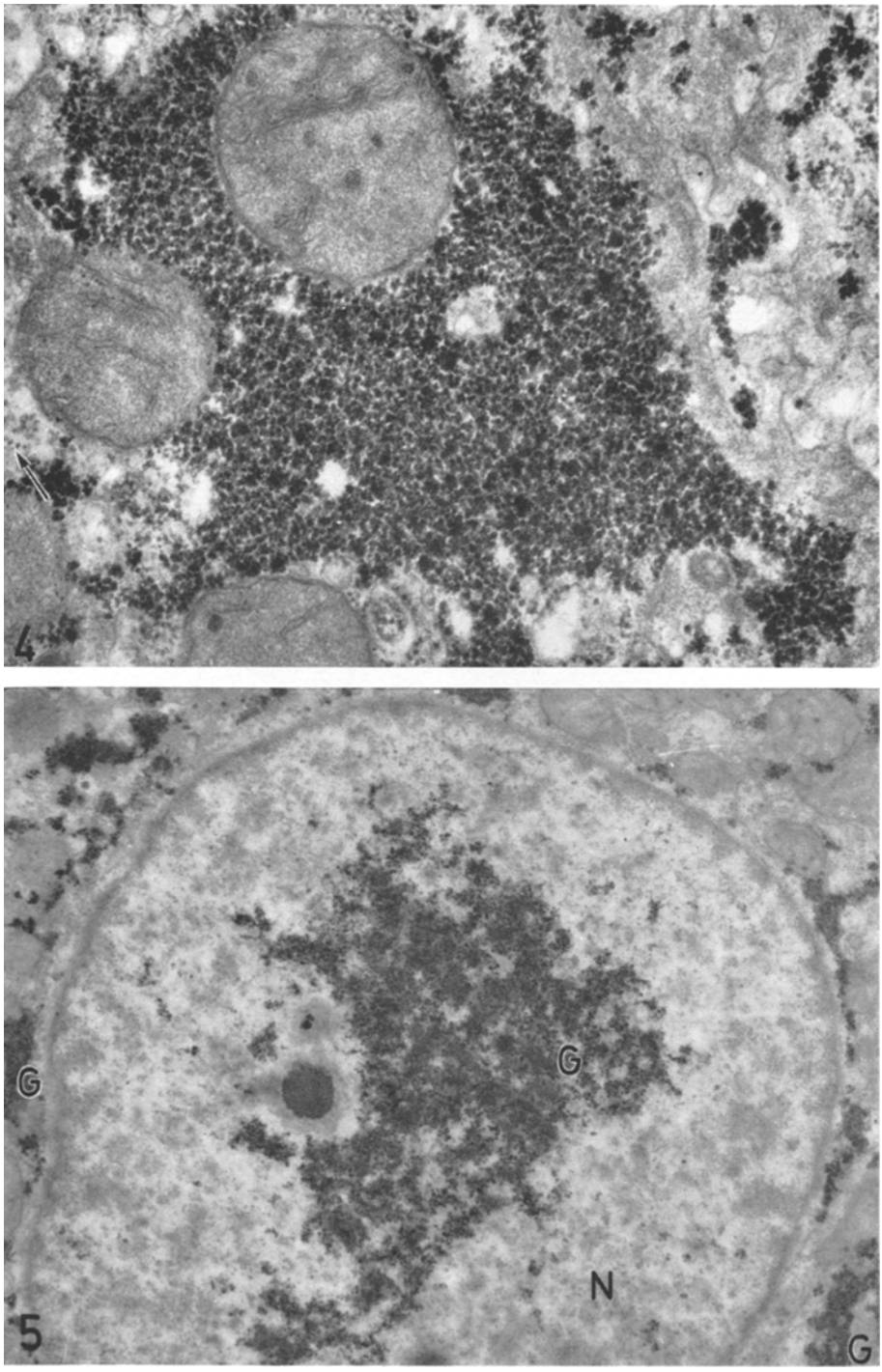



Fig. 4. A large glycogen deposit, composed of rosettes, of rosettes with fewer component units and $\beta$ particles is visible in the cytoplasm of an hepatocyte. Mitochondria and few ribosomes arranged in clusters (arrow) are present. Osmium fixation, lead hydroxide staining, $\times$ 40,000

Fig. 5. Part of the nucleus (*N*) and cytoplasm of an hepatocyte. The intranuclear and cytoplasmic glycogen (*G*) are specifically stained by PTA. Osmium Fixation, 10% PTA staining, $\times$ 15,600

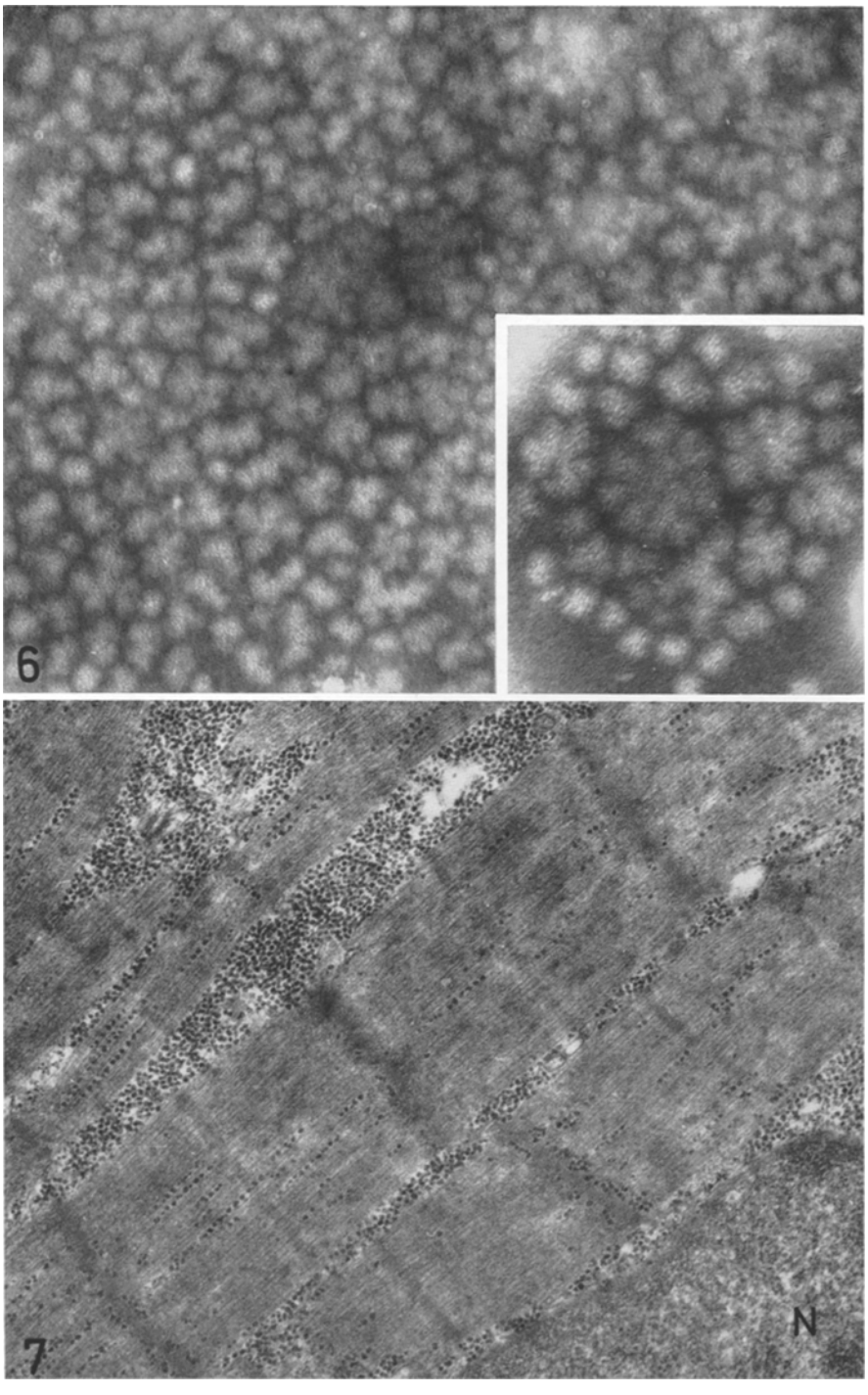



Fig. 6. Isolated hepatic glycogen. The glycogen particles are represented by a few rosettes and by numerous $\beta$ granules and rosettes with fewer component units, $\times$ 120,000. The inset shows glycogen particles at higher magnification, $\times$ 170,000. 2% PTA negative staining

Fig. 7. The picture shows part of a skeletal muscle cell. Deposits of glycogen are visible between the myofibrils, which appear in longitudinal section, and around a nucleus ($N$). Osmium fixation, lead hydroxide staining, $\times$ 28,000

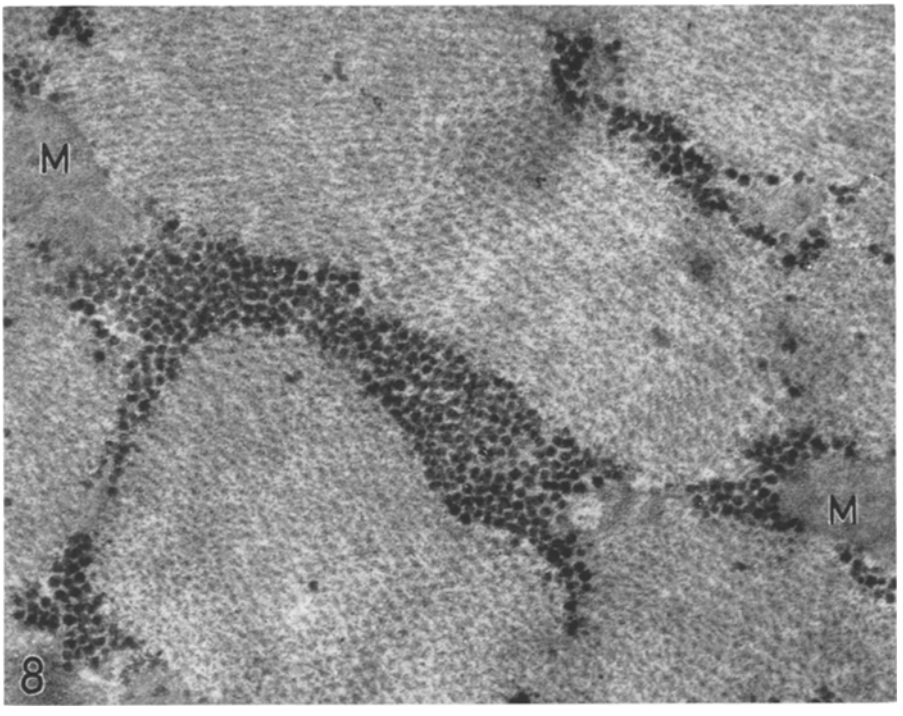



Fig. 8. Deposits of glycogen and two mitochondria (*M*) are present between the myofibrils which appear cross sectioned. Osmium fixation, uranyl acetate and lead hydroxide staining, × 60,000

*Skeletal Muscle*

Morphologic Data

*Light microscopy.* The examination of thick sections stained with toluidine blue shows that the myofibrils of muscular cells are displaced from their normal position and in some cases surrounded by a large amount of amorphous material.

*Electron Microscopy.* The study of sections stained with lead hydroxide or with uranyl acetate and lead hydroxide does not show changes in the nuclei of muscle cells (Fig. 7). The muscle cells contain lipid vacuoles and a few mitochondria (Figs. 8, 9). The elements of longitudinal and transversal sarcoplasmic reticulum are scarce (Figs. 7—9). Large deposits of glycogen, composed of $\beta$-particles (220 Å), are localized in the peripheral areas of muscle cells (Fig. 7). $\beta$-particles are also present between the myofibrils, along the Z-lines and interposed between the myofilaments (Figs. 7—9). A similar distribution and morphology of glycogen is seen in sections in which polysaccharides are specifically stained with PTA (Fig. 10).

After "negative staining" the isolated glycogen appears composed only of $\beta$-particles (200—400 Å) (Fig. 11).

*Biochemical Data.* The biochemical study shows an absence of amylo-1,6-glucosidase activity in the muscular tissue. The glycogen content is 5.85 per cent. The acid maltase activity, is 0.04 μmoles/min/g and the neutral maltase activity

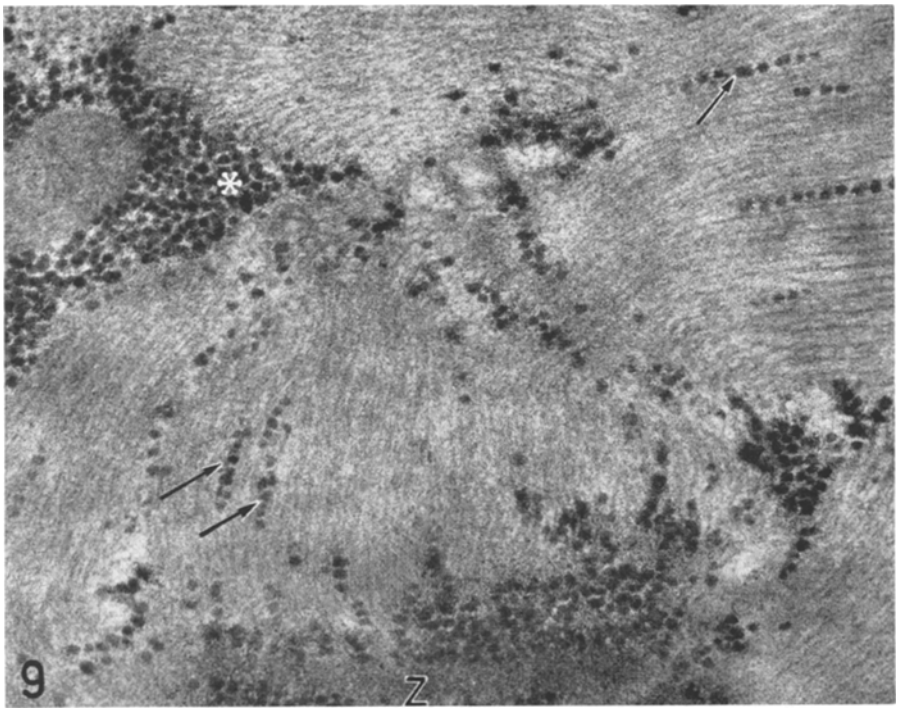



Fig. 9. In this picture the myofibrils appear in cross, in oblique and in longitudinal section. Glycogen $\beta$ particles are visible between the myofilaments (arrows), along a Z line (*Z*) and accumulated in the sarcoplasm (asterisk). Osmium fixation, uranyl acetate and lead hydroxide staining, $\times$ 60,000

0.016 μmoles/min/g; the levels of maltase activities in the normal subjects are 0.05 and 0.02 μmoles/min/g respectively, according to ILLINGWORTH and BROWN (1965).

*Leucocytes and Platelets*

Morphologic Data

The leucocytes and platelets have normal fine structure (Figs. 12—14). In leucocytes, glycogen is visible in the form of numerous $\beta$-particles (200—250 Å) scattered in the cytoplasm (Figs. 12, 13), whereas in the platelets the $\beta$-particles are mostly arranged in small, irregular aggregates (Fig. 14).

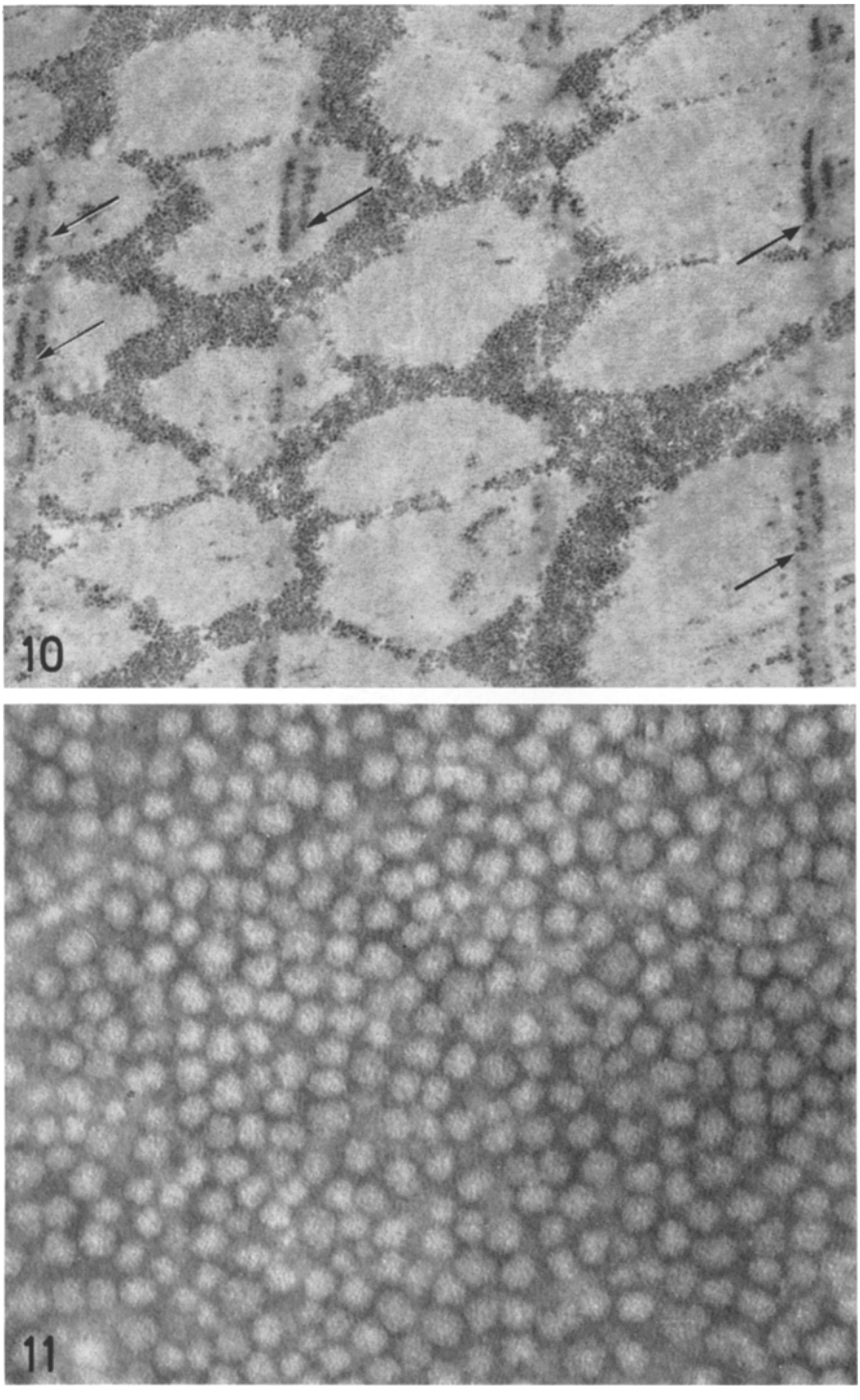
Biochemical Data

The glycogen concentration in leucocytes is 60 μg/$10^7$ cells. According to WILLIAMS *et al.* (1963) the glycogen concentration in leucocytes of normal humans is 35 $\pm$ 2 μg/$10^7$ cells.

**Discussion**

The diagnosis of type III A glycogenosis in this patient was confirmed by both the glycogen and amylo-1,6-glucosidase tests.

The increased content of intracellular glycogen represents the prominent feature observed in the hepatocytes as formerly stressed by several authors in




Figs. 10 and 11 (for legends see p. 173)

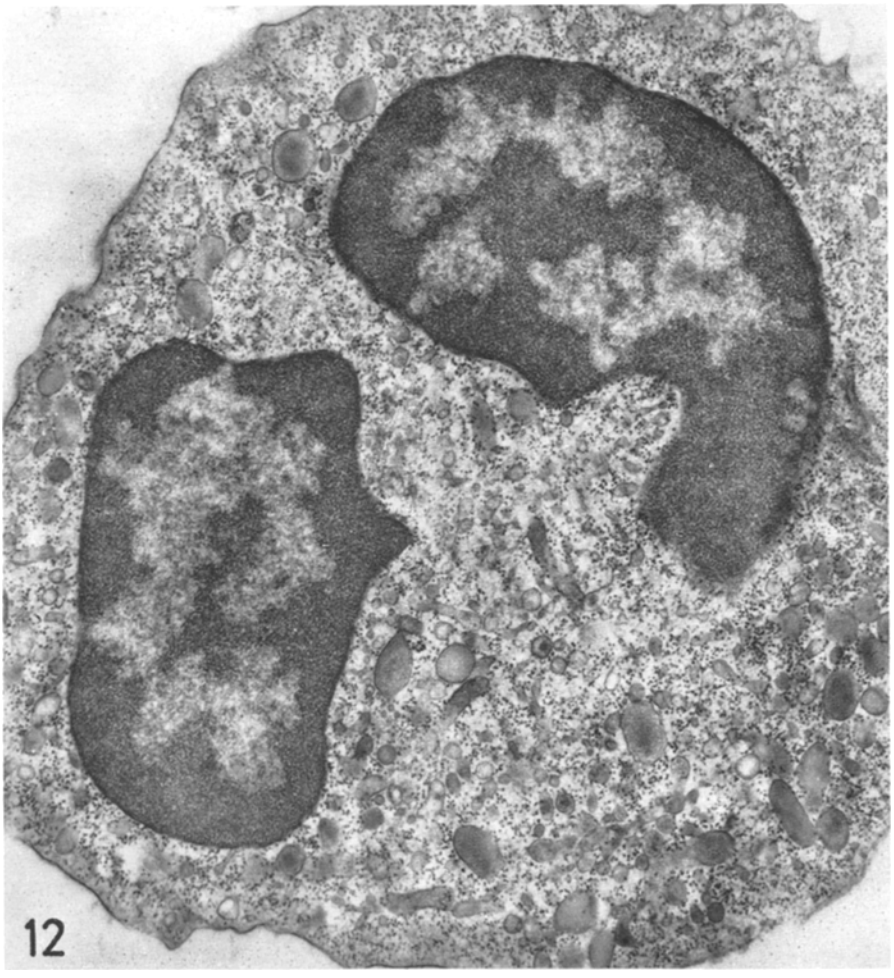


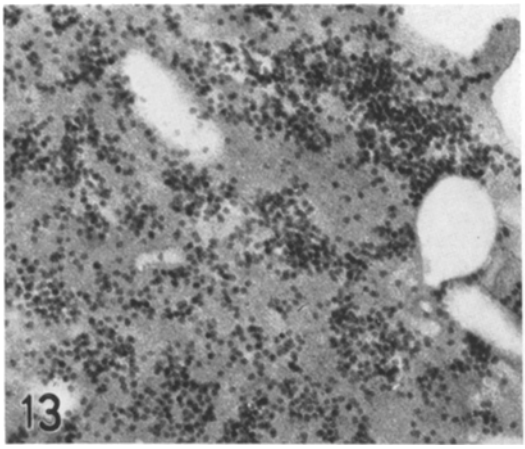

Fig. 12. A neutrophil leucocyte which contains numerous glycogen particles scattered throughout the cytoplasm. Osmium fixation, lead hydroxide staining, × 16,600

different types of glycogenosis (SALOMON, *et al.*, 1961; SHELDON *et al.*, 1962; BAUDHUIN *et al.*, 1964; CARDIFF, 1966; HUG *et al.*, 1966). The other morphologic changes are probably secondary to this abnormal accumulation.

The fibrillar and "vacuolar degeneration" of mitochondria cannot be considered specific of this disease, since similar aspects have been observed in normal subjects (WILLS, 1965) and in patients with other hepatic diseases (JEZEQUEL, 1959; LAGUENS and BIANCHI, 1963; MINIO and GAUTIER, 1967).

Fig. 10. PTA staining, specific for polysaccharides, not only reveals the same distribution of glycogen as in the preceding figures stained with uranyl acetate and lead, but actually accentuate the abnormal accumulation of glycogen on either side of the *Z* lines (arrows). Osmium fixation, × 22,000

Fig. 11. Isolated muscular glycogen. The morphology of glycogen is normal. Only $\beta$ particles are present. 2% PTA negative staining, × 140,000



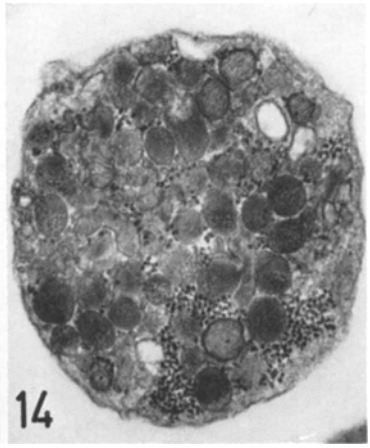




Fig. 13. Part of a neutrophil leucocyte at higher magnification. The cytoplasm contains deposits of glycogen particles. Glutaraldehyde and osmium fixation, lead hydroxide staining. × 35,000

Fig. 14. The picture shows the spotty localization of glycogen in the cytoplasm of a thrombocyte. Osmium fixation, lead hydroxide staining, × 25,000

The presence of glycogen in the nuclei of hepatocytes has been reported in type I (SHELDON *et al.*, 1962) and type III (SALOMON *et al.*, 1961) glycogenosis and in other pathologic conditions (ROUILLER and SIMON, 1962; BIAVA, 1963; LAGUENS and BIANCHI, 1963). In previous investigations (SHELDON *et al.*, 1962; BIAVA, 1963) the intranuclear glycogen was observed only in the form of $\beta$-particles. However, in this study, both $\alpha$- and $\beta$-particles were visible within the nuclei.

The intracytoplasmic and isolated glycogen is mostly composed of $\beta$-particles and of $\alpha$-particles with fewer component units. The rosettes, which normally represent the most frequent morphologic pattern of hepatic glycogen, are rare.

It is well known on the basis of routine electron microscopical methods that glycogen particles in the human liver are normally in the form of rosettes (BIAVA, 1963). It would have been desirable to compare the isolated glycogen particles from the liver in this case of glycogenosis III with glycogen from normal human livers, but unfortunately this was not possible. Two samples of surgical biopsies were made available but no glycogen was found in them, probably because the subjects had been fasting for 24 hours.

Anyway our findings are supported by an ultracentrifugation study performed by BUEDING *et al.* (1964). These authors observed that glycogen isolated from the liver of patients with type III glycogenosis has a prominent peak of low molecular weight material and a great polydispersity as compared with the controls.

The nature of the bonds that hold together the glycogen subunits ($\beta$-particles) in aggregates ($\alpha$-particles) is not known (ORRELL *et al.*, 1964). These forces are influenced by various physiopathological factors, such as changes in the nutritional state (ORREL *et al.*, 1964) as well as metabolic disorders in the glycogen synthesis and degradation (BUEDING *et al.*, 1964).

It is difficult to elucidate whether the peculiar pattern of glycogen distribution observed in this study is due to the biochemical disorders induced by the absence

of the debranching enzyme or to other unknown factors. In fact, similar patterns of glycogen distribution were also found in type VI glycogenosis (BUEDING *et al.*, 1964) in which the glycogen structure is normal.

In the muscle cells the number of mitochondria and the elements of sacroplasmic reticulum are reduced but these findings may be only apparent and merely due to the abnormal accumulation of glycogen. The morphology of both isolated glycogen and of that present in tissue sections appear to be normal. These findings are in agreement with the study of BUEDING *et al.* (1964) who observed that the ultracentrifugal pattern of glycogen extracted from the muscle of patients with type III glycogenosis is similar to that of glycogen extracted from normal human muscle.

In the leucocytes of this patient the finding of an increased glycogen concentration and of numerous glycogen granules within the cytoplasm is supported by biochemical studies performed in patients affected by the same disease (WILLIAMS *et al.*, 1963).

In type II glycogenosis (POMPE'S disease) acid maltase activity is lacking (HERS, 1963) whereas neutral maltase is present in normal amounts (ILLINGWORTH and BROWN, 1965). Therefore the two enzymatic activities seem to be genetically unrelated. The reduction in the hepatic tissue of both activities does not support, in this patient, the hypothesis of the coexistence of the enzymatic defect responsible for type II glycogenosis. The low levels of these activities may be due to the severe changes in the hepatocytes and to their partial substitution with connective tissue. The hypothesis is supported by the observation that in the muscle, which presents slight morphologic changes in comparison with those of the liver, the levels of the maltase activities are normal. Furthermore glycogen deposits bounded by a membrane, as described in the liver and in the muscle of patient lacking acid maltase activity (BAUDHUIN *et al.*, 1964; CARDIFF, 1966; HUG *et al.*, 1966), were not observed in this study.

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