

## RABBIT SKELETAL MUSCLE GLYCOGEN

### A Morphological and Biochemical Study of Glycogen $\beta$ -Particles Isolated by the Precipitation-Centrifugation Method

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#### ABSTRACT

Glycogen in its particulate  $\beta$ -form is localized in the sarcoplasm close to the sarcoplasmic reticulum. Some particles are in close contact with the membranes, on the outer side of the vesicles. The mild technique of differential precipitation-centrifugation has been adapted to the preparation of glycogen from adult skeletal muscle. A preliminary low-speed centrifugation which eliminates the contractile protein structures and the cell debris is followed by a high-speed centrifugation which produces pellets containing glycogen mixed with smooth-walled vesicles, the glycogen-sarcovesicular fraction. The glycogen obtained after treatment of this fraction with deoxycholate and two washings contains 3% protein. A similar protein content contaminates glycogen banded in a linear sucrose gradient. The glycogen-sarcovesicular fraction and the purified glycogen have been examined, under the electron microscope, in sections of fixed and embedded material or with the negative staining technique. The glycogen  $\beta$ -particles in negatively stained preparations have an average diameter of 39.4  $\mu$ . The largest particles present irregular outlines, suggesting the presence of conglomerated subunits, about 20  $\mu$  in diameter. These subunits seem to fall apart under the influence of concentrated potassium hydroxide. The mean sedimentation coefficients calculated for infinite dilution vary from 115 to 135S. The spectrophotometric analysis of the glycogen-iodine complex indicates the presence of long end-chains in the molecule.

#### INTRODUCTION

Studies on the macromolecular structure of glycogen and on the properties of proteins and other components which are bound to the polysaccharide particles require the use of a very mild technique of extraction. Many methods proposed in the literature have to be discarded because they degrade or disturb the macromolecular arrangement of the polymer and denature the bound proteins; other techniques, such as the classical differential centrifugation, may be applied to the fractionation of tissue homogenates only if the tissues are rich in glycogen. Revel et al. (1960) have isolated glycogen from turtle cardiac muscle,

toadfish muscle, and other organs in which glycogen is accumulated in large masses in the cytoplasm. In striated muscle of rabbits, rats, and other laboratory animals, however, the glycogen content represents less than 1% of the total wet weight. The difficulty in extracting this small amount of glycogen from muscle tissue has been faced by the biochemists who have applied the trichloroacetic acid (TCA) extraction procedure to muscle tissue. The proportion of the glycogen, called desmoglycogen, which is resistant to TCA extraction depends upon the glycogen content of the muscle and amounts to more than 60% of the total wet

weight, when the glycogen content is low (Cori, 1957). Roe et al. (1961) have shown that the residual desmo-glycogen, extracted only after potassium hydroxide (KOH) digestion of the muscle tissue, corresponds to a fraction which is entrapped in the mass of contractile proteins. Glycogen prepared by KOH extraction (Somogyi, 1957) or by other methods (Stetten and Stetten, 1957; Bueding and Orrell, 1964; Laskov and Gross, 1965) still contains impurities, namely proteins. The latter are discarded by treating the glycogen extract in different ways, depending upon the extraction method used: in Somogyi's method, the glycogen in solution is made acid and precipitated with alcohol; in Bueding's cold water extraction, it is shaken for long periods with chloroform; in Laskov's method, phenol is used to purify the extract. Such purifications eliminate impurities, but also eliminate substances normally bound to the particles. These substances may play a role in keeping the macromolecular organization intact or may bear an enzyme activity in direct relation with the polysaccharide formation or breakdown.

Many proteins present a great affinity for glycogen. For example, concanavalin, extracted from soya beans, shows a specific affinity for glycogen (Cifonelli et al., 1956) and is used as a test for the presence of glycogen. The enzymes concerned with synthesis and breakdown of the branched molecule, synthetase and phosphorylase, are fixed on the glycogen molecules and are found in the glycogen fractions of tissue homogenates. The uridine-diphosphoglucose-glycogen transglucosylase (synthetase) has been isolated by Leloir and Goldemberg (1960) from a cell fraction rich in glycogen. Luck (1961) has confirmed this finding and concluded from the synthetase activity measurements, carried out on fractions rich in glycogen, and on others mainly formed of smooth endoplasmic reticulum, that the distribution of the enzyme can be correlated with the glycogen content of each fraction. The enzyme is found free in the supernatant of liver homogenates when no glycogen is present. The phosphorylase shows a similar affinity for the glycogen particles. Madsen and Cori (1958) have determined the mean number of enzyme molecules which may cover the surface of a phytoglycogen particle. Tata (1964) has shown in liver homogenates of fed rats that the phosphorylase is firmly associated with glycogen; a redistribution occurs after starvation in glycogen-depleted livers.

In the present work, particular care has been taken to obtain from rabbit striated muscle a glycogen fraction in which the morphology of the particles has been preserved, by means of the precipitation-centrifugation method. It will be shown that the macromolecular structure of the purified preparations of muscle glycogen  $\beta$ -particles, examined in negative contrast, appears not elementary but composed of subunits. By avoiding protein-denaturing agents in the course of the isolation, a constant protein residue remains strongly linked to the particles.

## MATERIAL AND METHODS

### *Electron Microscopy*

*Muscle specimens* taken from the adductor magnus were fixed in 3% distilled glutaraldehyde (Fahimi and Drochmans, 1965), in 0.1 M phosphate buffer, pH 7.2, for 3 hr at 4°C, washed for 3 hr in 0.15 M sucrose, and postfixed for 1 hr in 1% osmium tetroxide dissolved in the same buffer. Some specimens were treated with an aqueous solution of uranyl acetate. The progressive dehydration in graded ethanols was followed by a classic embedding in Epon 812. Ultrathin sections were cut on a Porter-Blum microtome and stained with lead hydroxide.

*Pellets* were fixed in the same way by dropping minute samples of material taken with a microspatula. All manipulations were similar to those applied on tissue specimens, as specified above.

*Negative staining* was used for the examination of the different fractions containing glycogen, as specified in an early paper (Drochmans, 1962). Phosphotungstic acid, obtained from Riedel-de Haen (Hanover), was used because it dissolves without leaving an insoluble residue and gives a clear solution with no turbidity.

All preparations for electron microscopy were examined in a Siemens Elmiskop I.

### *Extraction and Purification of Glycogen*

Adult rabbits were anesthetized by an intravenous injection of Nembutal and immediately bled by sectioning the blood vessels of the neck. The adductor magnus muscles, composed predominantly of fibers of the white type, were excised from both hind legs. In some experiments, we added other muscles of the mixed type to the adductors to increase the mass of tissue. The excised muscles were quickly cooled by immersion in 0.033 M phthalate buffer, pH 4.8, at 2°C. We added phthalate buffer to obtain a final proportion of tissue to buffer solution of 1:3 (w/v).

The technique of differential precipitation-centrifugation was adapted to the preparation of a

glycogen-sarcovesicular fraction from a muscle homogenate. This fraction was subsequently treated with sodium deoxycholate (DOC) (Difco Laboratories, Detroit, or Merck, Darmstadt) or was submitted to a centrifugation on a sucrose-density gradient. The successive steps will be described in detail under Results. The density gradients used for further purification of the isolated glycogen fraction were prepared with a triple-outlet gradient mixer. The liquid delivered by the three outlet tubes was controlled by an Autotechnicon pulsation pump. The linearity of the gradient and the density at particular places along the gradient were measured with a Zeiss refractometer. After centrifugation, the gradients were fractionated into 22 fractions of 0.25 ml each by the injection of a 2.05 M sucrose solution at the bottom of the tube through a piercing device. The fractions collected from top to bottom in a sample collector were analyzed for glycogen and protein content.

Lyophilization was carried out as follows: 0.5 ml portions of the purified glycogen preparations at a final concentration of 5% were distributed in antiactinic tubes, frozen at  $-60^{\circ}\text{C}$ , and dried in a vacuum of  $10^{-5}$  mm Hg. The tubes were sealed under nitrogen and stored in the refrigerator at  $3^{\circ}\text{C}$ .

#### *Size Measurements of Glycogen Particles*

Particle size measurements of glycogen extracted from muscle were performed with the Zeiss TGZ 3 particle size analyzer (Drochmans, 1965; Drochmans and Dantan, 1968). The electron microscope plates were enlarged and printed on Copyline 071-m, 010-mm sheet films (Gevaert) so that the final magnification was 100,000. The semiautomatic recording of the diameters measured were transcribed in the form of size-frequency histograms. The cumulative frequency values were plotted on probability grids, in arithmetic and logarithmic scales. The average diameter and the corresponding standard deviation were determined.

#### *Analytical Ultracentrifugation*

All analytical ultracentrifugation work was carried out in the laboratory of J. Dirks, on the model E Spinco ultracentrifuge equipped with Schlieren optics. Rotor An-D was used; the KEL-F cell with a 12-mm path was completely filled. All centrifugations were done at  $20^{\circ}\text{C}$  at 17,980 rpm and photographs were taken every 4 min. For each sample analyzed, two to four different runs at different glycogen concentrations were carried out. The sedimentation coefficients were evaluated by extrapolation of the values obtained for finite concentrations (2.5–10 mg/ml) at infinite dilution. The sedimentation coefficient distribution was established by choosing on the Schlieren sedimentation curve successive points which

were converted into sedimentation coefficients according to Nichols and Bailey (1960).

#### *Chemical Analysis*

*Glycogen* in the different fractions obtained after centrifugation was detected by the classical iodine-iodide solution (Lugol). Glycogen content was determined by colorimetric methods: the anthrone method of Roe (1955) was applied when no reducing substances other than glycogen were present; the Krisman method (1962) was used when sucrose was present, namely in the fractions collected from a sucrose-density gradient. A total balance was established for the purpose of specifying the recovery obtained at each step of the fractionation experiment. For further characterization of the polysaccharide, spectrophotometric analysis of the complex formed with iodine (Larner, 1951) was necessary. In cases specified in the legends of the figures, the intensity of the absorbance was increased with calcium chloride at half or total saturation (Krisman, 1962). To obtain an appreciation of the external chain length of the polysaccharide, we added ammonium sulfate to the iodine complex (Schlamowitz, 1951). A DB Beckman spectrophotometer equipped with a recording system was used for following the increase and shift of the maximum absorption ( $\lambda$  max) between 360 and 600  $m\mu$  wavelength.

*Protein content* was estimated in the successive separation steps by Lowry's colorimetric method (Lowry et al., 1951). The data obtained for the DOC-treated glycogen samples were verified by determination of the protein nitrogen content with the micro-Kjeldahl method.

*Nucleic acid contamination* was tested by submission of the glycogen-sarcovesicular fraction and the purified glycogen to the orcinol technique for ribonucleic acid determination (Schneider, 1945) and the drawing of a UV absorption curve for each sample.

## RESULTS

### *Localization and Morphology of Glycogen in the Adductor Magnus*

The observations on the localization and morphology of the glycogen particles in the particular type of muscle studied are essential for appreciation of the difficulties which may be encountered during the extraction procedure. A general view in the electron micrograph of Fig. 1 shows clearly the enormous mass of contractile and vesicular structures to be discarded before the glycogen particles are obtained in a pure form.

Glycogen appears as distinct electron-opaque



FIGURE 1 Electron micrograph of a longitudinal section of the adductor magnus of rabbit. The dense, round, glycogen particles (*gl*) are accumulated in the sarcoplasm at the level of the I band; the boundaries of this band correspond to the flattened tubules of the transverse (*T*) system. Some particles are regularly aligned and form beadlike strings which infiltrate the myofibrils in between the myofilaments. The particles which cover part of the surface of the sarcoplasmic vesicles (*sv*) show in some rare cases (arrows) an intimate contact with the outer side of the smooth membrane.  $\times 60,000$ .

particles found free in the sarcoplasm (Fig. 1, *gl*) and not bounded by a membrane or enclosed in cell organelles. When the particles are relatively abundant, as in white fibers, they are present predominantly in the sarcoplasm, in the region of the I bands, close to the sarcotubular system. Most of the particles which are localized along the smooth-surfaced sarcoplasmic vesicles or tubules seem to be free of any firm or intimate connection with the outer side of the membranes. In rare cases, particles lie in contact with the surface of the sarcoplasmic membrane (arrows), although it is difficult to assess the respective positions of the membrane and the particles in material examined in sections. In some regions, particles are packed in a more or less regular pattern. When aligned, the particles are very close to one another. Although the various arrangements could be dictated

by the restricted space available, the presence of a small bridge between the units cannot be excluded. Similar arrangements in single file, like beads in a string, are present in between the myofilaments.

These elementary particles show a striking regularity in size and shape, and they do not tend to form clusters or rosettes as do the  $\alpha$ -particles found in liver cells or in some other tissues. The average diameter, calculated from the measurements of 500 particle diameters, is  $27.3 \text{ m}\mu$  and the standard deviation is  $3.0 \text{ m}\mu$ . A similar disposition, morphology, and size distribution will be described below for sections of the glycogen-sarcovesicular fraction, illustrated for comparison in Fig. 2.

More precise size measurements and the corresponding graphic representations were carried out after negative staining of the purified material

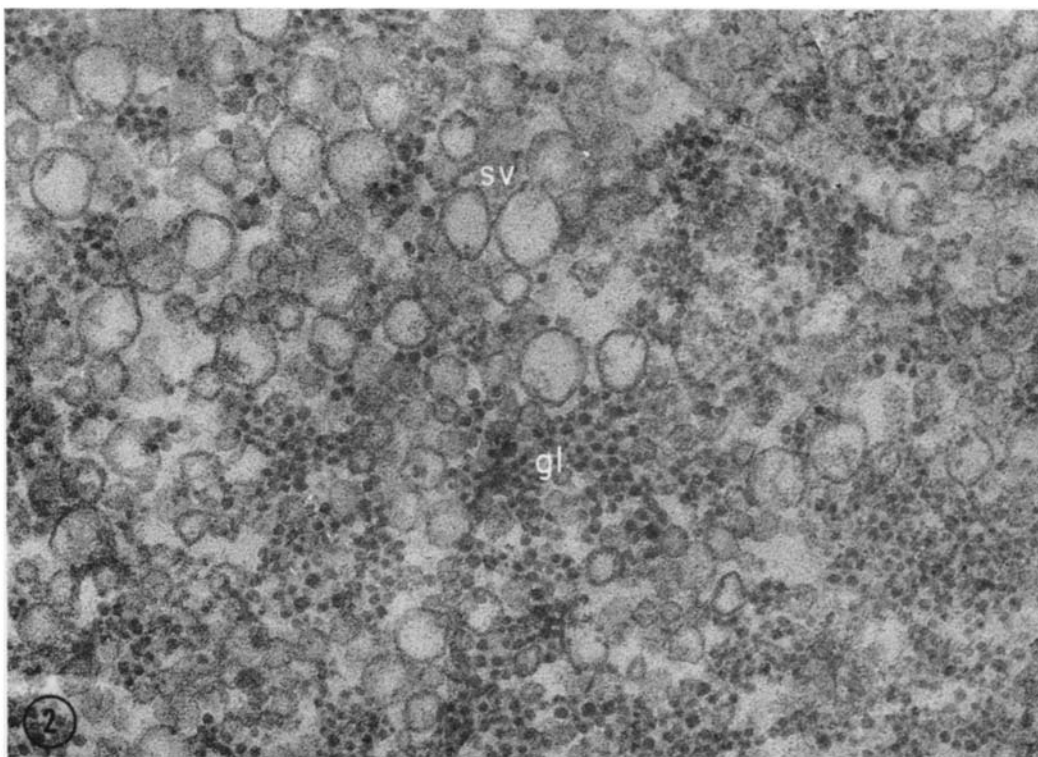


FIGURE 2 Electron micrograph of the glycogen-sarcovesicular fraction obtained after a preliminary centrifugation of the muscle homogenate. In this region of the fixed and sectioned pellet, the glycogen particles (*gl*) are abundant. Smooth-walled vesicles are present (*sv*).  $\times 60,000$ .

(see below). The relatively homogeneous size and shape of the particles observed in the sections are favorable to a simple sedimentation behavior of these particles during centrifugation.

#### *Preparation of a Glycogen-Sarcovesicular Fraction*

Different methods of homogenization of muscle tissue were tested: namely, the use of a mortar, a mortar followed by a Potter homogenizer, and a Waring Blender. The efficiency of the two first procedures is low compared to the use of a Waring Blender. Used for short periods of homogenization, not exceeding 1 min, this instrument does not alter mechanically the glycogen particles. 30 sec for homogenization were found optimal; longer times result in increased difficulties during the purification of the final glycogen pellets.

The technique which was adopted in our experiments is summarized in Fig. 3 and is described here. Fragments of muscle were homogenized in

an ice-cooled Waring Blender with an appropriate proportion of phthalate buffer at top speed for 30 sec. The pH of the homogenate was maintained at  $6.0 \pm 0.1$  (this pH value is critical, since slight changes cause massive agglutination of the myofibrils or myofilaments and may carry away a high proportion of glycogen). All manipulations were carried out in a cold room, instruments and solutions being kept constantly in ice. The homogenate was distributed in 250-ml centrifuge bottles and centrifuged for 15 min in an International Centrifuge, model PR 1, head 284, at 1,400 *g*. The sediments examined with the phase-contrast microscope consisted of agglutinated fragments of myofibrils and cell organelles and were discarded. The supernatant, containing 75-80% of the total glycogen present in the homogenate, was filtered through folded gauze for elimination of part of the solidified fat. It was centrifuged in a No. 30 rotor (Spinco, model L centrifuge) at 67,500 *g* (calculated at the bottom of the tubes) for 150 min. The

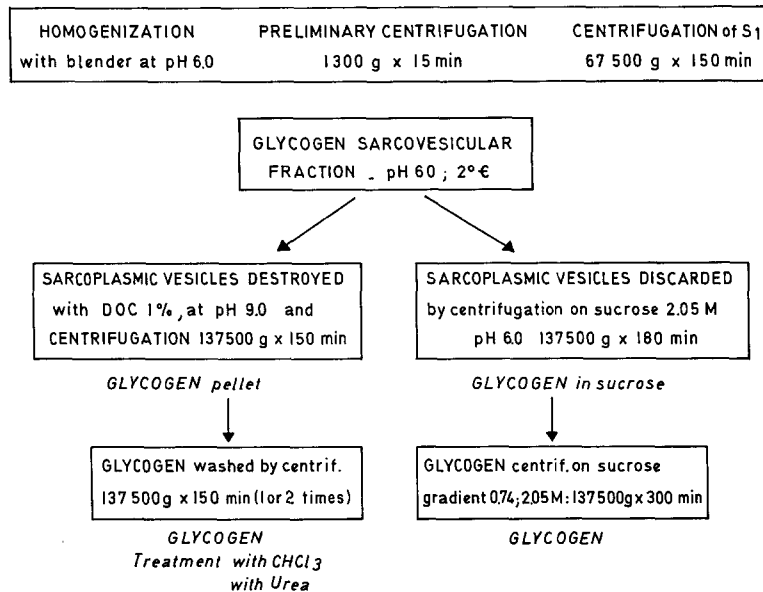


FIGURE 3 Schematic representation of the successive steps in the preparation of a purified glycogen fraction.

pellet, called *glycogen-sarcovesicular fraction*, is composed of two parts: a sticky, opalescent bottom part and a dense, milky, superficial layer which easily slides off when the centrifuge tube is inclined. These two parts of the glycogen-sarcovesicular fraction are different in composition, as shown in the morphological observations, but both of them contain glycogen. They were, therefore, mixed and resuspended in distilled water. 70% of the glycogen present in the first supernatant was recovered in the total glycogen-sarcovesicular fraction. At this stage of the fractionation, the preparations contain equivalent amounts of glycogen and protein, so that the protein-to-glycogen ratio is 1 (Table I).

**MORPHOLOGY OF THE GLYCOGEN-SARCOVESICULAR FRACTION:** Fig. 2 illustrates the morphology of a section through a small sample of a glycogen-sarcovesicular pellet. Two structured components are present: smooth-walled vesicles (*sv*) and particles (*gl*) which correspond to glycogen. This view is probably representative of the deep regions of the pellet, where glycogen is relatively abundant. In order to determine the proportion of these two components and the specific characteristics of the two parts of the glycogen-sarcovesicular pellet, we examined negatively stained preparations of each part. Fig. 4 corresponds to the superficial layer and is com-

posed mainly of vesicles (*sv*) and of particles (*gl*) having the characteristics of  $\beta$ -glycogen particles. The surface of these particles is irregular, probably loaded with contaminating material. Some particles form beadlike assemblies (Fig. 4, arrows). The presence of polysomes in this fraction is excluded, however, since the orcinol test is negative and no absorption occurs at  $260\text{ m}\mu$  in the UV spectrum. In the bottom part of the glycogen-sarcovesicular fraction (Fig. 5) the proportion of particles found is reversed: vesicles (*sv*) and other cell debris (*mi*) are less frequent, but glycogen particles (*gl*) are the main component.

A closer examination of the superficial layer of the pellet, rich in sarcoplasmic vesicles, reveals the existence of particle-membrane relationships, which are too characteristic and too frequent to be neglected. Figs. 6-11 represent areas which have been selected for the illustration of different examples of particles closely associated with membranes. Particles may be partly enclosed in depressions of the vesicle surface (Fig. 6, arrows) or may be connected to a protrusion of the membrane, suggesting a sucking spot (Figs. 8 and 9, arrows). In these same preparations, some particles are linked one to another and form strings of two to four beads.

Similar observations have been made on glyco-

TABLE I  
Glycogen and Protein Content of Different Fractions obtained by Centrifugation of a Muscle Homogenate

Fractions	Glycogen content		Protein content		Protein:glycogen ratio
	mg/g muscle	% of total	mg/g muscle	% of total	
Supernatant of preliminary centrifugation	5.0-8.0	100	10.0-15.0	100	—
Glycogen-sarcovesicular fraction	3.5-5.5	70	3.5-5.0	30-35	1
Glycogen after:					
DOC	3.00-4.80	60	0.15-0.50	1.5-3.0	0.05-0.15
DOC/washing	2.75-4.00	50-55	0.05-0.15	0.5-1.0	0.03-0.05
Centrifugation on 2.05 M sucrose	1.75-3.00	30-35	0.75-1.50	7.5-10.0	0.30
Centrifugation on sucrose gradient	—	—	—	—	0.05
Glycogen treated with:					
Chloroform	—	—	—	—	0.025-0.030
8 M Urea	—	—	—	—	0.025
KOH 33%	—	—	—	—	0.002

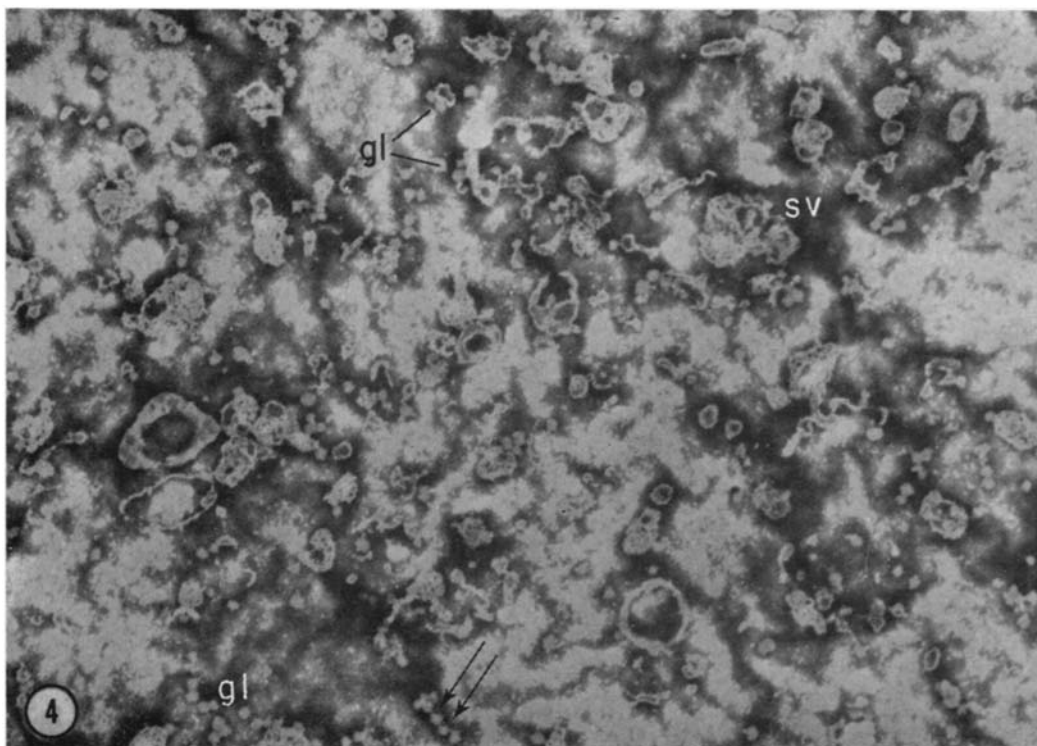


FIGURE 4 Negative staining of the superficial layer of the glycogen-sarcovesicular pellet. The vesicles predominate (*sv*). The particles which have the size and shape of glycogen particles (*gl*) are often in close contact with the vesicle walls. They form little agglomerates or are assembled in short chains (arrows). Note that no nucleic acid is present in this fraction.  $\times 37,500$ .

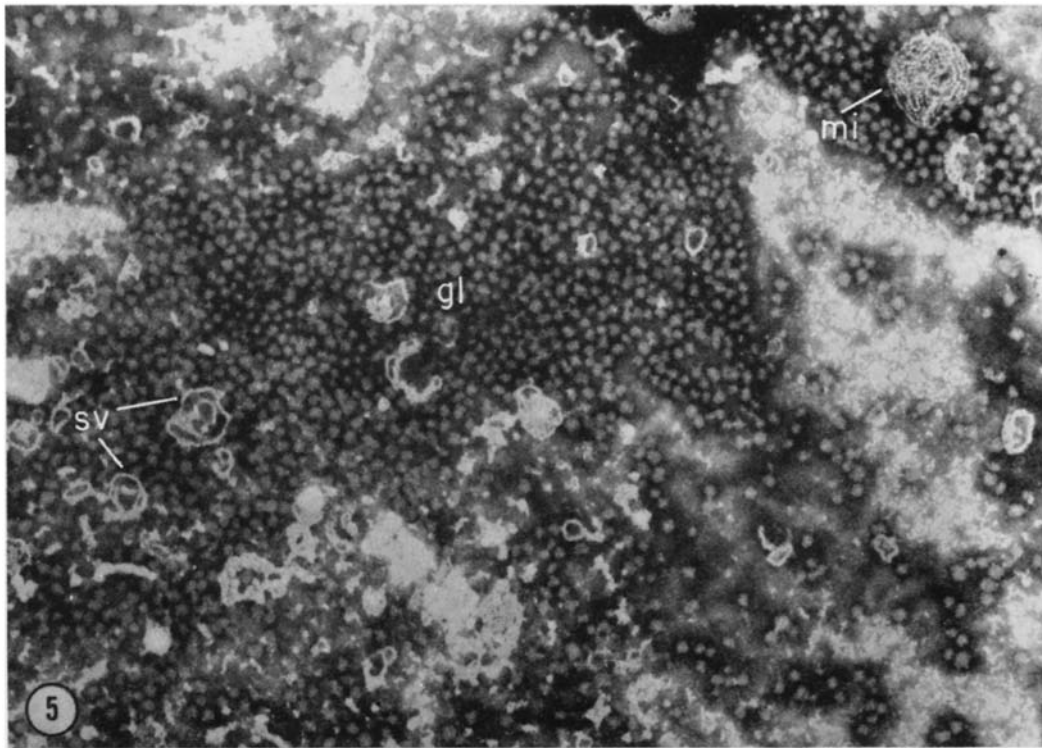


FIGURE 5 Negative staining of the bottom layer of the glycogen-sarcovesicular pellet in which the particles (*gl*) are predominant. Most vesicles are smooth-walled (*sv*), typical of the sarcoplasmic reticulum, and some (*mi*) are dotted with elementary particles, typical of mitochondrial membranes.  $\times 37,500$ .

gen-sarcovesicular fractions isolated after centrifugation on a 2.05 M sucrose solution (Figs. 10 and 11).

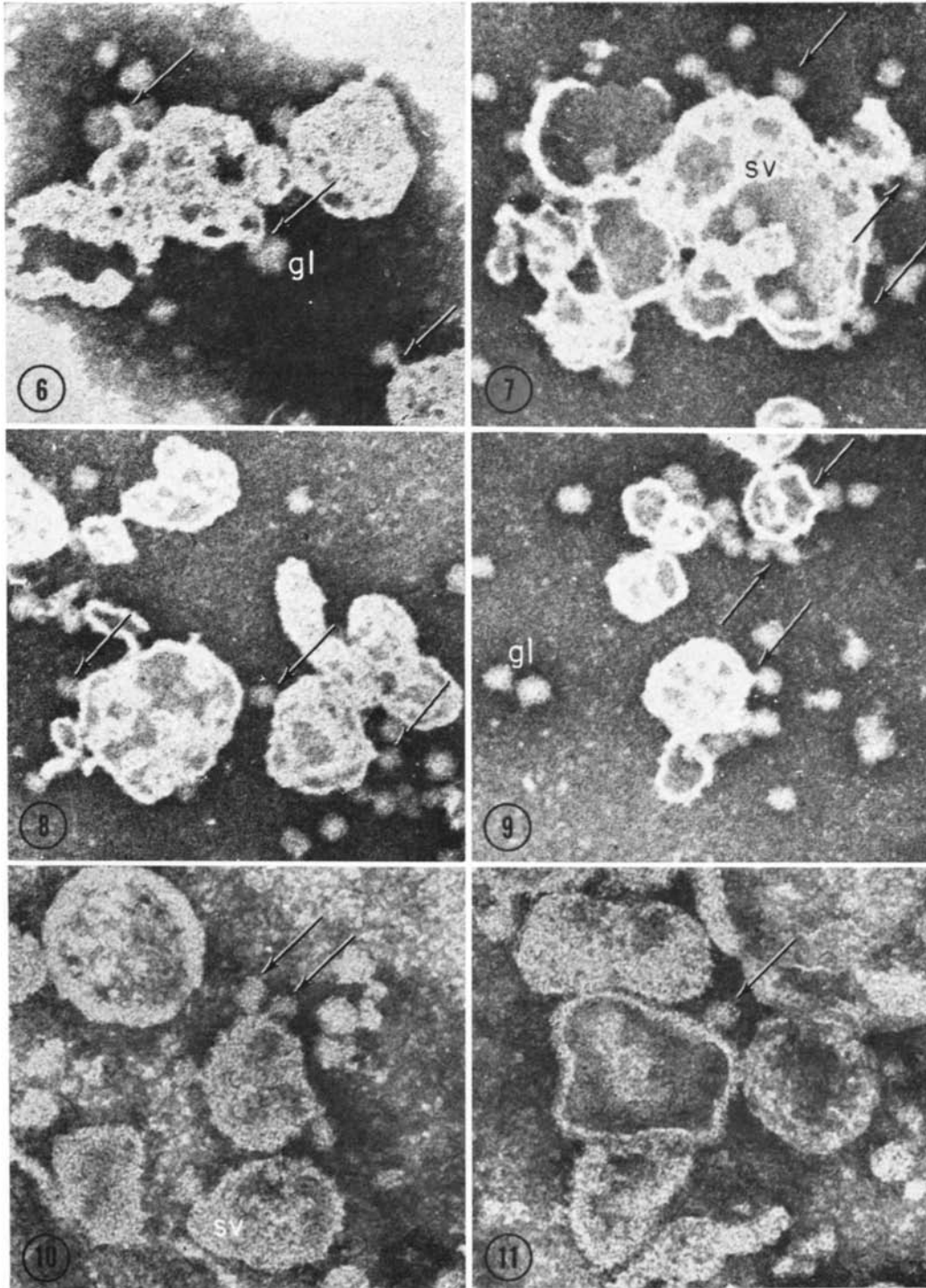
#### *Extraction of Glycogen*

To eliminate the sarcovesicular component of the glycogen-sarcovesicular fraction, we used two different types of treatment: (a) deoxycholate (DOC) at pH 9.0 was added to the suspension; (b) the diluted suspension was centrifuged on a sucrose gradient. In the first case, the glycogen-sarcovesicular pellet was resuspended in 0.1 M glycine at pH 9.0. In the second case, it was resuspended in a 0.25 M sucrose solution.

The separation of glycogen from the glycogen-sarcovesicular fraction by DOC treatment consists of adding sodium deoxycholate at a final concentration of 1% to the resuspended pellet. The suspension appeared immediately less opalescent and was centrifuged in a No. 40 rotor at 137,500 *g* for 150 min. The sedimented material appeared at the bottom as a translucent pellet of glycogen

covered with a thin fluffy layer, easily removed with a Pasteur pipette. Two washings were carried out by successive resuspensions in distilled water and centrifugations. The final sediment appears as a clean, colorless, translucent pellet, and its glycogen content accounts for 60% of that present in the original supernatant of the preliminary centrifugation (Table I). Since successive washings of this glycogen pellet do not change the content of contaminating proteins, further purification could be achieved only by treating the resuspended glycogen with more drastic methods: with chloroform-octanol, with urea at high concentration, or with hot potassium hydroxide. Samples of glycogen were submitted to strong shaking with chloroform-octanol (3:1) according to Sevag (1934), and for long periods, up to three treatments 8 hr each, as recommended by Orrell and Bueding (1958). Other samples were treated with 8 M urea for 3 hr and then dialyzed against distilled water at 2°C. To correlate the morphological observations and the chemical data obtained by the described





FIGURES 6 TO 11 High magnification of selected areas which illustrate contacts (arrows) between particles (*gl*) and vesicle membranes (*sv*). Figs. 6-9 were chosen in preparations similar to that of Fig. 4; Figs. 10 and 11 are negative stainings of sarcoplasmic vesicles obtained by centrifugation of the glycogen-sarcovesicular fraction on 2.05 M sucrose.  $\times 120,000$ .

methods with those published in the literature on glycogen extracted by potassium hydroxide digestion of tissues, we treated particulate glycogen by boiling it with 33% potassium hydroxide for 30 min. The chemical determinations reported in Table I indicate that the protein contamination of glycogen which remains constant after successive washings decreases slightly by the same amount after treatment with chloroform and urea. To obtain glycogen with less than 0.5% protein contamination, a partial degradation with concentrated potassium hydroxide is necessary.

The separation of glycogen from the glycogen-sarcovesicular fraction by centrifugation on a sucrose gradient avoids the use of DOC and was carried out in two steps, as summarized in Fig. 3. First, the sarcovesicular fraction, containing the glycogen, was resuspended in 0.25 M sucrose. 7 ml of this suspension was layered over 3.5 ml of 2.05 M sucrose in tubes of the No. 40 Spinco rotor. After centrifugation at 137,500 *g* for 180 min, the bottom phase (2.05 M sucrose) was collected with a pipette connected to an aspirating syringe (Perfusor, B. Braun, Germany); the upper phase and the material accumulated at the interface of the two solutions of different densities were then discarded. Glycogen was present in the interface material, but 30–40% of the glycogen layered on top of the gradient was recovered in the bottom phase. The latter was diluted with distilled water to give a final 0.25 M sucrose concentration. 0.5

ml of this diluted suspension was layered on top of a linear sucrose-density gradient (the extreme sucrose molarities being 0.75 and 2.05 M) prepared in 5-ml centrifuge tubes of the SW 39 rotor. After a centrifugation at 137,500 *g* for 300 min, the glycogen was banded and distinctly localized, but the contaminating proteins could not be visually distinguished at the concentration which they reached in these experiments. By the collection of successive fractions of 0.25 ml each, from top to bottom, glycogen and protein contents were determined at different levels.

An example of dissociation of protein and glycogen along the gradient is shown in Fig. 12. Most of the protein remains in the upper layers or is detached and tends to “float” in the gradient. At the level at which glycogen is banded, a small peak of protein is present. Within the limits of precision afforded by the technique used, the peaks of both glycogen and protein seem to correspond. The protein:glycogen ratio at this region of maximum glycogen concentration is 0.05 (Table I).

#### *Morphological Characteristics and Size Measurements of the Extracted Glycogen*

Glycogen obtained in a pure form, after DOC treatment and two washings of the glycogen-sarcovesicular fraction, was examined with the negative-staining technique. Fig. 13 illustrates an example of the evenly distributed particles at low magnification. Large fields of this type may be observed and allow counts of particles in sufficient number to determine average diameters and graphic representations.

The particles appear at first sight as elementary round bodies. A closer examination, at high magnification (Fig. 14), reveals the existence of irregularities in the contours of some particles (arrows), most frequently in the larger ones, suggesting that the largest particles are composed of smaller units. Sometimes four to six subunits can be delineated in such compound particles. A fine structure can be observed that remains difficult to be distinguished from the granularity produced by the electron beam. These densifications correspond to  $\gamma$  structures.

The glycogen collected from sucrose gradients presents the same morphological characteristics as that obtained from the DOC-treated fraction described above. Similar subunits may be distinguished within the larger particles.

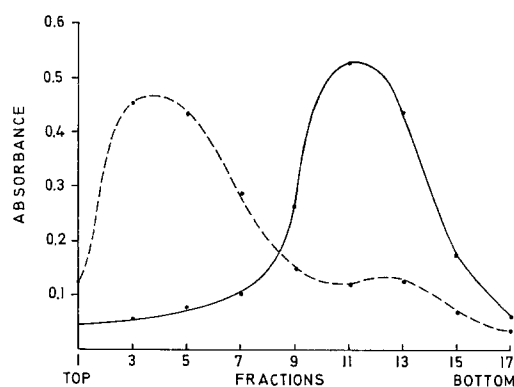


FIGURE 12 Diagram of distribution of glycogen (continuous line) and protein (discontinuous line) in successive fractions of a linear sucrose gradient (0.74 to 2.05 M), centrifuged for 5 hr at 137,500 *g*. The fractions are plotted on the abscissa. The absorbance of the glycogen-iodine reaction and of the Lowry reaction on proteins are plotted on the ordinate.

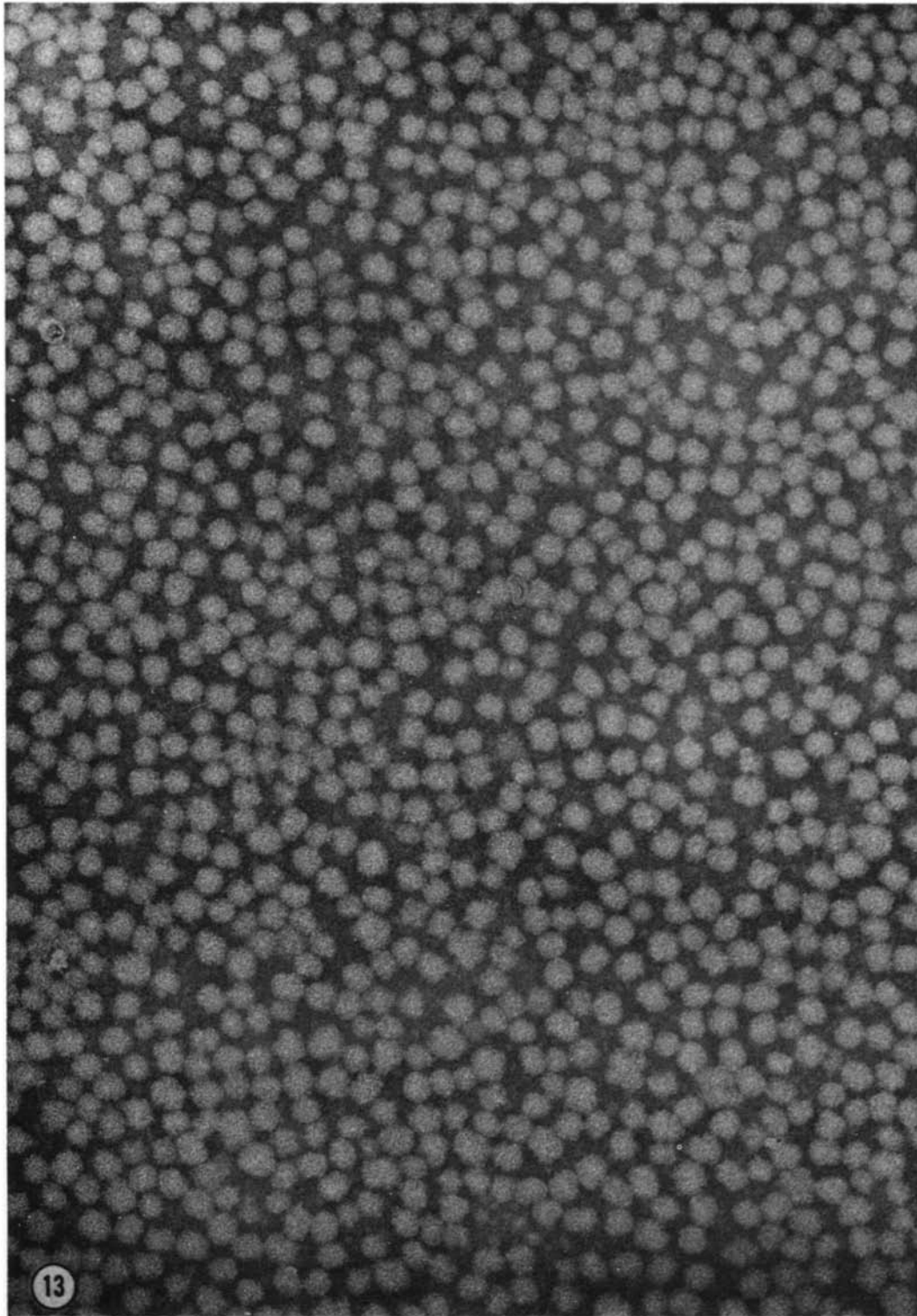


FIGURE 13 Negative staining of purified muscle glycogen  $\beta$ -particles forming a uniform monoparticulate layer.  $\times 100,000$ .

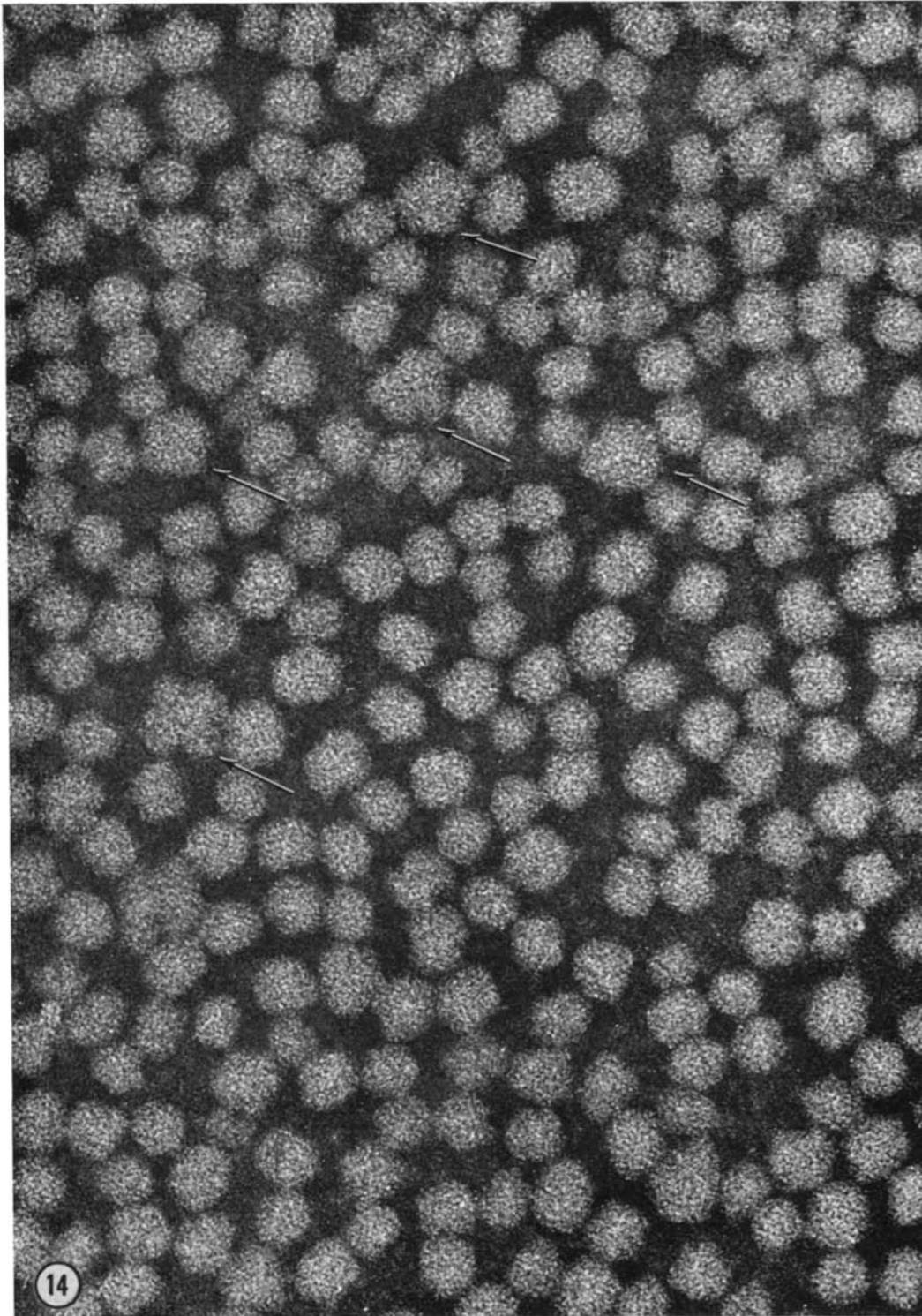


FIGURE 14 Enlargement of a region of Fig. 13. The arrows point to particles in which the subunits may be distinguished. The limits of these subunits are only visible at the periphery of the compound particle, but not in the center.  $\times 200,000$ .

Size-frequency histograms were obtained by measurement of the particle diameter on fields of electron micrographs characterized by a uniform dispersion of the particles, such as those illustrated in Figs. 13 and 14, with a Zeiss particle size analyzer. The values of 2,500 counts, expressed in diameters and divided in contiguous classes, are represented in the histogram of Fig. 15. By plotting the cumulative frequency values on a probability grid (Fig. 16), all experimental points, except the two which correspond to the smallest diameter, fall on a straight line. This is characteristic of a Gaussian distribution. The average diameter, calculated or derived from the probability curve, is  $39.4 \mu\mu$ . The standard deviation,  $\sigma$ , is  $6.8 \mu\mu$ .

Particle measurements carried out on glycogen treated with boiling potassium hydroxide for 30 min give a distribution with a reduced mean diameter and a lesser dispersion (Fig. 17, shaded area). The alkaline treatment seems to reduce the largest compound particles to smaller units. The average diameter is  $34.3 \mu\mu$ .

#### *Analytical Centrifugation Data*

Fig. 18 represents the analytical centrifugation pattern that is characteristic of glycogen extracted by the differential precipitation-centrifugation method used in the present work.

The sedimentation coefficients, calculated for the maximum concentration of particles (peak) on the sedimentation curves, were determined by centrifuging glycogen samples at different concentrations. The values extrapolated to infinite dilution vary between 115S and 135S. No difference was observed between freshly prepared glycogen and glycogen which was lyophilized and stocked for different periods. A glycogen preparation analyzed in the analytical ultracentrifuge, 2 hr after its isolation, had a sedimentation coefficient of 115S; after lyophilization and resuspension, the same value of 115S was determined.

The sedimentation coefficient distribution, corresponding to the Schlieren diagrams of Fig. 18, is represented by the curve in Fig. 19. Its relatively low polydispersion and symmetrical shape are in agreement with the distribution characteristics of the diameters measured on the electron micrographs, although, a serious discrepancy exists in the mean values obtained by the two methods. The peak at 135S of the distribution curve corresponds to  $20\text{-}\mu\mu$  particle diameter compared to the  $39.4\text{-}\mu\mu$  mean diameter measured on negatively stained

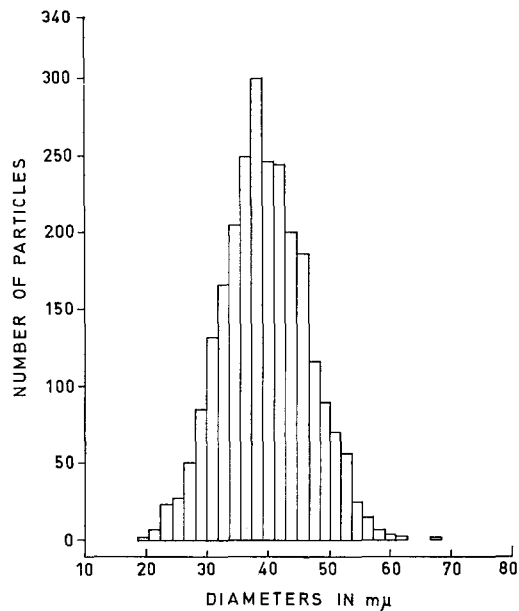


FIGURE 15 Size-frequency histogram of muscle glycogen  $\beta$ -particles. 2,500 particle diameters have been measured and distributed in successive contiguous classes with the aid of a Zeiss particle size analyzer.

particles. The extreme values of the sedimentation coefficients are about 60S for the smallest particles and 200S for the largest.

Particulate glycogen, treated with boiling, concentrated potassium hydroxide, undergoes a reduction in size (see size measurements), and its sedimentation coefficient decreases to about 100S, which is lower than the values obtained for the untreated samples studied.

#### *Spectrophotometric Analysis*

The spectral analysis of glycogen-iodine complexes, carried out on our purified glycogen preparations, shows differences in the absorbance and response of the iodine color reaction upon the addition of ammonium and calcium salts. A typical absorption curve of a glycogen-iodine complex is given in Fig. 20. The peak of absorption is not sharp and may be estimated to be about  $515 \mu\mu$ . The presence of contaminating proteins interferes with the iodine spectrum and may considerably weaken the color reaction. The characterization of glycogen samples, therefore, is possible only when glycogen is in its purified form and not when it is in its crude form mixed with an excess of other cell organelles or muscle proteins.

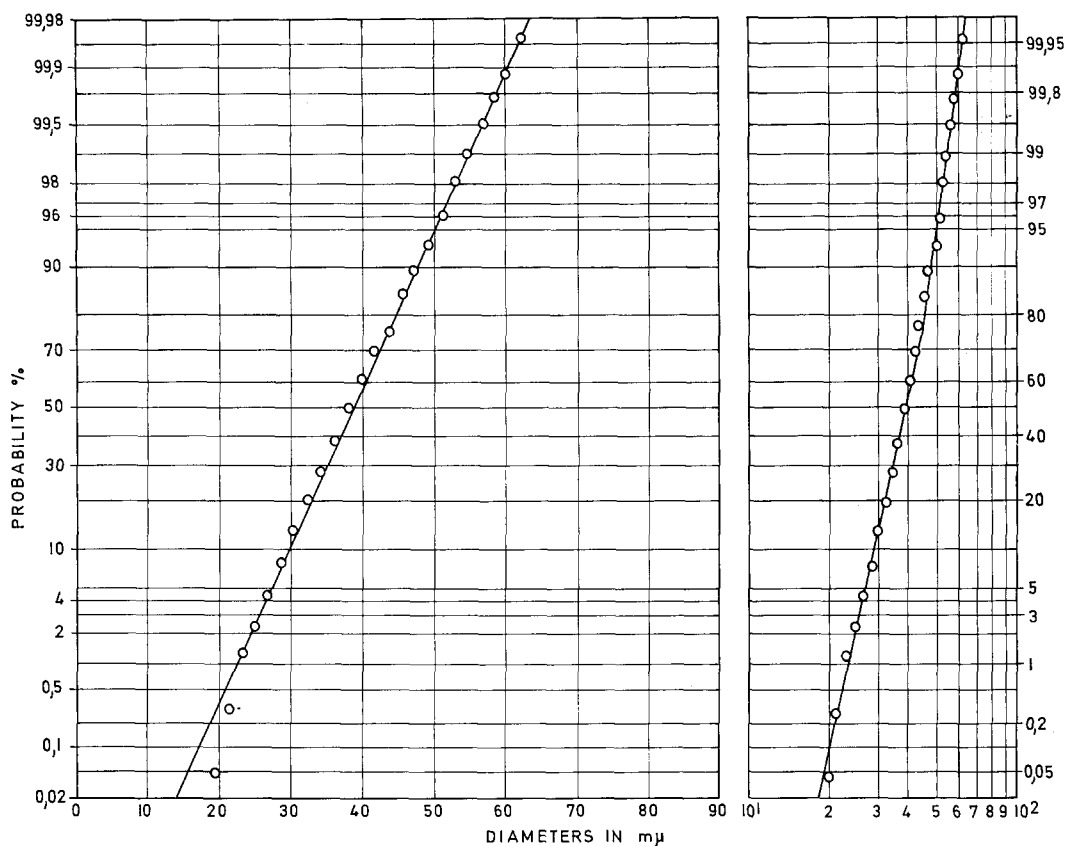


FIGURE 16 Probability plot of the cumulated size-frequencies plotted on an arithmetic abscissa (left diagram) and on a logarithmic abscissa (right diagram).

The addition of ammonium sulfate to the iodine reagent produces a considerable increase of the absorption in the visible light spectrum, between 400 and 600  $m\mu$  (Schlamowitz, 1951; Archibald et al., 1961). This is only valid for glycogen which has not been degraded to its  $\beta$ -dextrin. Thus this difference in reaction has been used to evaluate the degree of preservation of the molecules. The usual response of muscle particulate glycogen to iodine, in the presence of ammonium sulfate, is illustrated in Fig. 21. The increase of the absorbance varies with the glycogen preparation used (curves *II* and *III*). In one of our early preparations, made under conditions in which temperature was not carefully controlled, a partial degradation had taken place: this resulted in a lack of response of the glycogen-iodine complex to saturated ammonium sulfate (curve *I*, Fig. 21). Only a slight absorption exists around 410  $m\mu$ , which is

characteristic of glycogen with reduced outer chains.

The effect of calcium chloride on the glycogen iodine complex is also dependent upon the structural characteristics of the glycogen molecules (Krisman, 1962). The particulate glycogen prepared from muscle responds to calcium chloride by an increase of the absorption in the visible light region of the glycogen-iodine spectrum and by the appearance with saturated calcium chloride of a peak located between 475 and 485  $m\mu$ . A second, less marked peak exists in the region of 410–420  $m\mu$  (Fig. 22). This is characteristic of unaltered glycogen (Drochmans, 1965). The partially degraded glycogen, which did not respond to ammonium sulfate (curve *I* of Fig. 21), also behaves differently in the presence of different concentrations of calcium chloride (Fig. 23). The increase in absorption over the entire spectrum is less marked and the absorp-

tion peak at 485  $m\mu$  is of lower intensity than the peak at 410  $m\mu$ .

#### DISCUSSION

The localization of glycogen particles in the sarcoplasm, in between the myofibrils, has been

described frequently in the striated skeletal muscle tissue of various species. The frequent topographical relation existing between glycogen particles and the sarcoplasmic reticulum leads us to search for the type of morphological and biochemical significance which may be invoked for the justification of a particle-membrane relationship. From the morphological point of view, the evidence of a close particle-membrane contact, in negatively stained material, is put forward only with the following restriction. It is known that negative staining may give a higher probability of demonstrating more intimate connections between organelles than ultrathin sections would offer, but fortuitous associations may also occur, namely after adsorption of particulate material on membranous structures. If we accept the theory that in muscle tissue the sarcoplasmic reticulum is an extension or a derivative of the endoplasmic reticulum (Porter, 1961; Ezerman and Ishikawa, 1967), the comparison with other cases of a particle-smooth membrane relationship may be pertinent to remember here. The most classical example is that of the smooth endoplasmic reticulum in liver cells. Porter and Bruni (1959), examining liver of rats treated with 3'-methyl-diaminobenzene, have described the development of smooth-surfaced vesicles, which are derived from the rough endoplasmic reticulum and are present in close relation with the glycogen particles. Since this early discovery, many other similar observations in normal livers (Drochmans, 1960; Millonig and Porter, 1960) and in drug-treated livers (Orrenius and Ericsson, 1966; Jones and Fawcett, 1966) have been reported. A less known example is that described in endometrial cells (Drochmans,

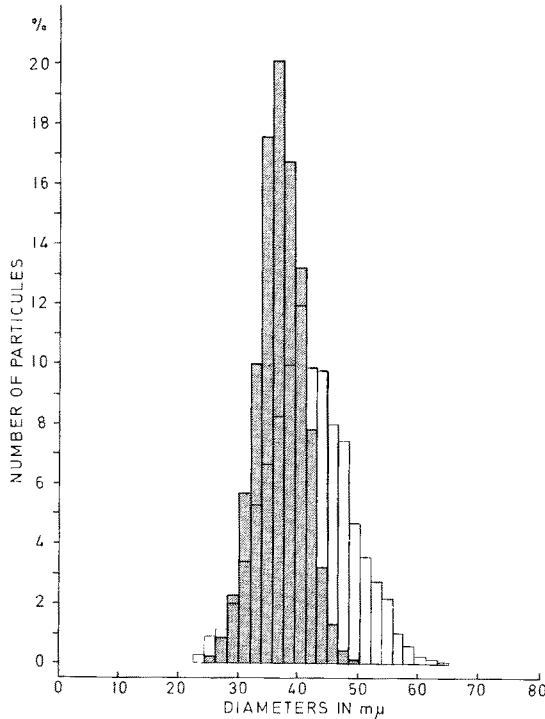


FIGURE 17 Comparison of the size-frequency histogram of KOH-treated glycogen particles (shaded area) with that of the control untreated sample (nonshaded area).



FIGURE 18 Analytical centrifugation pattern of purified glycogen particles. A 1% glycogen aqueous solution was centrifuged in a Spinco model E ultracentrifuge at 17,980 rpm. Photographs were taken at 4-min intervals.



1960), in which mitochondria are wrapped with an ergastoplasmic cisterna. The inner membrane (visceral) of this vesicular envelope which is close to the mitochondria is covered with ribosomes,

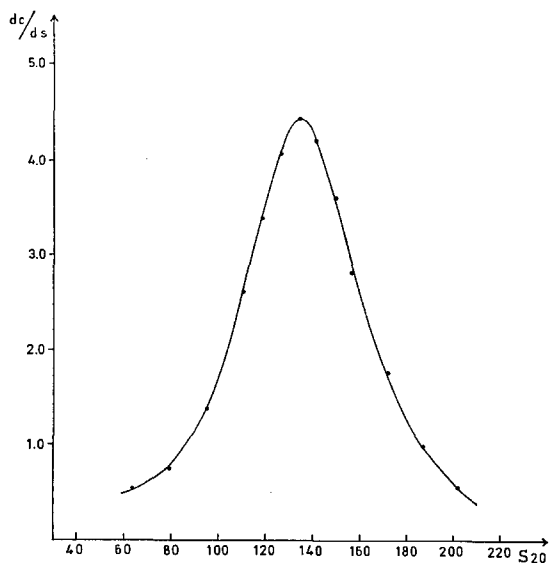


FIGURE 19 Sedimentation coefficient distribution derived from the analytical centrifugation pattern. On the abscissa, the values of the sedimentation coefficients are expressed in Svedberg units (*S*).

whereas the outer (parietal) membrane appears smooth and is in contact with glycogen particles. More recently, "glycogen bodies," which consist of concentric whorls of smooth endoplasmic reticulum cisternae enclosing glycogen particles, have been mentioned in liver cells treated with various drugs (Steiner et al., 1964). A similar glycogen-bearing structure has been found in normal embryonic muscle (Heuson-Stiennon and Drochmans, 1967) at the period of rapid differentiation. All these examples tend to eliminate the possibility that the relation between glycogen particles and their frequent partner, the smooth extensions of the endoplasmic reticulum, is only incidental.

The hope of finding a biochemical dependence between both of these cytoplasmic elements was seriously weakened by Luck's careful work (1961) on the location of the uridine diphosphate glucose-glycogen transglucosylase in liver cell fractions. Further investigations are necessary to determine whether other factors, essential to the synthesis of glycogen particles (i.e. a primer molecule), may be present on particular membrane sites.

The method of glycogen extraction described in the present work is relatively easy and efficient compared to other methods, when one takes into account the difficulty of extraction due to the nature of the skeletal muscle tissue. The final

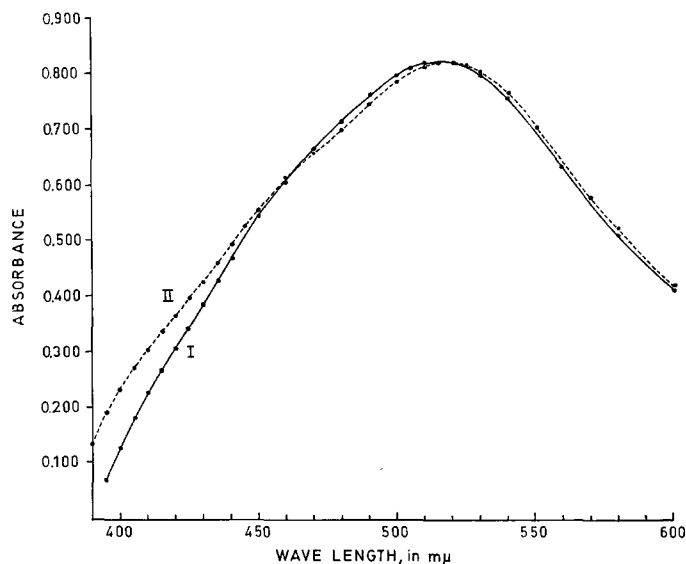


FIGURE 20 Glycogen-iodine spectrum. *I*. Freshly prepared glycogen. *II*. Same glycogen preparation, but after lyophilization.



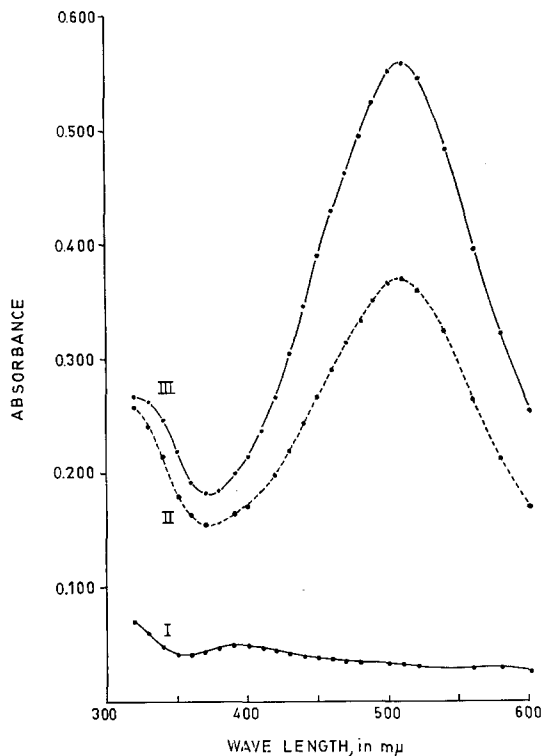


FIGURE 21 Effect of ammonium sulfate at half-saturation on the glycogen-iodine spectrum of three different glycogen preparations: curve I, spectrum of a partially degraded glycogen; curves II and III, spectra typical of well preserved glycogen preparations.

washed fraction of glycogen yields 55–60% of the glycogen present in the original supernatant, and contains only 3% protein, firmly bound to the glycogen particle. It has been shown that these bound proteins have partially kept their phosphorylase activity (Wanson and Drochmans, 1968).

The glycogen particles present in the tissue sections or isolated from the muscle appear as  $\beta$ -type structures according to Drochmans' nomenclature (1962). The particles examined in the electron microscope, with negative contrast, and measured with a Zeiss size-analyzer, form a homogeneous population, which obeys a Gaussian distribution. Subunits may be distinguished within the apparently elementary particles. Two to six subunits have been counted, depending upon the incidence of the electron beam with respect to the particles. Since we deal with a typical normal distribution, we may suppose that the particles are formed of randomly aggregated subunits, so that

the smallest particles measured (20  $m\mu$  in diameter) correspond to monomer and the largest (maximum diameter of 62  $m\mu$ ) to polymer particles. A comparable situation has recently been described by Barber et al. (1965) in glycogen particles isolated from a ciliate, *Tetrahymena pyriformis*, and by Gutman et al. (1967) in glycogen extracted from adipose tissue. Although the morphology of the muscle glycogen  $\beta$ -particles is quite different from that of the  $\alpha$ -particles typical of liver, both types are composed of subunits of a common size, about 20  $m\mu$  in diameter (Drochmans and Dantan, 1968). An intermediate type of particle has been described by Heuson-Stiennon and Drochmans (1967) in glycogen extracted from embryonic muscle tissue with the precipitation-centrifugation technique. This form seems to be labile and is only visible during a short time at the end of the embryonic life.

The sedimentation coefficient of 115S–135S determined for rabbit muscle glycogen at the peak of the sedimentation Schlieren curve is equal to or slightly higher than that mentioned generally in the literature. A value of 100S can be deduced from the sedimentation diagrams published by Orrell et al. (1964). Determinations carried out on glycogen  $\beta$ -particles extracted from other sources, e.g. *Ascaris* muscle (Orrell et al., 1964), *Tetrahymena* (Barber et al., 1965), and adipose tissue (Gutman et al., 1967), give similar values. Only phytyglycogen, which also consists of  $\beta$ -particles, has been found to present higher mean sedimentation coefficients, of the order of 220S (Madsen and Cori, 1958).

The treatment of the glycogen particles with concentrated potassium hydroxide reduces the size of the particles. This confirms earlier morphological observations (Revel, 1964; Barber et al., 1965) and well established biochemical findings on molecular weight determinations. Stetten and Stetten (1957) have compared, by light-scattering measurements, glycogen extracted from rabbit muscle with cold TCA and glycogen extracted with potassium hydroxide. Cold TCA extraction produces glycogen with a molecular weight of  $11.9 \times 10^6$ ; with potassium hydroxide extraction, the molecular weight is  $3.1 \times 10^6$ . The exposure to strong alkali may cause a dissociation of the particles into their constituent subunits by breaking glucosidic bonds. This might be the case in our assays, because only the largest particles were reduced in size. This reduction in size and in molecular weight

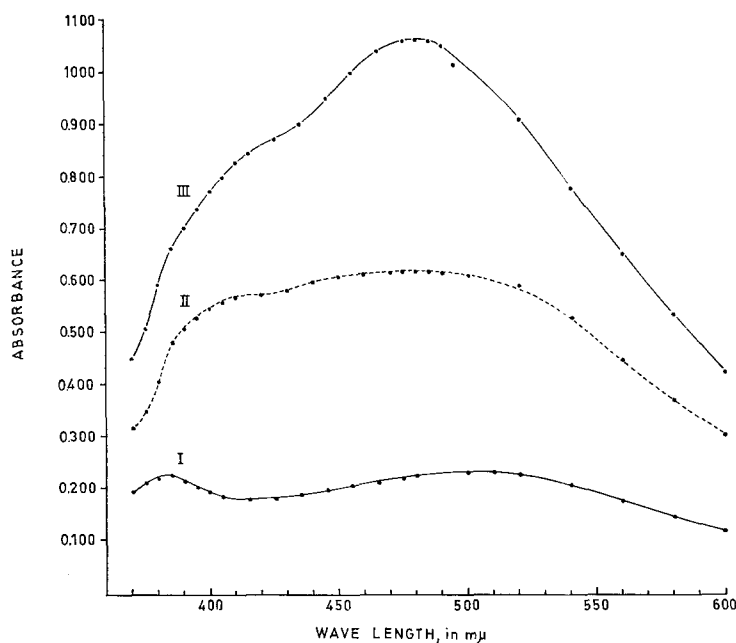


FIGURE 22 Effect of calcium chloride on the glycogen-iodine spectrum of well preserved glycogen preparations: curve *I*, reference, no salt added; curve *II*, half-saturation of calcium chloride; curve *III*, saturation of calcium chloride.

of the particles, essentially by dislocation of the macromolecular structure at the weakest points, may be complicated by the splitting of chains at other sites. This may explain the further breakdown of the molecules when potassium hydroxide treatment is applied to glycogen for longer periods of time (Barber et al., 1965).

The comparison of molecular weights derived from sedimentation coefficient determinations and particle sizes determined by diameter measurements reveals serious discrepancies between the two methods of size evaluation. Laskov and Gross (1965) have proposed a model for  $\alpha$ -particles extracted from liver and a formula to correct the flattening of the particles in shadowed preparations. These corrections may help in fitting together the values obtained by morphological observations and physical determinations; but for small-size particles, such as  $\beta$ -particles in muscle, we have not introduced the proposed correcting factors. The 50% difference in average diameters obtained by the two methods in the present work cannot be corrected only on the basis of a flattening of the particle. Other factors interfere, such as the shrinking of the film of stain during drying and, perhaps to a greater extent, the effect of the

irradiation by the electron beam on the dense film of phosphotungstate.

The absorption spectrum, with a maximum absorbance around 515  $m\mu$ , obtained for  $\beta$ -muscle glycogen treated with iodine characterizes the rabbit glycogen (Archibald et al., 1961). It is well known that rabbit glycogen has particularly long end-chains of mean length of 16–18 glucose units, compared to usual values of end-chains of 12 glucose units giving a maximum absorption of the glycogen iodine complex in the range of 390–420  $m\mu$ . Particulate glycogen extracted by centrifugation with the technical precautions proposed above also differs from chemically extracted glycogen and from glycogen slightly degraded during preparation (see curve *I*, Fig. 21 and curves of Fig. 23), in that it gives a clear response to the addition of ammonium sulfate or calcium chloride to the iodine reagent. This observation confirms previous similar findings reported for the  $\alpha$ -particles (Drochmans, 1965).

The 3% protein contamination of the glycogen preparations which resists washings with water seems firmly bound to the muscle  $\beta$ -particles. This result is in opposition to the very easy purifications of liver  $\alpha$ -particles (Lazarow, 1942; Drochmans,

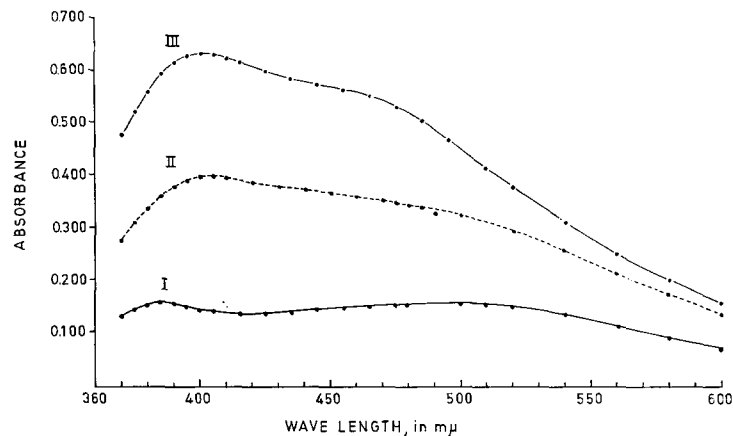


FIGURE 23 Effect of calcium chloride on the glycogen-iodine spectrum of a partially degraded glycogen preparation: curve *I*, reference, no salt added; curve *II*, half-saturation of calcium chloride; curve *III*, saturation of calcium chloride.

1963), since three successive washings with water or aqueous buffer solutions eliminate practically all protein residues from the glycogen pellets. Protein-denaturing agents, urea and chloroform, shaken for a few hours in the presence of the glycogen solutions reduce the protein content only slightly. In order to obtain less than 3% protein contamination, treatment for some days with chloroform, or eventually with concentrated potassium hydroxide, is necessary. It may be concluded that the proteins bound to the muscle  $\beta$  glycogen particles free of debris of cell organelles (checked with the electron microscope) are more

abundant and more firmly bound than the protein residues in liver  $\alpha$ -particles.

The authors wish to thank Dr. J. Dirckx for having carried out the analytical ultracentrifugation and Dr. M. P. Beumer-Jochmans for the lyophilization, of different glycogen specimens. They are indebted to Mr. J. Verheyden and Mrs. A. Popowski for valuable technical assistance.

This work was supported in part by the grant No. 862/1967 of the "Fonds de la Recherche Scientifique Médicale".

Received for publication 4 January 1968.

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