MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GLYCOGEN PARTICLES ISOLATED FROM RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

The fine structure of ascitic cells, consisting of 87–92% heterophil, 5–10% eosinophil leukocytes, and 3% macrophages, is well preserved by glutaraldehyde-osmium tetroxide fixation only when the osmolality of the fixative is appropriately balanced. The β-glycogen particles, 35–45 μm in diameter, are found either as large accumulations in the perinuclear region or in a dispersed form in peripheral cytoplasm. In the heterophils, they are embedded in a coarse-grained ground substance. Extraction and purification of the glycogen were performed by differential precipitation-centrifugation. Yield (35% recovery), purity (4% protein contamination), and preservation of a high sedimentation coefficient (240S) represent the main advantages of the proposed procedure. The analysis of the profile of the sedimentation curve, together with an analysis of the particle size measurements and of particle fine structure, leads to the conclusion that the β-particles form a homogeneous population with a gaussian distribution curve. Each particle consists of smaller units which increase in number with increasing size, the largest ones taking on the appearance of small rosettes. The glycogen particles of the microsomal fraction, still loaded with phosphorylase, were submitted to a synthetic activity by incubation in the presence of glucose-1-phosphate. The analysis of the particle growth shows that particles of all sizes respond equally well.

INTRODUCTION

The polymorphonuclear (PMN) leukocyte, because of its physiological importance in the living body and its relatively easy accessibility, represents an interesting cell to know in its different aspects. Previous electron microscope studies have described in much detail the maturation of the heterophil (Bainton and Farquhar, 1966) and eosinophil (Bainton and Farquhar, 1970) leukocytes, the morphological characteristics of their granules (Baggiolini et al., 1970), and also the modifications encountered as a result of various disease states. Most of these works mention the presence of glycogen but place no special emphasis on this particular cytoplasmic component. But other studies deal more specifically with the glycogen macromolecular state (Anderson, 1966; Watanabe et al., 1967; Scott, 1968; Scott and Still, 1968) or with metabolic disturbances which affect the glycogen content of the white blood cells (Wagner, 1946; Valentine et al., 1953).

It is the purpose of the present study to analyze further the location and fine structure of the glycogen particles in ascitic leukocytes. It will be shown that the precipitation-centrifugation
method applied to the leukocyte homogenates allows a good recovery of the polysaccharide and preserves the morphological, physical, and biochemical characteristics of the particles.

**MATERIALS AND METHODS**

**Separation and Electron Microscopy of Leukocytes**

Rabbit leukocytes were obtained from glycogen-induced peritoneal exudates, according to the method of Hirsch and Church (1960). The glycogen (commercial GBI glycogen, General Biochemicals, Chagrin Falls, Ohio) used was of very low molecular weight. The ascitic fluid was collected directly in an equal volume of 2% chilled distilled glutaraldehyde buffered with 0.2 M phosphate, pH 7.2. The final osmolality of the fixative was 315 milliosmols. Volumes of this suspension were spun at low speed (40 g for 3 min at 4°C) to obtain cell sediments about 1 mm thick. Fixation of the cell pellets was continued for 3 hr in renewed glutaraldehyde fixative. After 2 hr postfixation with 1% osmium tetroxide, buffered at pH 7.2 with 0.1 M phosphate, they were cut into small blocks and progressively dehydrated in graded ethanol solutions. The selected specimens were embedded in Epon 812. Ultrathin sections, prepared on an ultratome III (LKB Produkter, Stockholm, Sweden), were stained with lead citrate (Reynolds), after 30 min of treatment with an aqueous solution of 1% uranyl acetate, and examined in a Siemens Elmiskop I.

The fixation technique as recommended above was derived from a series of preliminary experiments in which different technical conditions were tested: glutaraldehyde concentrations and final osmolality of the fixative, ranging from 250 to 550 milliosmols; and buffers of different concentrations, 0.05, 0.075, and 0.1 M phosphate, and also 0.1 M cacodylate.

**Isolation of Glycogen Particles from Leukocytes**

The leukocytes separated by low speed centrifugation (see above) were resuspended in 0.1 M phosphate, pH 6.0, containing 0.06 M sodium fluoride (Merck, Darmstadt, Germany) and homogenized in a Potter, size B, homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) for 10 min at 4°C. One or two centrifugations at 2500 rpm for 15 min (International centrifuge, model PR 1, head 269, International Equipment Co., Needham Heights, Mass.) were carried out to discard cell debris, nuclei, and mitochondria. The supernatant was centrifuged at 30,000 rpm (Spinco, model L centrifuge, No. 30 rotor, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) for 150 min in order to obtain a microsomal fraction rich in glycogen particles: the so-called glycogen-vesicular fraction (G.Ve.F.). These pellets were resuspended in 0.1 M glycine buffer at pH 9.0 to protect them against enzymic degradation. A purified glycogen preparation was obtained by treating the G.Ve.F. by two different means: (a) by addition of sodium deoxycholate (DOC; Merck, Darmstadt, Germany), and (b) by zonal centrifugation on a linear sucrose density gradient (0.7–2.0 M, containing 0.01 M imidazole buffer, pH 7.0), at 25,000 rpm for 5 hr in 30-ml centrifuge tubes of the SW 25 rotor (Spinco, model L centrifuge).

**Biochemical Determinations**

The glycogen content was estimated by the Krisman method (Krisman, 1962) in different subcellular fractions obtained by differential centrifugation of the homogenate and along the density gradient after zonal centrifugation of the G.Ve.F. On the same fractions the protein content was determined by the Folin-phenol colorimetric method (Lowry et al., 1951).

The phosphorylase activity, expressed in micromoles of free phosphorus liberated from the glucose-1-phosphate per milligram of glycogen tested per minute, was measured by the Hers technique (1964), which requires the addition of standard commercial glycogen as primer.

The morphological demonstration of the remaining phosphorylase activity localized on the glycogen particles was carried out on glycogen preparations without the addition of extraneous primer. Each assay was controlled by determining the inorganic phosphorus (Fiske and SubbaRow, 1925) released by the reaction and by submitting the synthesized products to a spectrophotometric analysis (Larner, 1951).

**Negative Stain**

Different fractions containing glycogen were resuspended in 3% sodium phosphate buffer (Rietel de Haan, Hanover, Germany) adjusted to pH 7.2 with NaOH. Diameter measurements were performed with the Zeiss TGZ 3 particle size analyser (Carl Zeiss, Oberkochen, West Germany), previously used for rabbit skeletal muscle glycogen (Wanson and Drochmans, 1968). The recorded data were used to draw size distribution histograms and to calculate the average diameter and the standard deviation. Probit curves were established by plotting the cumulative frequency values on probability grids.

**Sedimentation Coefficient Determinations**

The sedimentation coefficients of three types of β-glycogen particles (commercial GBI glycogen, native muscle glycogen, and native leukocyte glycogen) were calculated as proposed by de Duve et al.
from the distribution glycoprotein profiles obtained after centrifugation on linear sucrose gradients. The scale of sedimentation coefficients ranging from 40S to 280S was determined along the gradients.

RESULTS

Morphology and Location of Glycogen in the Ascitic Cells

Among the cells isolated from the aseptic peritoneal exudates, two types of leukocytes, the heterophil and the eosinophil, may be distinguished. They form 97% of the total cell population.

The heterophil leukocyte (Fig. 1) constitutes the major cell type of this preparation. Its classical morphological characteristics have been described in many previous works: a multilobulated nucleus rich in condensed granular chromatin; azurophil and specific granules; scarce, small mitochondria; and few endoplasmic reticulum cisternae dispersed in a coarse-grained "ground substance" (gs, Fig. 1) in which glycogen particles are embedded. The eosinophil leukocyte is also a well-known characteristic cell (Bainton and Farquhar, 1970).

What seem to be worth a renewed description are the cytoplasmic structures which may have some bearing on glycogen metabolism or may interfere with the isolation technique described later in the present paper. In the heterophil leukocyte the coarse "ground substance" which fills the spaces between the cytoplasmic organelles also contains the glycogen particles when they are dispersed or aggregated. It represents a constant feature which is found in all mature heterophils throughout the experiments and is maintained in the same form in spite of technical modifications of fixation and staining. The eosinophil leukocyte also contains this ground substance, but the endoplasmic reticulum is much more prominent compared to that in the heterophil leukocyte. In the perikaryon region (Fig. 2) both rough and smooth cisternae are present. The agranular segments are more developed and form long tenuous tubules which meander among the granules (Fig. 2, arrows). No particular relationship exists between this membranous system and the glycogen particles. In the more peripheral regions of the cytoplasm the smooth endoplasmic reticulum (Fig. 3, ser) is more abundant and forms branched tubules 40 mµ in diameter.

The glycogen particles, of the β type, found in both types of cells have the same morphological properties. Large quantities of particles are assembled in irregularly shaped agglomerations, wherein each particle keeps its individuality (Fig. 1, inset). No membrane bounds these gatherings of polysaccharide particles. It might be interesting to note here that similar aggregates of glycogen particles were observed in blood leukocytes of control animals which were not injected with commercial glycogen. In some regions of the periphery of the cell (Fig. 3, gl), the particles are preferentially dispersed randomly throughout the cytoplasm. The β-particles measure 35-45 mµ in diameter, are strongly stained, and show a regular outline. The γ-subunits, visible at high magnification, may be an expression of the molecular structure or may result from a diffraction of the electron beam.

Isolation of Glycogen from the Exudate Cells

About 100 ml of peritoneal exudate was usually drained off by one operation. The exudate contains an average of 10⁴ leukocytes/mm³. The different glycogen and protein determinations carried out on the fractions isolated by differential centrifugation are expressed, as mean values from five experiments, in Table I, in micrograms per 10⁶ cells. In spite of the small starting quantity present in the extract, glycogen was recovered with a relatively good yield: 63% of the glycogen present in extract was recovered in the microsomal fraction and 35% in the purified product. Purification obtained by treating the G.Ve.F. with DOC and by separating the particles by centrifugation can also be accomplished by centrifuging the G.Ve.F. on sucrose density gradients. Both techniques result in a final contamination of the glycogen with 4% proteins.

Physical and Biochemical Properties of the Glycogen

The profile of the glycogen distribution along the centrifuge tube, after centrifugation of the G.Ve.F. layered on top of the density gradient, may be used to determine the sedimentation properties of the particles and to separate a purified fraction on which an iodine reaction and phosphorylase tests may be performed.

Sedimentation Curves and Calculations of Sedimentation Coefficients:
The glycogen distribution curve (Fig. 4, curve 3,
FIGURE 1  Rabbit heterophil leukocyte. The cytoplasm contains two types of granules: the large, dense azurophil granules (a) and the smaller, less dense specific granules (s). Large accumulations of glycogen particles can be distinguished especially in the perinuclear area. A region (demarcated rectangular area) of the accumulated particles is illustrated at higher magnification in the inset. It shows the individuality of the particles and fine $\gamma$-substructures. The endoplasmic reticulum (er) is very scarce. All organelles are embedded in a coarse-grained ground substance (gs). $\times 38,500$; inset, $\times 98,000$. 
FIGURE 2 Portion of a rabbit eosinophil leukocyte. Some of the numerous specific granules contain needle-like crystals. Glycogen particles (gl), 35–45 nm in diameter, are irregularly assembled, as in the heterophil. The endoplasmic reticulum is more developed than in the heterophil. Smooth endoplasmic tubules meander among the granules (arrows), without apparent relationship to the glycogen particles. Rough endoplasmic reticulum (rer) is sparse. × 50,000.
FIGURE 3 Peripheral cytoplasm of an eosinophil leukocyte. In this region glycogen particles are dispersed throughout the cytoplasm (gl). Note the presence of a predominant smooth endoplasmic reticulum (ser) appearing like branched tubules, 40 µm in diameter. × 44,000.
**Table I**

Glycogen, Protein and Phosphorylase Content of Different Fractions Obtained by Centrifugation of a Leukocyte Homogenate

| Fractions           | Glycogen (µg/10⁶ cells) | Protein (µg/10⁶ cells) | Protein:glycogen ratio | Phosphorylase activity (units enzyme/mg glycogen) |
|---------------------|-------------------------|------------------------|------------------------|--------------------------------------------------|
| Extract             | 15.9                    | 23.5                   | 1.5                    | 0.30-0.70                                         |
| Microsomal fraction | 10.2                    | 2.0                    | 0.2                    | 0.10-0.25                                         |
| Purified glycogen fraction | 4.8                   | 0.2                    | 0.04                   | -.*                                              |

The phosphorylase activity is expressed in units enzyme which correspond to the µmoles of inorganic phosphorus liberated per minute of incubation at 37°C.

* No phosphorylase activity was detected in the purified glycogen fraction, after resolubilization at pH 9.0 and DOC-treatment.

*Figure 4* Comparative glycogen sedimentation curves: leukocytic glycogen (curve 3, PMN), striated muscle glycogen (curve 2, M), and commercial glycogen (curve 1, GBI) obtained after centrifugation on linear sucrose gradient (0.7-2.0 M) at 24,000 rpm for 300 min. The glycogen content was estimated in the successive fractions (No. of tubes) by the Krisman colorimetric method. The gradient density was measured along the gradient by refractometry (see upper part of the diagram) and the sedimentation coefficients (s₂₀) were established.
PMN), reproduced with a remarkable constancy, shows a relatively low polydispersity and a good symmetry, except for the higher values. Compared to the distribution of striated muscle glycogen (curve 2, M), prepared by the same method and centrifuged under the same conditions, a much higher sedimentation rate is obtained for leukocyte glycogen. The commercial glycogen (curve 1, GBI), used to induce the ascites and as primer in the phosphorylase determinations, has a much lower sedimentation rate.

The sedimentation coefficients of the three glycogen preparations have been calculated: the commercial GBI glycogen presents a sedimentation coefficient of 80S; the value of 140S determined previously for the striated muscle glycogen particles at the peak of the sedimentation Schlieren curve (Wanson and Drochmans, 1968 a) was confirmed by the present method of calculation; and the leukocyte glycogen particles are characterized by an average sedimentation coefficient of 240S at the peak of the glycogen particle distribution on the sucrose gradient (Fig. 4).

**The Glycogen-Iodine Complex:** The isolated glycogen gives a characteristic brown-red color with a maximum absorption around 520 mµ (Fig. 5, curve I).

**Phosphorylase Activity:** The phosphorylase activity was measured in the successive fractions (Table I) and found to be very low. In the microsomal fraction, 0.1-0.25 units of enzyme/mg of glycogen was recovered; this is about 100 times less than what was found in the skeletal muscle glycogen. Such low activity is completely lost when the microsomal fraction is treated with DOC.

The glycogen of the microsomal fraction was incubated in the presence of 0.1 M glucose-1-phosphate without addition of primer. In such conditions, 16-22.4% of the glucose molecules of the substrate were transferred to the glycogen particles. This affects the glycogen-iodine reaction by giving a purple-red color to the complex with a maximum absorption at 550-560 mµ (Fig. 5, curve II).

**Electron Microscopy**

**Negative Staining of the Microsomal Fraction:** Only two elements are clearly distinguished in the microsomal fraction after negative staining: β-glycogen particles and membrane-bounded structures (Fig. 6). In many regions of the preparations, a fine irregular precipitate (Fig. 6, p) contaminates the background.

The glycogen particles represent the most important part of the visualized structures; they remain free from the other membranous structures. At some places, a fine precipitate of unknown nature is attached to the particles, which gives an irregular outline to their surface and, eventually, simulates small bridges (arrow). A further description of the particles obtained in a purified form and their size measurements will be given in the next section.

The membrane-bounded elements form vesicles (Fig. 6, ve), tubules, and complex structures consisting of vesicles extended by tubules. The vesicles, 100-150 mµ in diameter, are delineated by a smooth membrane of variable thickness depending on its position in the section. The tubular formations, 35-45 mµ in diameter, extend from the vesicles as one or two tubules. Their form will have to be related to similar tubular structures found in the cytoplasm of the eosinophils, but they may also derive from other organelles, namely the mitochondria or the plasma membrane.

**Negative Staining of the Purified and Incubated Glycogen:** Purified glycogen dispersed in a monoparticulate layer was examined by the negative staining method. Fig. 7 illustrates a typical field of glycogen particles, which have little contaminating membranous debris. This material appears favorable for the study of the fine structure of the particles: subunits of the γ type, defined in Drochmans' nomenclature (1962), are detected and are characterized
FIGURE 6  Negative staining of the glycogen-rich microsomal fraction showing β-glycogen particles, vesicles (ve), tubules, and vesicles extended by tubules. One may note the presence of a fine precipitate (p) in the background, which contaminates the particles and sometimes forms small bridges between glycogen particles (arrow). × 40,500.
Figure 7 Negative staining of purified β-glycogen particles dispersed in a monoparticulate layer. A little contaminating membrane debris is still detectable in this preparation. × 66,000.

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FIGURE 8 Higher magnification of the purified glycogen fraction. The ultrastructure of the β-particles appears clearly in the form of filamentous γ-units disposed in a complex pattern. The largest particles present irregular contours and constitute small rosettes (circles). X 100,000.

FIGURE 9 Glycogen particles of the G.Ve.F. incubated at 37°C in the presence of 0.1 M glucose-1-phosphate, 0.003 M adenosine monophosphate (AMP), and 0.2 M NaF, pH 6.10, for 60 min. The synthesis induced by the endogenous phosphorylase is characterized by an increase in size of the particles, which present more irregular contours and sometimes a fusion due to the growth of the γ-subunits. X 100,000.

by filamentous structures disposed in a complex pattern. These subunits are better observed at the final magnification of 100,000 (Fig. 8). Furthermore, the largest particles (Fig. 8, circles) present irregular contours, suggesting the existence of aggregates of small β-particles. A similar observation was made in the case of striated muscle glycogen (Wanson and Drochmans, 1968 a, b).

Fig. 9 illustrates the morphological characteristics of the glycogen particles after synthesis induced by endogenous phosphorylase. The particles incubated at 37°C for 60 min show an increase in size and have more irregular contours. Some of them fuse together by the growth of the γ-units. This increased size was measured and compared to the size of particles of the original glycogen.

PARTICLE SIZE MEASUREMENTS: Size distribution histograms of the isolated glycogen particles were established by measuring the diameters of 1500 particles on micrographs of negatively stained preparations. The homogeneous population of particles (Fig. 10) of an average diameter of 47 μm shows a low dispersity, expressed by a standard deviation (SD) of 6.4 μm. In the same figure, the size distribution of the glycogen extracted from muscle (nonshaded area) is represented for reference. The mean diameter is smaller, but the standard deviation (6.8 μm)
is of the same order of magnitude. A straight line is obtained by plotting the cumulative diameter frequencies, ranging from 25 to 70 μ, on a probability scale (Fig. 11). This normal distribution denotes no significant heterogeneity.

Similarly, 1500 particle diameters were measured for a glycogen preparation which was incubated with glucose-1-phosphate. The distribution curve is shifted to higher values (Fig. 12, shaded area), compared to the control, untreated glycogen (nonshaded area). The mean diameter is now 51.5 μ and the standard deviation remains constant, 6.4 μ.

DISCUSSION

It was noticed by several authors that the structure of the mature polymorphonuclear leukocytes, particularly of the heterophils, is difficult to preserve (Bainton and Farquhar, 1966; Hirsch and Fedorko, 1968). After several tests of fixation, we came to the conclusion that the classical two-step method of fixation, glutaraldehyde followed by osmium tetroxide, may be applied with success to this material if the final osmolality is carefully maintained at 300 milliosmols and if the fixed material is collected by very low speed centrifugations.

The granular appearance of the cytoplasmic matrix remains a constant feature in the different observations published (Bainton and Farquhar, 1966; Hirsch and Fedorko, 1968). This substance, in which the glycogen particles are confined, represents the main mass of the cytoplasm. It contaminates probably the G.Ve.F. and forms the background in the negatively stained preparations. Its nature remains unknown.

The isolated and purified glycogen can not be confused with the commercial glycogen used to induce the formation of ascites. This latter glycogen is very small in size (Drochmans, 1965), reacts only slightly with iodine, and is, according to Hirsch (personal communication), rapidly lost in the peritoneal cavity. It could still be argued that some of the intracellular particles, namely those which constitute agglomerates, may derive from the injected material. But this seems unlikely because similar glycogen agglomerates were observed in blood leukocytes of untreated animals. However, the fundamental unanswered question remains whether foreign primer molecules of small molecular weight may be incorporated by a cell to constitute new glycogen particles.

The determination of sedimentation coefficients and the measurement of particle size are complementary means to appreciate the degree of homogeneity of the population of particles. In the present case, particles originate in more than one type of cell and from different locations within the cell. Despite this, the profile of the sedimentation curve and that of the size distribution curve are remarkably symmetrical, denoting no heterogeneity except for the highest values. These latter correspond to the aggregates of small β-particles which give the appearance of a rosette structure.

The phosphorylase activity found in the glycogen-rich microsomal fraction (G.Ve.F.) compared to that of the sarcovesicular fraction of muscle is very low, about 100 times lower. However, compared to the values given in the literature (Yunis and Arimura, 1964; Hujing, 1968), the activity, expressed in terms of units per milligram of glycogen, appears high. The next step of purification, which consists of treating the particles
with DOC at pH 9.0, destroys, of course, the largest part of the enzyme.

The good preservation of the glycogen particles with a high sedimentation coefficient, the high purity of the preparations, and the reproducibility of the results make the method of separation suitable for further applications.

The authors are extremely grateful to Professor P. Drochmans for stimulating discussions during the course of the work and for his help in the preparation of the manuscript. They wish to thank Professor Beaufay for his advice in the calculation of the glycogen sedimentation coefficients. They are indebted to Mr. R. Mosselmans for his valuable technical assistance, and to Mr. J. Verheyden for the help in the preparation of the electron micrographs.

This work was partly supported by a grant of the "Nationaal Fonds voor Wetenschappelijk Onderzoek."

Received for publication 19 August 1970, and in revised form 3 December 1970.

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