Glycogen in Human Peripheral Blood Leukocytes

II. THE MACROMOLECULAR STATE OF LEUKOCYTE GLYCOGEN

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A B S T R A C T Glycogen of normal human blood
leukocytes was studied in cell suspensions con-
taining chiefly neutrophiles.

In electron micrographs of neutrophiles stained
with lead the glycogen particles appear to be
relatively uniform with a diameter of 20 mμ. At
high magnification the 20 mμ particle appears to
be composed of at least eight subunits.

Leukocyte glycogen released by lysis or homo-
genization sediments as a single peak of high
molecular weight material. The great majority of
the cell glycogen can be accounted for in the large
molecular weight material. The large molecular
weight material is degraded to small fragments by
α-amylase and partially degraded by β-amylase.
Purification of cell glycogen by alkali extraction
and ethanol precipitation produces a relatively uni-
form particle smaller than the original native
macromolecule.

Native glycogen was prepared in pure form by
a sucrose density gradient technique and its purity
demonstrated by its susceptibility to purified α-
amylase and by analytical ultracentrifugation.

INTRODUCTION

Peripheral blood leukocytes contain considerable
amounts of glycogen (1, 2), and since they are

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readily accessible, provide a convenient system in
which glycogen metabolism can be studied. In a
preceding paper (3) studies involving suspensions
of leukocytes (chiefly neutrophiles), were re-
ported and some aspects of glycogen synthesis,
degradation, and turnover were described.

In the present paper these studies were ex-
tended to demonstrate the particulate or macro-
molecular state of leukocyte glycogen. These stud-
ies show that glycogen exists in leukocytes as a
group of molecules of similar size and that syn-
thesis and degradation involve adding and sub-
tracting glucose residues uniformly from all mole-
cules rather than synthesizing entirely new
molecules or breaking down single molecules.
Electron microscopic evidence of a subunit struc-
ture of the predominant glycogen particle is also
presented.

METHODS

Preparation of leukocyte suspensions. Leukocyte sus-
pensions containing an average of 83% granulocytes were
prepared from normal adult donors by combined dextran
sedimentation and differential centrifugation as previously
described (3). Hypotonic lysis of contaminating erythro-
cytes was usually omitted. If erythrocytes contain gly-
cogen, the quantity is quite small (4) compared to granu-
locytes and its contribution was neglected. Cells were
suspended in glucose-free Hanks' (GFH) solution con-
taining penicillin and streptomycin for incubation.

Incorporation of glucose-^{14}C into glycogen. In order
to insure adequate incorporation of label during glycogen
synthesis, we preincubated the cells first in GFH solution
for 2 hr which resulted in a loss of about one-third of
the glycogen stores (3). Uniformly labeled glucose-^{14}C
(specific activity 172 mc/mmole) [1] was diluted in sterile
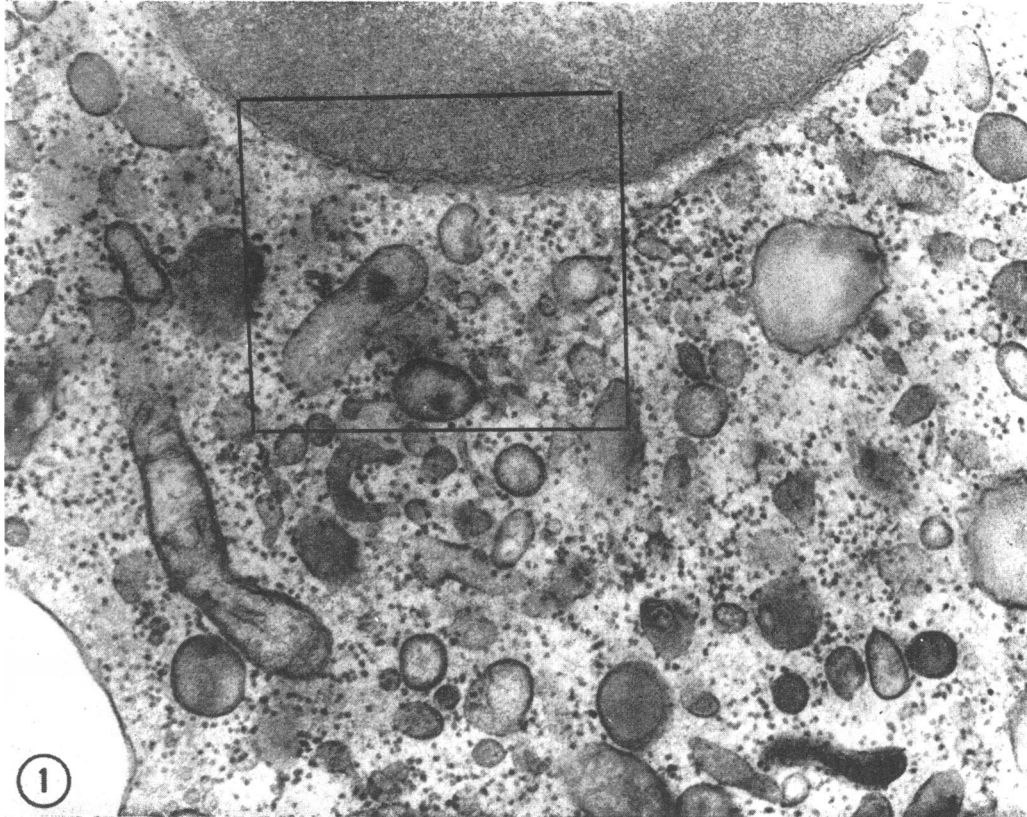
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[1] New England Nuclear Corp., Boston, Mass.

FIGURE 1 Electron micrograph of a portion of a neutrophile granulocyte. Osmium-fixed preparation showing a portion of the nucleus and numerous specific granules in the cytoplasm. Glycogen is seen as small, dense, uniform particles scattered throughout. × 40,000.

10% glucose. For long label (2–4 hr) the label was diluted to 0.172 mc/mmole and for shorter labels the dilution was 1.72 or 0.86 mc/mmole. Radioactive glucose was added to the suspensions in GFH in concentrations of 50–100 mg/10⁹ neutrophiles. Incubations were performed in air at 37°C without agitation.

Preparation of cell lysates and homogenates. After incubation in glucose-¹⁴C, the cells were iced, washed three times with cold isotonic saline, and lysed in 0.34 M sucrose by a procedure similar to that described by Cohn and Hirsch for rabbit leukocytes (5). The leukocyte pellet was resuspended in 0.5 ml of 0.34 M sucrose, 0.01 M Tris, pH 8 and vigorously agitated by aspirating and expelling the cells through a 20 gauge needle on a 1 ml syringe. It was necessary to repeat the procedure with three additional 0.5 ml volumes of sucrose in order to lyse most of the cells in the pellet as determined by phase microscopy. The mixture was then spun at 1000 g for 5 min and the supernate separated. The supernates were pooled and then diluted with 0.75–1.0 volumes of 0.01 M Tris pH 8 in order to be layered on top of sucrose density gradients. In some later experiments the cells were homogenized rather than lysed. For homogenization, the washed cells were suspended in 1 or 2 ml of 0.01 M Tris

pH 8 and broken with 15 strokes of a "no-clearance" homogenizer.[2] This was sufficient to release 80–90% of the cell glycogen; the remaining glycogen was apparently firmly bound to cell structures. Homogenates were centrifuged at 1000 g for 10 min and the supernate layered on sucrose density gradients. Identical results were obtained with both homogenization and osmotic lysis.

Radioactive glycogen was also purified from whole cells as previously described (3).

Ultracentrifugation procedures. Linear sucrose density gradients were prepared with 10–25% sucrose (w/w) in 0.01 M Tris pH 8 with total volume 28 ml. 1–1.5 ml of cell lysate or homogenate was layered on each gradient and spun at 24,000 rpm in an SW 25.1 rotor in a model L-2 ultracentrifuge[3] at 5°C. After centrifugation the bottom of the tube was punctured with an 18 gauge needle and 0.8 ml fractions collected. One drop of each fraction was removed and counted at infinite thinness in a low background gas-flow counter.[4]

Electron microscopy. Pellets of white cells were fixed in 1% buffered osmium tetroxide (6) for 1 hr at 4°C.

[2] Kontes Glass Co., Vineland, N. J.

[3] Beckman Instruments, Palo Alto, Calif.
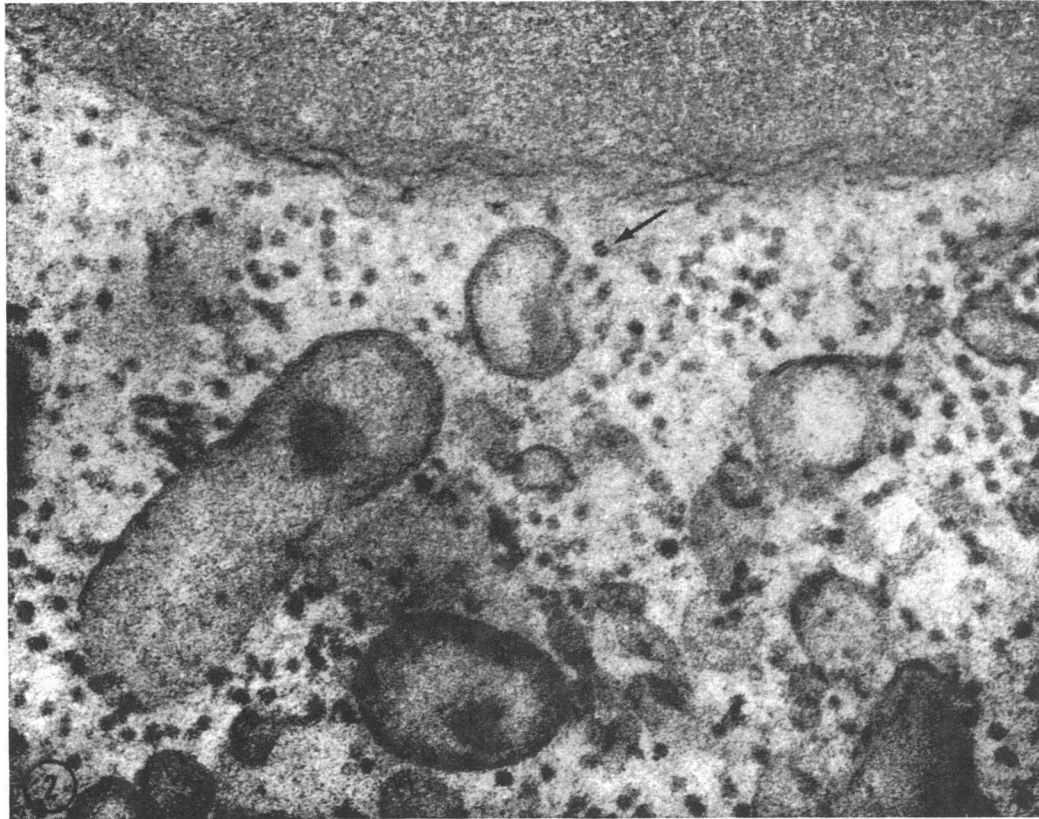
[4] Nuclear-Chicago Corp., DesPlaines, Ill.

FIGURE 2 Higher magnification of the area outlined in Fig. 1. Some of the particles appear to have a substructure composed of rounded units. The arrow points to one showing at least eight subunits. × 100,000.

After dehydration in graded ethanols the pellets were embedded in Maraglas D.E.R. (7). Thin sections were prepared, stained with lead acetate, and viewed in a Siemens Elmiskop I [5] at 80 kv. Purified α-amylase [6] and β-amylase [7] were obtained commercially.

RESULTS

The size and substructure of leukocyte glycogen particles. A suspension of leukocytes from a normal donor was prepared in which the glycogen content was found to be 7.1 mg glycogen/10^9 neutrophiles. The cells were fixed as described in Methods and visualized in the electron microscope. A portion of a granulocyte is reproduced in Fig. 1 at a magnification of 40,000 showing the glycogen particles scattered randomly throughout the cytoplasm. The particles were discrete except in rare instances some aggregation was seen. A portion

of the same cell sample was incubated in glucose-free medium to deplete glycogen, and the cells were unchanged except that these particles had become too small to be readily resolved in the electron microscope. Measurements of particle size were made at higher magnification (Fig. 2). The results of 528 individual measurements, which illustrate that the majority of the particles were 20 mμ in diameter, are shown in Fig. 3. At high magnification there appears to be a regular substructure to the particles composed of smaller subunits. The particle near the arrow in Fig. 2 shows at least eight subunits.

The sedimentation of particulate glycogen in sucrose density gradients. Leukocyte suspensions were incubated in glucose-^{14}C and lysates or homogenates prepared as described under Methods. Fig. 4 illustrates one such experiment in which leukocytes were labeled with glucose-^{14}C for 1 hr. The homogenate was spun through linear 10–25%
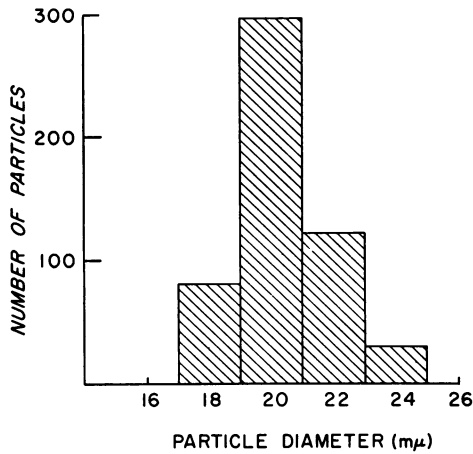
FIGURE 3 Size distribution of leukocyte glycogen particles. Measurements were made of 528 glycogen particles on the plate shown in Fig. 1. The greatest number of particles were 20 mμ in diameter.

sucrose density gradients for 210 min at 24,000 rpm. One-half of the sample was treated with crystallized pancreatic α-amylase (50 μl/ml for 20 min at 4°C) before layering on a gradient.

The untreated sample shows a relatively homogeneous group of large radioactive particles and additional radioactivity in smaller material at the top of the gradient. The total number of counts recovered under the large peak (samples 1–28) account for 78.5% of the total radioactivity in the gradient. Since under the conditions of labeling approximately 80% of the ultracellular radioactivity should be found in glycogen (3), the

great majority of the cell glycogen is accounted for by the material under the heavy peak. The particles are probably not completely uniform in size as evidenced by the spreading of the base of the large peak. The curve designated "α-amylase" shows that the material under the large peak was broken to smaller fragments by α-amylase treatment.

What proportion, if any, of the radioactive small molecular weight material in the untreated sample is glycogen cannot be determined. However, similar preparations were centrifuged for 13, 20, 60, and 80 hr at 24,000 rpm and failed to show any other peaks of lighter particulate material.

Effect of β-amylase or alkali extraction on sedimentation of glycogen. Alpha amylase will degrade glycogen almost entirely while β-amylase will remove glucose units down to a branch point of the chain but no farther. Fig. 5 illustrates the change in sedimentation after β-amylase treatment. Leukocytes were incubated in glucose-¹⁴C for 3 hr. One-half of the lysate was treated with sweet potato β-amylase (50 μl/2 ml for 20 min at 4°C) after which the samples were layered on sucrose density gradients. A sample of ¹⁴C-labeled glucose, alkali-extracted glycogen was run simultaneously to show the characteristic change in particle size induced by alkali. Centrifugation time was 210 min. The treated sample sedimented more slowly than the "native" glycogen but ahead of the peak of alkali-purified glycogen. The majority (65%)
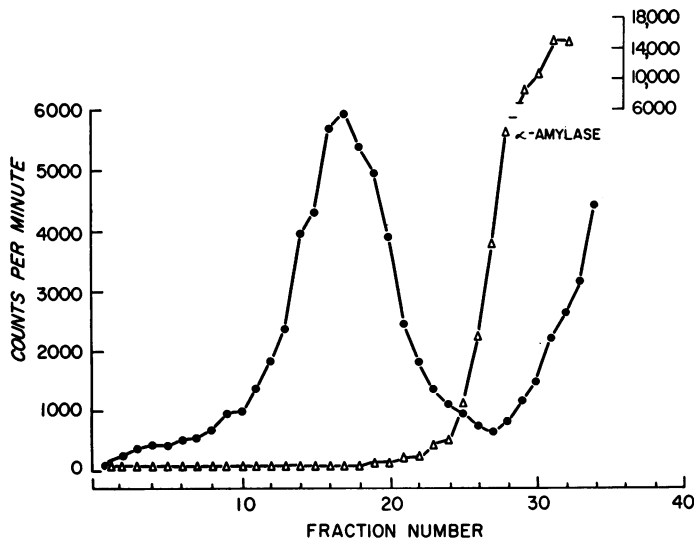

FIGURE 4 Sedimentation of native leukocyte glycogen in sucrose density gradient. Leukocyte glycogen was made radioactive by incubation in glucose-¹⁴C. The homogenate was centrifuged through a 10–25% sucrose density gradient and sequential fractions collected as described in Methods. Direction of sedimentation to the left. Control preparation (●), and a simultaneously run sample treated before centrifugation with α-amylase (△).
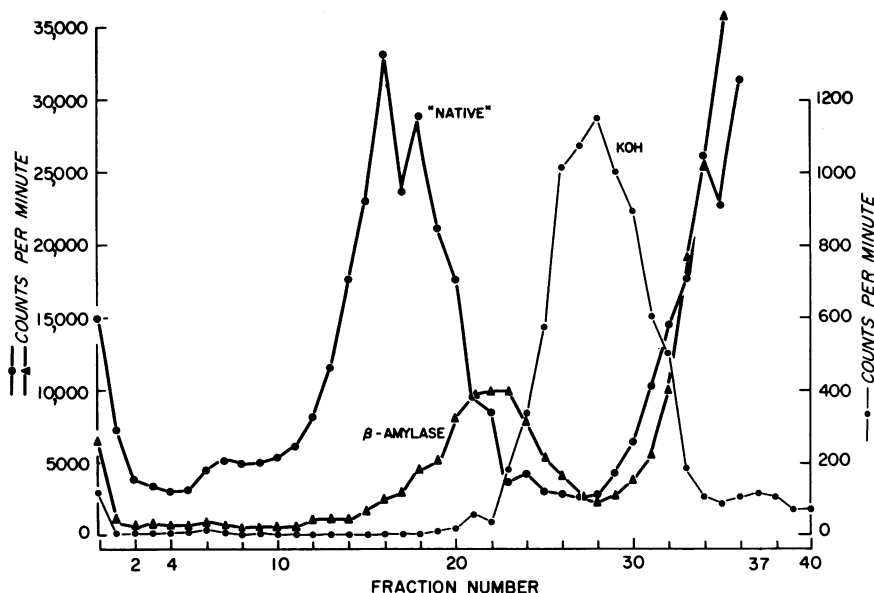
FIGURE 5 Effect of beta amylase or alkali extraction on the sedimentation of leukocyte glycogen. One-half of a ^{14}C-labeled homogenate was treated with β-amylase and run simultaneously with the control homogenate and a sample of alkali-purified leukocyte glycogen. Beta amylase acts to remove glucose units down to a branching point on the chain, thus removing the outer shell of the molecule.

of the counts in the "native" glycogen peak were solubilized by β-amylase treatment.

Sedimentation coefficient of "native" glycogen. 400 ml of blood was collected from a normal donor and leukocyte suspensions prepared and counted. Small aliquots were removed for glycogen estimation. The remaining cells were homogenized and the homogenate divided between three sucrose
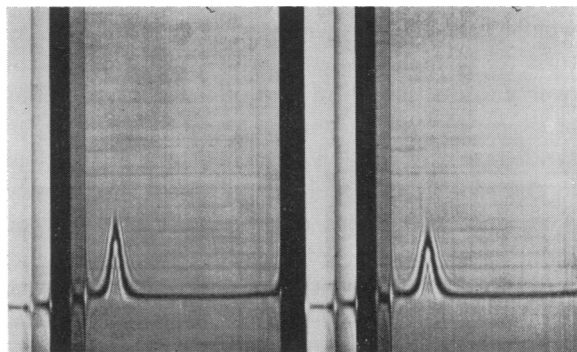

FIGURE 6 Schlieren patterns of leukocyte glycogen purified in sucrose density gradients. Frame on the left was taken 8.5 min after reaching speed of 15,220 rpm and, on the right, after 12.0 min. Glycogen was suspended in 0.01 M Tris pH 8, concentration 2.2 mg/ml. The single peak seen was completely degraded by α-amylase treatment.

gradients. The gradients were centrifuged as in Fig. 4. After collection of fractions, fractions corresponding to the large peak including its broad base (fractions 8–24) were pooled. The pooled fractions from the three gradients were then centrifuged at 28,000 rpm in a No. 30 rotor for 6 hr. The glycogen pellet was resuspended in 0.6 ml of warm 0.01 M Tris pH 8. The slightly cloudy solution was then centrifuged in the model E analytical ultracentrifuge [8] at 15,220 rpm at 25°C. The results are pictured in Fig. 6. The original leukocytes contained 6.2 mg glycogen/10^9 neutrophiles. The solution of "native" glycogen contained 2.2 mg/ml. The single peak seen in Fig. 6 had a sedimentation coefficient of 125S and was completely broken down by α-amylase treatment.

DISCUSSION

The glycogen macromolecule, being in a state of constant turnover, varies in size depending on the relative rates of synthesis and degradation. Characterization of the molecule, as presented here, shows an order of magnitude for the dimensions and sedimentation coefficient of the molecule in

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leukocytes and correlates these with the glycogen content of the cells from which it was extracted.

Glycogen structure in liver differs from that in leukocytes in that glycogen molecules in liver appear as a spectrum of sizes rather than a relatively uniform particle size as in leukocyte glycogen. Drochmans (8) studied rat liver glycogen by electron microscopy and was able to disaggregate the large (40–200 mμ) molecules into smaller units of uniform size by lowering the pH below 5.0. The uniform particles produced were spheres of 30 mμ diameter. These particles appeared to have subunits which he described as being filaments 3 mμ in diameter and 20 mμ long. The largest particles were called α-, the 30 mμ particles designated β-, and the smallest subunits γ-particles. Barber, Harris, and Anderson (9) studied rat liver glycogen by electron microscopy and centrifugation in density gradients and described a spectrum of particle sizes up to 200 mμ with sedimentation coefficients up to 10,000. They observed, like Drochmans, that the α-particles were aggregates of varying numbers of uniform β-particles of 20–25 mμ diameter. They believed, however, that the γ-particle was a sphere 2.5–3.5 mμ in diameter rather than a filament as Drochmans described. Leukocyte glycogen appears as uniform size particles 20 mμ in diameter and probably corresponds to the β-particles described in rat liver. The leukocyte particles likewise contain subunits, at least eight in some particles. Why liver β-particles aggregate is unknown. The nature of the binding of γ-particles into a β-particle is likewise unclear.

Labeling of glycogen with glucose-^{14}C during a period of active glycogen synthesis, as in the sucrose gradient experiments, permits some conclusions as to the assembly of the macromolecule. If the predominant particle (β-particle) is produced by a stepwise addition of subunits (γ-particles) then a spectrum of particle sizes in the density gradient would appear. This is not the case, which indicates that existing β-particles are enlarged uniformly during glycogen synthesis. New glucose units are probably added individually although some small subparticle could conceivably add to the β-particles. Extended centrifugation (up to 80 hr at 24,000 rpm) revealed no lighter peaks, so if smaller subunits exist they must exist very transiently. If smaller particles exist free in the leukocyte homogenates, they are not likely to exist in large amount since, as illustrated in Fig. 4, the large glycogen peak accounts for nearly all of the ^{14}C counts to be expected from labeling under the conditions of the experiment.

It is of interest that extraction of glycogen by alkali produces a discrete particle, smaller than the original β-particles (Fig. 5). The exact nature of the change in the molecule which makes it smaller than a β-particle but still larger than the presumed γ-particle size is not known.

The experiment (Fig. 5) illustrating the action of β-amylase on glycogen shows an incomplete degradation of the molecule. Beta amylase action stops at a branch point in the glycogen chain and the action of the "debrancher" enzyme, dextrin-1, glucosidase, is required before further β-amylase activity is possible. Although the molecules appear to be only slightly smaller, judging from their sedimentation, the radioactivity associated with the peak is only about one-third as great as the control. This rough comparison suggests, as is known to be the case with liver glycogen (10), that the outer chains of the molecule are the most metabolically active.

From these studies of the macromolecular form of leukocyte glycogen, and from the studies of the dynamic state of the molecule in the preceding paper (3), one can begin to understand the functional or "native" state of the molecule in the cell. Glycogen synthesis and degradation proceed rapidly even when the total amount of glycogen in the cell is constant. When endogenous glucose is needed, the glycogen level falls rapidly and when glucose is replaced, the glycogen stores return to normal. Despite the constant rapid turnover and fluctuation in size of the glucose chains of glycogen, the enzymatic machinery (glycogen synthetase and phosphorylase) which is attached to the molecule (11, 12) is turned over slowly if at all. The number of glycogen molecules (or β-particles) in the cell probably is fixed since degradation and resynthesis involve all molecules equally, and during resynthesis, no sequential assembly of new molecules was detected. It is, of course, possible that β-particles might be completely assembled so rapidly that intermediate size particles are undetectable, but this would require a sizable pool of subunits (γ-particles) which were not detected.

What fixes the number of glycogen molecules

in the cell? How are the molecules first assembled during the differentiation of the leukocyte? Are the enzymes of glycogen synthesis and degradation fixed to some structural component of the glycogen β-particle, or do they attach and reattach as needed? These are some of the important questions posed by the present knowledge of glycogen in leukocytes and other tissues.

Particulate or "native" glycogen as visualized in the cell is not a molecule in the ordinary static sense, but a dynamic "organelle" whose product is fuel in the form of glucose. These organelles, for which we have applied the term "glycosomes," are probably fixed in number but varying in size, with discrete substructure and stable enzymatic components, held together by still undetermined structures or bonds.

ACKNOWLEDGMENTS

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