Glycogen in Human Peripheral Blood Leukocytes

I. CHARACTERISTICS OF THE SYNTHESIS AND TURNOVER OF GLYCOGEN IN VITRO

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ABSTRACT In the present paper a model system is described utilizing suspensions of peripheral blood leukocytes in which glycogen synthesis and degradation can be studied.

Leukocyte suspensions containing 72–94% granulocytes were prepared essentially free of platelets and erythrocytes and consisted almost entirely of neutrophile granulocytes. Initial glycogen content averaged 7.36 ± 2.05 mg/10^9 neutrophiles. In a glucose-free medium glycogenolysis took place with glycogen losses averaging 38% in 2 hr. When adequate glucose was added to the medium, glycogen was resynthesized to the original level.

Glycogen resynthesis was studied with varying glucose "loads" to determine (*a*) the glucose level which was adequate for cell maintenance without utilization of glycogen stores, and (*b*) the glucose level which provided maximal glycogen resynthesis. With cell densities of $20–50 \times 10^6$/ml the minimum glucose load which allowed maintenance of glycogen stores was 2 mg and 5.3 mg/10^9 neutrophiles for 30 and 60 min, respectively.

During resynthesis with glucose-^{14}C, as much as 88.9% of the intracellular radioactivity could be found in glycogen. Leukocyte glycogen was made radioactive by a "pulse" of glucose-^{14}C followed by a "chase" with nonradioactive glucose. Specific activity changes in glycogen isolated during the "chase" showed that glycogen was in constant turnover.

When glycogen was made radioactive by a "pulse" of glucose-^{14}C and the cells placed in glucose-free medium, the specific activity of isolated glycogen fell rapidly. Thus, the most recently added glucose units of the molecule were also the first to be removed when conditions favoring synthesis were changed to conditions favoring degradation.

Even though glycogen is constantly turning over, the enzymatic "machinery" for its synthesis is relatively stable and not dependent on continuous protein or RNA synthesis, as shown by experiments with puromycin and actinomycin.

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INTRODUCTION

Among circulating blood cells in man, glycogen occurs primarily in leukocytes and platelets (1–3). Erythrocytes may contain minute amounts of glycogen, but the levels reported are over a 1000-fold less per cell than in leukocytes (4). Of the leukocytes, normal lymphocytes contain little or no glycogen (1, 2), and monocytes contain relatively small amounts of stainable glycogen (1–3). There is controversy as to whether eosinophile and basophile granulocytes contain glycogen (1, 3), but these cells are present in relatively small numbers in peripheral blood. Thus neutrophile granulocytes account for the vast majority of leukocyte glycogen.

It has been shown (5, 6) that the glycogen content of leukocyte suspensions decreases when the cells are incubated in medium without glucose.

Glycogen is resynthesized to the original level when adequate glucose is added. This phenomenon provides a useful in vitro system in which glycogen metabolism of leukocytes can be studied readily. The studies reported in this paper delineate some basic characteristics of glycogen synthesis and degradation in this system to provide a basis for more detailed studies of the nature of the glycogen molecule and the control of its utilization.

Studies of the macromolecular state of glycogen synthesized under these conditions are reported in a second paper (7).

METHODS

Preparation of leukocyte suspensions. Venous blood was collected from normal adult male and female donors into sterile graduated cylinders containing 10 ml of 2% disodium ethylenediaminetetraacetate (EDTA) and 20 ml of 6% dextran in 0.9% NaCl [1] for each 100 ml of blood. The contents were mixed by inversion and the cylinder allowed to stand in an incubator at 37°C for 1 hr. The plasma was drawn off and centrifuged at 225 *g* at 5°C. Remaining erythrocytes were lysed (8) by the addition of 2 ml of cold 0.24% saline to the pellet. The mixture was agitated by repeated pipetting with a Pasteur pipette for no more than 30 sec after which 4 ml of 0.9% NaCl was added and mixed before centrifugation as before. Two additional washes were carried out with glucose-free Hanks' (9) solution (GFH) prepared in the laboratory and sterilized before use by Millipore filtration. 1000 U each of penicillin and streptomycin and 0.01 ml of 2% EDTA were routinely added to each 10 ml of Hanks' solution. The leukocyte pellet was finally resuspended in GFH (2.5 ml/100 ml of original volume of blood) and leukocytes were counted in duplicate (10). Differential counts of neutrophiles vs. mononuclear cells were made from the counting chambers at a magnification of 450. The results were found to be no different from differential counts made on Wright's-stained smears of the preparations. Eosinophiles, like lymphocytes, tended to remain with the platelets so the counts were expressed as neutrophiles rather than granulocytes. Erythrocyte contamination was five red cells or less/100 neutrophiles. Platelets contamination was negiligible as determined by stained smears and phase microscopy. Viability of cells as determined by trypan blue exclusion (8) showed 1% stained cells.

Incubation procedure. Cell suspensions were adjusted when necessary to a cell density of $20–50 \times 10^6$ neutrophiles/ml. At concentrations less than 15×10^6/ml glycogen synthesis was about half that at concentrations between 20 and 50×10^6/ml. The original suspension was mixed and divided into paired aliquots containing at least 10×10^6 neutrophiles and incubated in separate sterile tubes. Incubation was carried out without agitation in a 37°C water bath in air. Incubation on a rotating platform had no effect on glycogenolysis or glycogen resynthesis compaired to the unagitated suspensions. To study glycogen resynthesis, cells were preincubated in glucose-free medium for 2 hr. Glucose was then added from a stock 10% solution (in distilled water) which had been sterilized by Millipore filtration immediately before use. Testing of cell viability after a 4 hr incubation with glucose showed 2–4% of the nuclei to be stained with trypan blue. Incubations were ended by icing the tubes.

Glycogen assay. Glycogen was extracted from cell suspensions by adding an equal volume of 60% KOH and by heating in a boiling water bath for 20 min followed by precipitation of glycogen with 1.1 volume of absolute ethanol. The precipitates were allowed to form for a minimum of 2 hr at −15°C and centrifuged at 1000 *g* for 20 min. The glycogen was then dissolved in 1 ml of distilled water and reprecipitated. After redissolving a second time in distilled water we used an aliquot for glycogen assay by the anthrone method of Seifter (11) with glucose standards. All assays were performed in duplicate and results expressed as mg glycogen/10^9 neutrophiles.

Incorporation of radioactive precursors. Uniformly labeled glucose-^{14}C (specific activity 172 mc/mmole) [2] was diluted with sterile nonradioactive glucose to a specific activity of 0.172 mc/mmole. This mixture was then added to leukocytes suspended in GFH. At least 10×10^6 neutrophiles were used for each sample, and always in duplicate. After incubation the cells were chilled, centrifuged briefly in an International Clinical centrifuge, and washed three times with chilled isotonic saline. One ml of 30% KOH was added to the pellet and glycogen extracted as described. Of the final 2 ml solution, 0.2 ml was dried on a copper planchet and counted in a low background gas-flow counter.[3]

Also, the supernates were pooled after both alcohol precipitations, the volume measured, and an aliquot plated for counting. This fraction represents glucose-derived radioactivity which existed in the cell but not in the form of glycogen.

Radioactive amino acids-^{14}C (specific activity 1 mc/mg) [2] were incorporated into leukocyte protein by their addition to leukocyte suspensions in Hanks' solution at a concentration of 2.8 μc/ml and a cell density of 12.4×10^6 neutrophiles/ml. At intervals of incubation at 37°C duplicate aliquots containing 7.8×10^6 neutrophiles were removed and treated with equal volumes of cold 10% trichloroacetic acid (TCA). The resulting precipitate was deposited on Millipore filters and washed with cold 5% TCA. The filters were then glued to aluminum planchets and counted in the low background gas-flow counter.

Uridine-2-^{14}C (specific activity 30 mc/mmole) [2] was incorporated into RNA by incubating leukocytes in Hanks' solution with the isotope at a concentration of 5 μc/ml, labeling 10×10^6 neutrophiles per duplicate sample. The

[1] Clinical Grade H, Pharmachem Corporation, Bethlehem, Pa.

[2] New England Nuclear Corp., Boston, Mass.

[3] Nuclear-Chicago Corp., Des Plaines, Ill.

macromolecules were precipitated and counted as described above and the incorporated radioactivity considered to represent newly synthesized RNA.

Materials. Puromycin [4] and anthrone [5] were obtained commercially. Actinomycin D [6] was prepared in a stock solution of 60 μg/ml in Hanks' solution and sterilized by Millipore filtratiaon before use. Mathematical analyses of data were performed with a computer using standard techniques (12).

RESULTS

Glycogenolysis in glucose-free medium. Leukocyte suspensions prepared as described under Methods averaged 83% neutrophiles (range 72–94%) and represented a recovery of an average 99.6×10^6 neutrophiles/100 ml of blood (range $33–186 \times 10^6$). When suspensions were incubated in GFH at 37°C, glycogenolysis began immediately. The initial rate of glycogen degradation was more rapid than at later time intervals in the 2 hr incubation period. The initial mean content of glycogen was 7.36 ± 2.05 SE mg glycogen/10^9 granulocytes (range 3.30–11.0). At the end of the 2 hr incubation, in the absence of glucose, the average glycogen content was 4.53 ± 0.21 SE mg glycogen/10^9 granulocytes (range 2.19–6.19). Thus, the average loss of glycogen during the incubation period was 38.5% and the difference was highly significant ($P < 0.001$).

Effect of varying glucose "loads" on glycogen resynthesis. Cells suspended in glucose-free medium, or in medium supplemented with inadequate glucose, will degrade intracellular glycogen stores. If cells are presented increasing amounts of glucose, and glycogen content is measured before and after incubating with glucose, it would be possible to determine a glucose "load" at which the cell has enough glucose for immediate needs and glycogen levels remain stable. This type of experiment also allows an estimation of the glucose load which would permit maximal glycogen synthesis for this system.

Leukocyte suspensions were prepared and first incubated for 2 hr in Hanks' solution without glucose, so that glycogenolysis would take place. Samples were then taken for glycogen determination. At the end of 2 hr, varying amounts of sterile glucose were added to aliquots containing $10–15 \times 10^6$ cells and the glycogen content of the cells was measured again at the end of either 30 or 60 min of further incubation at 37°C. The results of these experiments are shown in Figs. 1 and 2. For each individual suspension, the change in glycogen content after adding glucose is recorded on the ordinate and the glucose load is recorded logarithmically on the abscissa. The curves were fitted to the data by the method of least squares. The best fit proved to be a simple third-order polynomial for the 30-min incubation times. The equation of the curve is $y = -0.334 + 0.532x + 2.278x^2 - 1.085x^3$. The multiple correlation coefficient (r^2) for the curve is 0.77. The dashed lines indicate the 95% confidence limits. The glucose load at which there was no change in glycogen content during the 30 min period was found by setting the equation equal to zero and resulted in a value of 2.00 mg glucose/10^9 neutrophiles. The 95% confidence limits for the zero change are 1.50–2.56 mg of glucose/10^9 neutrophiles. A maximum value for glycogen synthesis on the 30 min curve was obtained by differentiating the equation and resulted in a value of 32.2 mg of glucose/10^9 granulocytes.

For the 60 min incubation period, the polynomial obtained by the least squares method was $y = 1.936 + 1.918x + 1.720x^2 - 0.794x^3$. The multiple correlation coefficient (r^2) of this fitted curve is 0.72. The glucose load which would give a zero net change in glycogen for 60-min incubation was found to be 5.3 mg of glucose/10^9 neutrophiles with 95% confidence limits at 4.20 and 6.55 mg of glucose/10^9 neutrophiles. The maximum change in glycogen content was obtained at a glucose load of 74.9 mg/10^9 neutrophiles.

When the glucose "loads" for each experiment were converted to mg/100 ml of glucose in the medium, the concentrations corresponding to zero change in glycogen averaged 17.6 mg/100 ml for 60 min and less than 10 mg/100 ml for 30 min. Glucose concentrations resulting in maximum glycogen synthesis were in the range of 200 mg/100 ml for 60 min and slightly above 100 mg/100 ml for the 30 min experiments.

The partition of intracellular glucose between glycogen synthesis and other pathways of utilization. Glucose-^{14}C was used to determine the fate of the isotope which entered the leukocytes and either remained as glucose or became incorporated

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Fisher Chemical Corp., Silver Spring, Md.
[6] Supplied by Merck, Sharpe & Dohme, West Point, Pa.

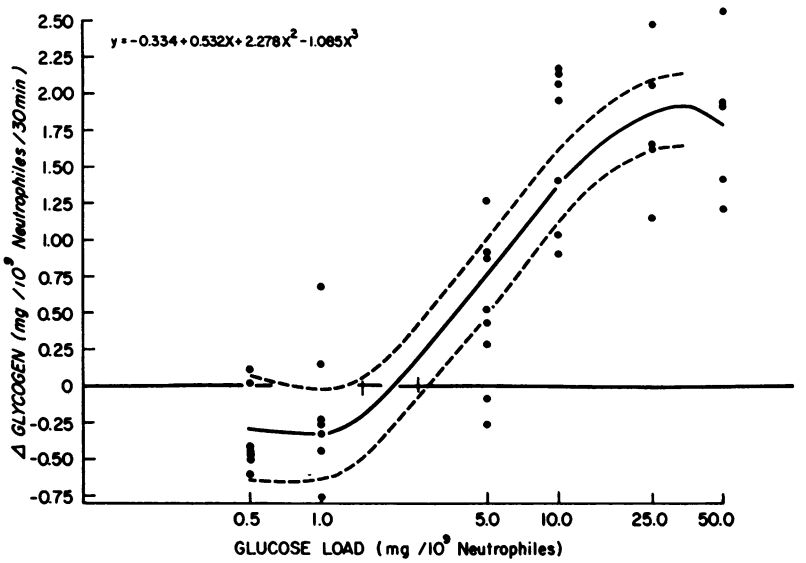

FIGURE 1 Change in glycogen content during a 30 min incubation of glycogen-depleted leukocytes after the addition of varying amounts of glucose. The solid line was fitted by the least squares method with 95% confidence limits represented by the dashed line. The vertical bars on the zero change line are the 95% confidence limits for the point at which there is no net glycogen change after 30 min. Details of the experiments are given in the text.

into cell structures. After preincubating the leukocyte suspensions in glucose-free medium, we stimulated glycogen resynthesis by adding glucose at various concentrations as in the previous experiments. After 1 hr in the glucose-containing medium the glycogen of the cells was isolated, purified, and radioactivity counted as described under Methods. The radioactive glucose within the cell

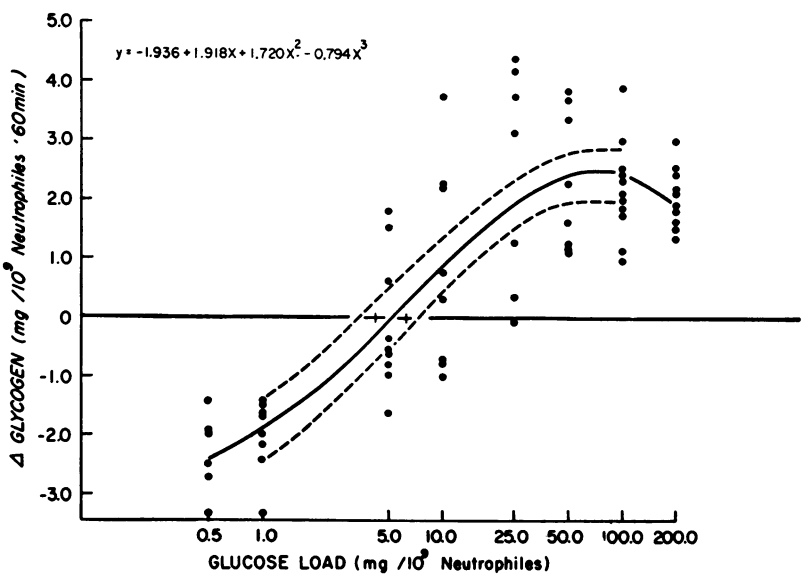

FIGURE 2 Change in glycogen content during a 60 min incubation of glycogen-depleted leukocytes after the addition of varying amounts of glucose. Curves drawn as in Fig. 1. See text for details.

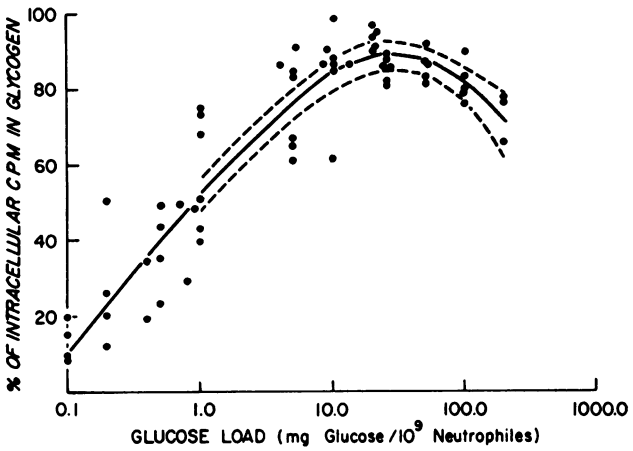

FIGURE 3 The proportion of intracellular glucose-^{14}C appearing in glycogen after a 60 min incubation of glycogen-depleted leukocytes supplemented with varying amounts of glucose-^{14}C. The ordinate represents the fraction (cpm in isolated glycogen/cpm in glycogen + alcohol supernatant) × 100. The solid curve was fitted by the least squares method; the dashed lines represent the 95% confidence limits. Experimental details given in the text.

would be utilized by a variety of cell processes and the excess would be stored as glycogen. The total amount of glucose which entered the cell and was incorporated into cell components would be reflected in the total of radioactivity found in the precipitated glycogen and in the supernate after alcohol precipitation. Fig. 3 illustrates a series of experiments performed with radioactive glucose. That fraction of the total radioactivity (glycogen cpm/glycogen + supernate cpm) which appeared in the isolated glycogen is plotted on the ordinate as a percentage and the glucose load added to the leukocyte suspension is plotted logarithmically on the abscissa. A third order polynomial was fitted to the experimental points by the method of least squares. The equation is $y = 52.63 + 40.77x - 5.20x^2 - 3.90x^3$. The multiple correlation coefficient (r^2) is 0.88. The dashed lines represent the 95% confidence limits. The data show that at increasing glucose loads, the fraction of intracellular glucose-derived radioactivity which appears in glycogen increases to a maximum and thereafter begins to fall off. Differentiation of the equation yielded a maximum point on the curve at 88.9% corresponding to a glucose level of 29.8 mg/10^9 neutrophiles. At the lower end of the scale of glucose loads the data show that as little as 10% of the intracellular glucose was found in glycogen.

Dynamic state of glycogen in leukocytes. Since liver glycogen has been shown to be in a constant state of turnover with both synthesis and degradation being carried out simultaneously (13), it was of interest to determine whether leukocyte glycogen behaved in a similar fashion. Leukocyte suspensions were incubated in radioactive glucose for a period of 30 min during which time the radioactive label appeared in the glycogen (Fig. 4). The cells were rapidly washed free of radioactivity and new medium was added which contained nonradioactive glucose in a concentration which was adequate only for the maintenance of glycogen stores at a nearly constant level. If leu-

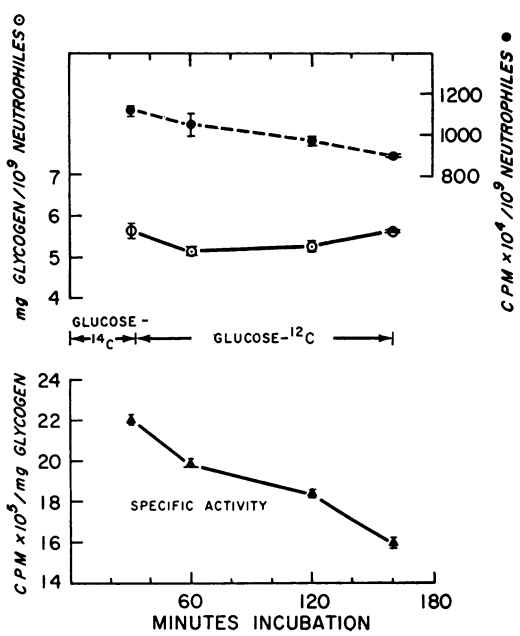

FIGURE 4 The turnover of leukocyte glycogen stores while the level of glycogen remains relatively constant. Glycogen was made radioactive by incubating cells in glucose-^{14}C. After removing the glucose-^{14}C from the medium, a limiting amount of nonradioactive glucose was added so that the glycogen content remained relatively constant. Points are means of duplicate determinations.

kocyte glycogen were in a constant state of turnover the radioactivity previously incorporated would be progressively washed out by the entrance of nonradioactive glucose into glycogen during the period of incubation with nonradioactive glucose. As can be seen from Fig. 4 the glycogen content during the period of observation changed very little whereas the radioactivity of the isolated glycogen decreased progressively as shown by the specific activity curve, which indicated that nonradioactive glucose was being incorporated into glycogen without appreciable change in the total glycogen level.

Other experiments were performed to determine whether newly acquired glucose units were added in a random fashion throughout the glycogen molecule or through some more selective mechanism. Pulse-chase experiments were performed as illustrated in Fig. 5. Leukocyte suspensions were incubated for 2 hr in adequate concentrations of radioactive glucose as shown in Fig. 5. After the leukocyte glycogen was radioactive, the cells were washed rapidly and resuspended in medium containing no glucose so that glycogenolysis took place. If new glucose units were added randomly

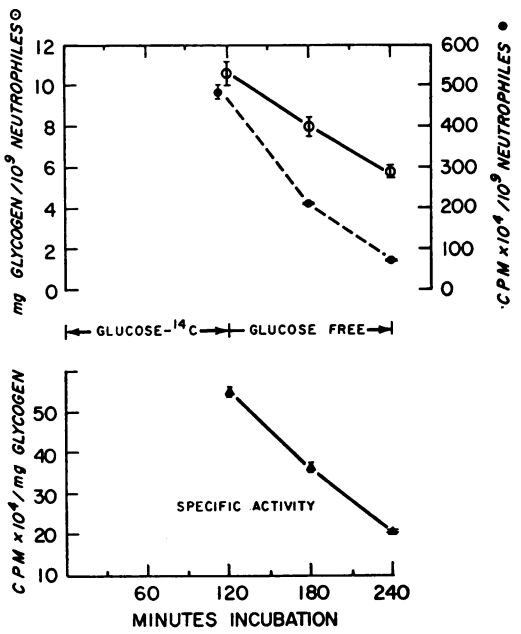

FIGURE 5 Preferential loss of more recently added glucose units of glycogen when conditions change from glycogen synthesis to degradation. See text for details. Points are means of duplicate determinations.

throughout the molecule and removed in a similar fashion, then the specific activity (cpm per mg glycogen) of the extracted glycogen would not change during a period of glycogenolysis. If the older glucose units (those present before the recent synthesis with radioactive glucose) were degraded first, then the specific activity of the glycogen would actually increase during a period of glycogenolysis. As can be seen in Fig. 5 the specific activity of the isolated glycogen decreased progressively during incubation in glucose free medium.

Stability of the protein "machinery" for glycogen synthesis. Both the enzyme which synthesizes glycogen (glycogen synthetase) and the glycogenolytic enzyme (phosphorylase) are known to be closely attached to the glycogen molecule (14, 15). It was of interest then to determine whether during conditions of glycogen synthesis and degradation these enzymes were degraded and resynthesized as well as the glycogen, or whether the "machinery" for glycogen metabolism was a stable structure. Puromycin at a concentration of 100 μg/ml was found to stop leukocyte protein synthesis immediately in this system. Leukocyte suspensions were then incubated in glucose-free medium for 3 hr to deplete glycogen and then adequate concentrations of glucose were added and glycogen content measured during the following 2 hr. The suspensions were divided into three parts: one part being a control, the second having puromycin added at the same time as glucose, and the third having puromycin added at the beginning of the incubation in glucose-free medium. When puromycin was added at the time of glucose addition, there was no loss of capacity to synthesize glycogen. When we added puromycin at the beginning of the glucose-free incubation, adding glucose at 3 hr did not result in increased glycogen content of the cells.

Other experiments were done with actinomycin to determine whether glycogen resynthesis in this system depended on new RNA synthesis; actinomycin D at a concentration of 12.5 μg/ml was found to be adequate to stop new RNA synthesis after 20–40 min. Leukocyte suspensions were preincubated for 3 hr in glucose-free medium and after 160 min actinomycin D was added to half of this suspension. At 180 min adequate glucose was added to both control and actinomycin-treated

cells and glycogen content measured during the following 2 hr. Cessation of RNA synthesis had no appreciable effect on the resynthesis of glycogen in the suspensions.

DISCUSSION

From these experiments a number of characteristics of the test system have been defined which enhance its usefulness for further study of glycogen metabolism. The initial glycogen content of the cells corresponds well with values previously reported in the literature (5, 16). Although the maximum level to which glycogen may be increased in the cells is not known, it was considered useful first to deplete a portion of the glycogen so that resynthesis then could be studied under conditions in which synthesis is very active.

Adding a given glucose "load" to the cell suspensions is useful in determining the levels at which glycogen synthesis is favored and the level at which maximal synthesis takes place, but this differs from the circulating blood where the glucose concentration remains relatively stable. When the data for glucose "loads" are converted in terms of mg/100 ml the level at which glycogen degradation is no longer necessary is 17.6 mg/100 ml for 60-min incubation and less than 10 mg/100 ml measured at 30 min. At glucose "loads" which provide maximal glycogen synthesis is this system the glucose concentration in the suspension was in the range of 200 mg/100 ml for the 60 min experiments and slightly above 100 mg/100 ml for the 30 min experiments. Since these concentrations are above that seen in the circulating blood normally, it is not surprising that further increases in glucose "loads" produced less glycogen synthesis.

The figures of 5.3 mg of glucose/10^9 neutrophiles/hr or 2.0 mg/30 min were an estimation of the minimal needs of the cell for glucose. Since the amount of glycogen in the cell is 7.6 mg/10^9 neutrophiles as measured in glucose units, the cell would be expected to have over an hour's supply of glucose in glycogen. However, the glycogen degradation studies show that in a 2 hr period the cells utilized only 2.8 mg of glycogen/10^9 neutrophiles. There are many possible interpretations of this discrepancy, but likely possibilities are that cells with inadequate glucose may slow down many aspects of their metabolism, or that gluconeogenesis may be increased under these conditions and act to spare the glycogen stores partially, or that only part of the glucose in the medium can be utilized.

The experiments illustrated in Fig. 3 show that cells which have partly depleted glycogen stores will shunt sizeable amounts of available glucose into making new glycogen. The data do not account for glucose-^{14}C which was converted to lactate and equilibrated with the extracellular compartment nor the ^{14}C which was lost as $^{14}CO_2$ but show the partition between glycogen and all other intracellular components. In the range of glucose "loads" at which maximum glycogen synthesis took place, over 80% of the cell-bound glucose is found in glycogen at the end of a 1 hr incubation. At lower glucose "loads" which were inadequate to cause an increase in glycogen stores, there was still a significant percentage of the counts appearing in glycogen. This was true even at glucose "loads" which allowed active glycogen degradation. This was no doubt due in part to the fact that there is a constant glycogen synthesis even though glycogenolysis predominates.

Also, as the amount of radioactive glucose was decreased, the total radioactivity in glycogen became lower, and when expressed as a per cent of the total the figure may appear high due to a small amount of isotope simply trapped in the glycogen pellet.

The experiment pictured in Fig. 4 documents the fact that leukocyte glycogen is in a constant state of turnover with simultaneous synthesis and degradation. The experiment was performed under conditions in which the glycogen level remained nearly constant. During that time the radioactivity of previously labeled glycogen decreased, which indicated that nonradioactive glycogen was displacing the radioactive molecules from the glycogen macromolecules. A similar turnover of glycogen has been shown for liver (13). It has also been shown in the platelet, under conditions in which glycogen is being degraded, that glycogen synthesis is still taking place, so a similar dynamic state can be inferred for platelet glycogen (17). One may assume that this is a general phenomenon for glycogen metabolism. The experiment illustrated in Fig. 5 shows that newly formed glycogen is the first part of the molecule to be degraded when conditions change and glycogen degradation exceeds the rate of synthesis. It is assumed that the

new glucose units are added to the outer shells of the glycogen molecule and the outer layers of the molecule are the regions of rapid turnover. This finding corroborates similar results of Seitz (5). The same holds true for liver glycogen (18). It is not known how rapidly or under what conditions the entire molecule might undergo a complete turnover. The experiment shows that after a 2 hr incubation glycogen was not uniformly labeled, indicating a turnover time in excess of 2 hr.

Glycogen exists in the cell not as a naked molecule but has at least two enzymes fixed to it. Glycogen synthetase and phosphorylase sediment with glycogen in the ultracentrifuge (14, 15). Thus, the glycogen molecule and at least part of its enzymatic components form an organelle of sorts—an energy storage organelle. This concept of glycogen as part of an organelle requires that the machinery for controlling the glycogen level is a stable apparatus which is renewed slowly if at all. The data from the actinomycin and puromycin experiments support this concept. At least glycogen synthesis does not require new RNA or protein synthesis in order to take place. The lack of glycogen synthesis after cells had been exposed to puromycin for 3 hr can be interpreted in various ways. It may indicate that proteins involved in glycogen synthesis are renewed so slowly that the short-term experiment did not show it. After 3 hr, the cells may have been injured in nonspecific ways which prevented glycogen synthesis. Perhaps puromycin affects the enzymes of glycogen metabolism by mechanisms independent of protein synthesis, as suggested by Søvik (19), and these effects are more delayed than the effects on protein synthesis. Søvik (20) has also shown that in muscle glycogen synthesis takes place normally in the presence of actinomycin.

A great deal more information needs to be learned about the glycogen-associated enzymes before a concept of a stable organelle can be substantiated. For instance, such a concept would require that the enzymes themselves be stabilized one to another and that they would remain associated with the last remaining chains of the glycogen molecule as it is degraded. Since glycogen begins to appear in the developing myelocyte (1, 2), the enzymatic machinery may be synthesized at that time or even earlier in cell differentiation.

Now that the conditions appropriate for glycogen synthesis in suspensions of granulocytes have been shown, and some aspects of the dynamic state of the molecule have been described, the system can be exploited further. The system should be useful in determining the activity and subcellular localization of enzymes associated with glycogen metabolism under conditions of varying glycogen stores, and methods of extracting glycogen can be perfected so that the physical state of the "native" molecule can be studied under various conditions.

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