Partly Ordered Synthesis and Degradation of Glycogen in Cultured Rat Myotubes*

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The following questions concerning glycogen synthesis and degradation were examined in cultured rat myotubes. 1) Is synthesis and degradation of the individual glycogen molecule a strictly ordered process, with the last glucosyl unit incorporated into the molecule being the first to be released (the last-in-first-out principle), or is it a random process? 2) Are all glycogen molecules in skeletal muscle synthesized and degraded in phase (simultaneous order) or out of phase (sequential order)? Basal glycogen stores were minimized by fasting and were subsequently replenished in two intervals, the first (0–0.5 h) with tritium-labeled and the second (0.5–3 h) with carbon-labeled glucose as precursor. Glycogen degradation was initiated by addition of forskolin. The kinetics of glycogen accumulation as well as degradation could be approximated by monoexponential equations with rate constants of 0.81 and 1.39 h⁻¹, respectively. The degradation of glycogen largely followed the last-in-first-out principle, particularly in the initial period. Analysis of the size of the glycogen molecules and the β-dextrin limit during glycogen accumulation and degradation showed that both synthesis and degradation of glycogen molecules are largely sequential and the small deviation from this order is most pronounced at the beginning of the accumulation and at the end of the degradation period. This pattern may reflect the number of synthase and phosphorylase molecules and fits well with the role of glycogen in skeletal muscle as a readily available energy store and with the known structure of the glycogen molecule. It is emphasized that the observed nonlinear relation between the change in glycogen concentration and release of label during glycogen degradation may have important practical consequences for interpretation of experimental data.

Glycogen is a branched polymer of glucose of spherical geometry composed of ~53,000 glucosyl residues for the fully synthesized molecule (β-glycogen). Glycogen plays a very dynamic role in the energy metabolism of skeletal muscle by serving as a readily available energy source during muscle contraction of high intensity (1, 2). The spherical form and branched structure of the glycogen molecule are optimally suited to fast breakdown by the glycogen phosphorylase and the debranching enzyme (2). The basic structure of glycogen consists of layers (tiers) of branched glucosyl chains with the protein glycogenin as the core. Each B-chain consists of 13 glucosyl residues linked by α[1→4] glucosyl bonds and gives rise to two new B-chains by way of the α[1→6] branch points (3). This design principle is repeated until 11 layers of B-chains are formed and the synthesis is completed by the addition of one tier of A-chains of the same length as the B-chain, but without branch points (2, 4). Further synthesis is not possible for steric reasons (2).

For the first eight tiers, the α[1→4] glucosyl bonds are synthesized by proglycogen synthase and the α[1→6] branch points by branching enzyme, and the molecule is defined as proglycogen. For the last four tiers, the α[1→4] glucosyl bonds are synthesized by glycogen synthase and the α[1→6] branch point by branching enzyme, and the molecule is defined as β-glycogen. It is assumed that glycogen synthesis and degradation under dynamic physiological conditions involve only the four outer tiers (tiers 9–12) (5–8), which, however, account for some 96% of the glucosyl residues of the full sized β-glycogen molecule. Proglycogen can therefore be considered a scaffold for the metabolically active four outer tiers of the β-glycogen molecule. For a full sized β-glycogen molecule, 35% of the glucosyl residues may be removed before the first debranching occurs (2).

Cognate with this highly ordered structure of the individual glycogen molecule (defined as intramolecular order), there may also be an intermolecular order, in the sense that all glycogen molecules are degraded/synthesized simultaneously. This has been investigated in adipocytes and in the liver (9, 10), but not in skeletal muscles. The aim of the present work was to investigate: 1) whether synthesis and degradation in skeletal muscle of the individual glycogen molecule is a strictly ordered process with the last glucosyl unit incorporated into the molecule being the first to be released (the “last-in-first-out” principle), or if it is a random process; and 2) whether all glycogen molecules are synthesized and degraded in phase (simultaneous order) or out of phase (sequential order).

One important practical consequence of this work is related to the interpretation of data, where the rate of glycogen degradation is estimated from the release of label from glycogen. With such data it is assumed that incorporation/release of label is proportional to actual incorporation/release of glucosyl units. Depending on the mechanism of synthesis and degradation, however, this may not have to be the case.

EXPERIMENTAL PROCEDURES

Materials

Fetal calf serum (FCS)† and horse serum were purchased from Biological Industries. Penicillin was obtained from Løvens Kemiske Fab-

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rrik, Ballerup, Denmark. Culture dishes were obtained from Nunc, Roskilde, Denmark. [6-3H]Glucose and [U-14C]glucose were purchased from Amersham Biosciences, Inc. Glycogen were obtained from Roche Molecular Biochemicals. Trypsin was purchased from Invitrogen. Dulbecco’s modified Eagle’s medium (DMEM), gentamycin, cytosine-1-β-β-arabinofuranoside (AraC), and all other chemicals were obtained from Sigma.

Cell Culture

Primary cultures of myotubes were prepared from the hindleg muscles of 21-day-old rat fetuses of Wistar rats by a modification of the method described by Küh et al. (11) and Daniels (12). Muscles from 10–15 fetuses were finely cut with a pair of scissors for 10 min in 2 drops of Dulbecco’s phosphate-buffered saline with 1% glucose and without Ca2+ and Mg2+ (CMDDPBS), and transferred to a centrifuge glass with 10 ml of 0.1% collagenase 1 0.15% trypsin with EDTA + 0.01% DNase I in CMDDPBS. After incubation on a water bath at 37 °C for 30 min with shaking every 5 min for the first 20 min and every 2 min for the last 10 min, the digest was triturated 15 times at room temperature with a wide bore pipette tip, diluted with 10 ml of 80% DMEM and 20% FCS, and centrifuged for 8 min at 1000 g. The cell pellet was suspended in 5 ml (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS) and filtered by syringe pressure through a 20-μm filter. The centrifuge tube was washed six times with 5 ml (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS), which was also passed through the filter. The filtrates were collected on a 150-mm uncoated culture dish and incubated for 45 min in a 5% CO2, 95% atmosphere air at 37 °C. The nonadhering cells were diluted to 1 × 106 cells/ml, and 25 ml were plated on a 150-mm gelatin-coated culture dish, prepared by coating the dish with 10 ml of 0.1% gelatin for 2–3 h at 37 °C and removed by suction twice immediately before plating. The myoblasts were confluent to confluence for 48 h at 37 °C in 5% CO2, 95% atmosphere air, and were released from the dish by dispase treatment; the medium was removed by suction and 10 ml of 0.1% collagenase I (Sigma) in CMDDPBS. After centrifugation for 5 min at 70 × g, the cell pellets were combined in a single centrifuge tube and incubated for 2 h at 37 °C, removing every 5 min for 5 min at 70 × g (to remove volume of 1% dispase in CMDDPBS). One volume (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS) was added, and the suspension was centrifuged for 5 min at 70 × g. The cell pellet was suspended in 5 ml (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS), and the cells were incubated for 5 min at 37 °C in 5% CO2, 95% atmosphere air. The medium was carefully removed and the myoblasts loosened by tapping the dish against the edge of a table for 1 min while rotating the dish. The cells were washed into a centrifuge tube by 5 ml (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS). The empty dishes were tapped against the edge of a table and washed into the centrifuge tube with 5 ml (80% DMEM with 25 mM NaHCO3 and 25 mM glucose + 20% FCS). After centrifugation for 5 min at 70 × g, the cell pellets were combined in a single centrifuge tube and incubated for 2 h at 37 °C. The cell pellets were removed from the dish by dispase treatment; the medium was removed by suction two times at 1–2 °C with Hanks’ balanced salt solution. 1000 ml of KB f buffer (15) with 1 mM dithiothreitol instead of 15 mM β-mercaptoethanol was added to each dish, and the cells were scraped loose with a rubber policeman and homogenized on ice by ultrasonication (Branson Sonifier B-12) at 50 watts for 5 s. The homogenates were kept frozen at –80 °C until analyses. Protein content was measured by the method of Lowry et al. (16) with bovine serum albumin as standard.

Preparation of Homogenates for Determination of Glycogen, Radioactivity in Glycogen and β-Dextrin, and Molecular Size Distribution of Glycogen—The dishes were placed on ice, the medium was removed from the dishes by suction and 1000 μl of 0.4 n KOH was added to each dish. After 5 min at room temperature, aliquots of the homogenates were frozen at –20 °C until analysis.

Glycogen Assay—Glycogen was determined as glucose after treatment with amyloglucosidase as described by Katz et al. (17).

Radioactivity in Glycogen and β-Dextrin—Aliquots for determination of β-dextrin limits were acidified to pH 4.6 by 1 m citric acid and digested with β-amylase (9). The β-dextrin digest were precipitated with 2 volumes of absolute ethanol for 1 h on ice, centrifuged, and washed twice with 70% ethanol. The final precipitate was hydrolyzed in 1 N HCl for 2 h at 80 °C and neutralized with 1 ml NaOH. 3 ml of Ultima Gold liquid scintillation solution was added, and the radioactivity was determined in a Packard Scintillator with external counting efficiency determination.

The procedure for determination of radioactivity in glycogen originating from 3H2O, two additional washing procedures with 70% ethanol were included.

Size Distribution of Glycogen Molecules—Homogenates in 0.4 mM KOH were heated on a boiling water bath for 10 min, cooled, and centrifuged for 2 min at 10,000 × g. The supernatant was applied on a 5-cm Dowex-50 (H+) on top of Dowex-1 (acetate) and the columns eluted with 25 ml water. The eluate was lyophilized and dissolved in 100 μl of water and kept at –20 °C until EM analysis. Glycogen granules were negatively stained (1% uranyl acetate in water) on 200-mesh formvar and carbon-coated nickel grids previously subjected to glow discharge just before use. Electron microscopy was carried out using a Zeiss EM 900 electron microscope operated at 80 kV.

Experimental Design and Calculations

Experimental Design—The mechanism of glycogen synthesis and degradation was elucidated by the following experimental design, as illustrated in Fig. 1A. From day 14 to day 15, cells were depleted of glycogen for 3 h. On day 15 at t = 0 h, the cell cultures were incubated under conditions favoring glycogen synthesis. Glycogen accumulation reached a maximum after ~3 h; the first interval of glycogen accumulation occurred from 0 to 0.5 h and was carried out in the presence of [6-3H]glucose; the second interval of glycogen accumulation from 0.5 to 3 h was carried out with [U-14C]glucose. At 3 h, incubation conditions were changed so glycogen degradation was favored, i.e. low glucose and forskolin.

The medium composition for the periods was as follows: depletion period, DMEM with 25 mM NaHCO3, 0 mM glucose, 10% horse serum; accumulation period, first interval, DMEM (19 mM NaHCO3, 19 mM Heps, pH 7.2, and 0.1 mM creatine), 10 mM glucose, and 1.6 mM [U-14C]glucose; second interval, DMEM (same composition as for first interval), 10 mM glucose, and 0.4 μCi/ml [U-14C]glucose; and degradation period, DMEM (same composition as for first interval, but without pyruvate), 1 mM glucose, and 10 μμ forskolin.

At the time points indicated in Fig. 1B (0, 0.5, 1.5, and 3 h for the glycogen accumulation period and 3, 3.25, 3.5, 4, 5, 6, and 7 h for the degradation period) total glycan and radioactivity in β-dextrin (for the estimation of degree of sequential synthesis/degradation, see “β-Dextrin Limit” below) and glycogen (for estimation of degree of the last-in-first-out principle, see “Calculations” below) were determined. The molecular size distribution of glycogen (for the estimation of degree of sequential synthesis/degradation) was determined at 0, 0.5, 3, 3.5, and 7 h.

Recovery of the 3H radioactivity, which was incorporated in glycogen from 0.5 h, was estimated by determination of the 3H content at t = 1.5 and 3 h. For 14C radioactivity, incorporation in glycogen from t = 0 to 3 h, recovery was determined at t = 4 h in parallel dishes where the accumulation period was extended to 4 h in the absence of radioactivity from t = 3 to 4 h.

Glycogen net synthesis during the degradation period from 3 to 7 h was determined in parallel dishes, which were treated as for glycogen accumulation in two intervals as described above, but without addition

Analytical Procedures

Protein Assay—The dishes were placed on ice, the medium was removed by suction, and the cells washed three times at 1–2 °C with Hanks’ balanced salt solution. 1000 ml of KB buffer (15) with 1 mM
of radioactive tracers. At the start of the degradation period at \( t = 3 \) h, 500 \( \mu \)Ci of \(^3\)H2O/ml of medium was added and \(^3\)H radioactivity in glycogen was determined at \( t = 4, 5, 6, \) and 7 h.

\( \beta \)-Dextrin Limit—The \( \beta \)-dextrin limit was determined as the amount (or radioactivity) of glycogen remaining after treatment with \( \beta \)-amylase divided by the total amount (or radioactivity) of glycogen. In glycogen particles with full sized A-chains, \( i.e. \) 13 glucosyl units, the size of the \( \beta \)-dextrin relative to the untreated glycogen molecule is \( (2^t - 1\) - 1\( )/(2^t\) - 1), where \( t \) is the number of glucosyl tiers, \( 2^t - 1\) - 1 is the number of glucosyl chains left in glycogen (\( \beta \)-dextrin) after treatment with \( \beta \)-amylase and \( 2^t - 1\) is the total number of glucosyl chains in glycogen (\( 2, 18\) resulting in a \( \beta \)-dextrin limit of 48–50\% for \( 5 \leq t \leq 12\). For A-chains shorter than 13 glucosyl residues, the \( \beta \)-dextrin limit was calculated as \((13(2^t - 1\) - 1\( )/(13(2^t - 1\) - 1\) + \(n(2^t - 1)\)), where \( n \) is the number of glucosyl residues in the A-chains. During glycogen synthesis, the \( \beta \)-dextrin limit will range from 65 to 50\%, assuming a minimum A-chain length of 7 glucosyl residues, and during degradation the range will be from 50 to 76\%, assuming a minimum A-chain length of 4 glucosyl residues.

If all full sized glycogen particles behave similarly during degradation, \( i.e. \) a fully simultaneous degradation pattern, it follows from the definition of the \( \beta \)-dextrin limit that the amount of glycogen that a given time point can be estimated as the \( \beta \)-dextrin limit in the full sized particles divided by the \( \beta \)-dextrin limit at that time point: glycogen = \( \beta \)-dextrin limit - \( \beta \)-dextrin limit. Calculations—The accumulation and degradation of both labeled and total glycogen could be described by monoexponential equations (see “Results,” Figs. 1 and 2, and Table I).

\[
\text{Glyc}_\text{acc} = \text{Glyc}_{\text{span-acc}} \times (1 - e^{-k_{\text{acc}}t}) + \text{Glyc}_{\text{c0}} \tag{1}
\]

and

\[
\text{Glyc}_\text{deg} = \text{Glyc}_{\text{span-deg}} \times (1 - e^{-k_{\text{deg}}t}) + \text{Glyc}_{\text{c0}} \tag{2}
\]

\( \text{Glyc}_\text{acc} \) and \( \text{Glyc}_\text{deg} \) are the glycogen concentration during the synthesis and degradation period, respectively; \( \text{Glyc}_{\text{span-acc}} \) and \( \text{Glyc}_{\text{span-deg}} \) are the span of glycogen concentrations for the synthesis and degradation period, respectively; \( t \) is time; and \( k_{\text{acc}} \) and \( k_{\text{deg}} \) are rate constants for the synthesis and degradation, respectively. The maximal glycogen concentration \( \text{Glyc}_{\text{max}} \) equals \( \text{Glyc}_{\text{span-acc}} + \text{Glyc}_{\text{c0}} \) or \( \text{Glyc}_{\text{span-deg}} + \text{Glyc}_{\text{c0}} \). The glycogen content was normalized to 1 at \( t = 3 \) h.

To quantify the degree of last-in-first-out, the value of the following expression was calculated for the time intervals where release of tracer is estimated to take place if 100\% degree of order is assumed (cf. Fig. 1A, Table II).

\[
\text{fraction of tracer released from labeled glycogen} = \frac{1 - \text{fraction of glycogen degraded}}{1 - \text{fraction of glycogen degraded}} \tag{3}
\]

The degree of order ranges from zero to one, corresponding to completely random and fully ordered degradation of glycogen, respectively. The degradation time intervals where release of tracer is estimated to take place if 100\% degree of order is assumed were calculated from Equations 1 and 2, and the release of \(^3\)H or \(^1\)C label was calculated from Equation 2 using these time points and the degradation constants for \(^3\)H and \(^1\)C, respectively.

**RESULTS**

The glycogen pool in the muscle cells was labeled over two intervals during the accumulation of glycogen, first with [6-\(^3\)H] glucose for 0.5 h and then from 0.5 to 3 h with [U-\(^1\)C]glucose (cf. Fig. 1A and “Experimental Design”). The subsequent release of radioactivity during glycogen degradation was intended to show the degree to which the synthesis and degradation of glycogen followed the last-in-first-out principle.

**Kinetics of Glycogen Accumulation and Degradation**— Prior to the initiation of glycogen accumulation, the myotubes contained 47 ± 8 nmol of glycogen/mg of protein. Glycogen accumulation was initiated by changing the medium to 10 mm glucose (\(^3\)H-labeled from \( t = 0 \)–0.5 h and \(^1\)C-labeled from 0.5–3 h) and after 0.5 and 3 h, glycogen content had increased to 151 ± 18 and 310 ± 35 nmol/mg of protein (\( n = 8 \), respectively. The glycogen accumulation for the 3-h interval could be well fitted by the monoexponential Equation 1 (see “Calculations”), with a rate constant \( k_{\text{acc}} \) of 0.81 ± 0.10 h\(^{-1}\), \( \text{Glyc}_{\text{span-acc}} = 0.944 ± 0.045\), \( \text{Glyc}_{\text{c0}} = 0.166 ± 0.023\), and \( \text{Glyc}_{\text{max}} = 1.109 ± 0.037\) (Table I).

Glycogen degradation was followed for 4 h and initiated by a medium containing 1 mm glucose and 10 \( \mu \)M forskolin, which is supposed to activate glycogen phosphorylase (15). Analogous to the synthesis, the degradation process could be well approximated by the monoexponential Equation 2 (see “Calculations”) with a rate constant \( k_{\text{deg}} \) of 1.39 ± 0.14 h\(^{-1}\), \( \text{Glyc}_{\text{span-deg}} = 0.913 ± 0.016\), \( \text{Glyc}_{\text{c0}} = 0.095 ± 0.018\), and \( \text{Glyc}_{\text{max}} = 1.080 ± 0.011\) (Table I).

**Estimation of the Degree of Simultaneous Glycogen Synthesis and Degradation**—Interpretation of the results would be complicated if significant glycogen recycling took place during the experiments. Glycogen degradation during the accumulation period and glycogen synthesis during the degradation period was therefore estimated. In the \([\text{14C}]\)glycogen accumulation interval (from 0.5 to 3 h), the \(^3\)H content of glycogen decreased linearly by 4 ± 1\% h\(^{-1}\) (\( p < 0.001 \)). The \(^1\)C content in glycogen decreased by 6 ± 6\% h\(^{-1}\) (\( p < 0.05 \)), as determined in parallel experiments where the glycogen accumulation period was extended to 4 h with unlabeled glucose in the period from 3 to 4 h.

Recycling of radioactive tracer may therefore have occurred. However, this is probably not a problem because released labeled glucose 1-phosphate and glucose would be diluted by the much higher concentration of labeled precursors (10 mm, see “Experimental Procedures”). During the degradation period (from 4 to 7 h), net glycogen synthesis was determined as incorporation of \(^3\)H2O into glycogen. The synthesis rate of glycogen under degradation conditions was extremely low and nonsignificant.

**The Degree of Order of the Glycogen Accumulation-Degradation Process**—Because the \(^3\)H label was introduced first during the glycogen synthesis, it could be expected to be released last and *vice versa* for the \(^1\)C label if the last-in-first-out principle applies and if all glycogen molecules are in the same phase of degradation. The data show that there was no significant release of \(^3\)H-labeled glucose from glycogen during the first 15 min of the degradation period, during which 25\% of the glucosyl units was released (Fig. 2). Thereafter, however, significant \(^3\)H release took place, although at a lower rate than \(^1\)C release as reflected in the increase in the \(^3\)H/\(^1\)C ratio from 3 to 7 h (Fig. 3). With the ratio normalized to 1 at \( t = 3 \) h, it increased linearly to ~7 at \( t = 7 \) h. The release of \([\text{14C}]\)glucose started immediately upon establishing degradation conditions and followed first order kinetics, as did the total glycogen pool, but the rate constant was ~40\% higher (Fig. 2).

The degree to which the last-in-first-out principle applies to glycogen synthesis and degradation in myotubes was calculated as described under “Calculations” and is shown in Table II. The time interval in which all \(^1\)C or \(^3\)H was expected to be released if a strict last-in-first-out principle applied was calculated from the time course of isotope incorporation in glycogen as described under “Calculations” and is given in Table II. The estimated release of isotope in these intervals allowed calculation of the degree of last-in-first-out to 0.03 ± 0.03 (mean ± S.E. (\( n = 8 \))) for \(^3\)H-labeled glycogen and 0.36 ± 0.03 (mean ± S.E. (\( n = 8 \))) for \(^1\)C-labeled glycogen (\( p < 0.001 \)), respectively. These results indicate a completely random degradation of the first synthesized (\(^3\)H-labeled) glycogen and a partially ordered degradation of the last synthesized (\(^1\)C-labeled) glycogen.

To check for possible procedural effects, the order in which the two tracers were administered was reversed, although with no effect (data not shown).
Glycogen Particle Size—The mean diameter of glycogen particles increased from 24.9 ± 1.8 nm (mean ± S.E. (n = 3)) at t = 0 h to 28.1 ± 1.1 nm at t = 0.5 h, remained constant during accumulation from t = 0.5–3 h (29.4 ± 1.6 nm at t = 3 h) and during degradation from t = 3–3.5 h (29.1 ± 1.1 nm at t = 3.5 h), and then declined to the initial value at the end of the degradation period (24.4 ± 0.1 nm at t = 7 h) (Fig. 4).

β-Dextrin Limits—β-Dextrin limits during the glycogen ac-
The glycogen molecules must have a mean A-chain length of approximately 13 glucosyl residues at 1.5 and 3 h. From 3 to 7 h, the \(14C\)-dextrin limit increased from approximately 48 to 67%, suggesting a decrease in the average A-chain length.

The \(3H\)\&\(14C\) activity in glycogen and glycogen content were normalized to 1 at \(t = 3\) h. Values are means ± S.E. \((n = 8)\).

**FIG. 3. Ratio of \(3H/14C\) activity in glycogen during degradation.** Glycogen in cultured myotubes was labeled by \(3H\) and \(14C\) as illustrated in Fig. 1A from \(t = 0–3\) h, at which time degradation conditions were initiated. The ratio between \(3H\) and \(14C\) activity in glycogen was calculated and normalized to 1 at \(t = 3\) h. Values are means ± S.E. \((n = 8)\).

**TABLE II**

**Calculated degree of last-in-first-out**

| Interval (isotope) | Estimated degradation interval if 100% degree of last-in-first-out | Estimated fraction of tracer released from glycogen in the degradation interval | Estimated fraction of glycogen degraded in the time interval | Degree of last-in-first-out |
|-------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-----------------------------|
| First (\(3H\))    | 3.75 ± 0.07–5.38 ± 0.43                         | 0.33 ± 0.05                                    | 0.29 ± 0.02                                    | −0.03 ± 0.03                 |
| Second (\(14C\))  | 3–3.75 ± 0.07                                   | 0.71 ± 0.05                                    | 0.57 ± 0.06                                    | 0.36 ± 0.03*                |

This increase was not the result of release of outer, tritium-labeled glucosyl residues because the decrease of \(3H\) in \(\beta\)-dextrin and in total glycogen from 0.5 to 3 h occurred at the same rate (results not shown). In the degradation period, the \(3H\)\&\(\beta\)-dextrin limit increased linearly to −0.87, but the S.E. of the values between \(t = 3–7\) h were big. In the accumulation period, the \(14C\)\&\(\beta\)-dextrin limit was −0.41 at \(t = 1.5\) h and remained at the same level to \(t = 3\) h. During degradation the \(14C\)\&\(\beta\)-dextrin limit increased gradually to −0.63 at \(t = 7\) h.
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FIG. 4. Size distribution of glycogen molecules in myotubes. The diameter of glycogen molecules was determined by EM (see "Experimental Procedures") and grouped into intervals corresponding to the closest number of tiers (3–12). For each time point, the relative number of molecules of each size is indicated by the length of the horizontal line. The total number of glycogen molecules measured for each time point was 240–431. Data are mean values from three experiments.

Discussion

The hypotheses tested in the present work were as follows. 1) The degradation of the individual glycogen molecule is an ordered process with the last glucosyl unit incorporated into the molecule being the first to be released (the last-in-first-out principle). 2) All glycogen molecules in skeletal muscle are synthesized and degraded simultaneously (in phase).

One of the methods applied to elucidate this question was to label the initially incorporated glucosyl units with 3H and the principle. 2) All glycogen molecules in skeletal muscle are degraded simultaneously with the last glucosyl unit incorporated into the molecule being the first to be released (the last-in-first-out principle). This is illustrated in Fig. 6, showing that a 5% fall in molecular diameter corresponds to a 40% increase in the β-dextrin limit in a full-sized glycogen molecule.

β-Dextrin Limits—The β-dextrin limit is defined as the fraction of the glycogen molecule that is resistant to degradation by β-amylase, and thus reflects the mean length of the outer A-chains in glycogen. The β-dextrin limit is a much more sensitive measure of the molecular size than the diameter of glycogen particles. This is illustrated in Fig. 6, showing that a 5% fall in molecular diameter corresponds to a 40% increase in the β-dextrin limit in a full-sized glycogen molecule.

The β-dextrin limit at t = 0.5 h was 61% (Fig. 5A), a figure close to that of glycogen molecules with the minimum A-chain length of 7 glucosyl residues (see "β-dextrin limits"). From 0.5 to 1.5 h, the β-dextrin limit fell to ~50% but remained constant for the rest of the accumulation period, suggesting that A-chains are elongated to the maximum length of 13 glucosyl residues from 0.5 to 1.5 h, and that the 37% increase in glycogen content from 1.5 to 3 h was the result of the synthesis of new molecules, i.e., a partially sequential pattern of synthesis.

The β-dextrin limits for tritiated glycogen (Fig. 5B) increased from 61% at 0.5 h to 72% at 1.5 h and then remained constant, in accordance with an increase in number of tiers in glycogen molecules from 0.5 to 1.5 h, and synthesis of new glycogen molecules from 1.5 to 3 h. The [14C]β-dextrin limit did not increase from 1.5 to 3 h as expected. In this time interval, however, only ~37% of the total 14C activity is incorporated in glycogen, and the effect on the [14C]β-dextrin limit will be beyond the limit of detection.

In the initial degradation period from 3 to 3.5 h, the β-dextrin limit increased from 48 to 57%. If all glycogen molecules were degraded simultaneously, this should have resulted in a 17% decrease in glycogen content (see "Calculations"), whereas a 44% decrease was actually observed. Thus, a purely simultaneous degradation pattern is unable to account for the result. From 3.5 to 7 h, the β-dextrin limit increased further to 67% and the total amount of glycogen decreased from approximately 56 to 9%. This also indicates a sequential degradation pattern, but with a larger simultaneous contribution than from 3 to 3.5 h.

From the analysis of β-dextrin limits and the size distribution of glycogen molecules, it is concluded that the process of synthesis as well as the process of degradation of glycogen molecules is sequential with a simultaneous component and that the latter component is largest at the beginning of the accumulation and at the end of the degradation period. This same mechanism of glycogen synthesis and degradation is also suggested by the increasing ratio between 3H and 14C with...
time (Fig. 3), because a fully synchronous synthesis combined with a sequential degradation of all glycogen molecules or vice versa will result in a constant $^{3}H/^{14}C$ ratio.

The interpretation of the present results is that groups of glycogen molecules are synthesized or degraded before the next group of molecules are synthesized or degraded, respectively. For the individual glycogen molecule, there is evidence for an ordered mechanism of degradation according to the last-in-
first-out principle. This model may reflect the number of synthase and phosphorylase molecules relative to the glycogen molecules and fits well with the role of glycogen in skeletal muscle as a fast available energy store (the most efficient model with respect to number of available attack points of especially phosphorylase is a simultaneous degradation pattern), and with the structure of the glycogen molecule (2).

An ordered sequential glycogen degradation mechanism may depend on the existence of glycogen binding molecule(s) that also may bind both the synthase and the phosphorylase and is necessary for the activity of the two enzymes. If such binding molecule(s) are targeted to glycogen molecules as they become available by synthesis, the result would be that the last synthesized glycogen molecules would be the first to be degraded. In skeletal muscle two glycogen-binding proteins with regulatory effect on glycogen synthesis and degradation has been identified. The two proteins are PTG (protein targeting to glycogen) (20) and RG-1/GM (muscle isoform of the regulatory subunit of protein phosphatase-1) (21). PTG has binding sites for both synthase and phosphorylase (22). However, the role of both proteins appears to be a stimulation of glycogen synthesis (21, 23, 24) and their properties do not appear to provide an adequate explanation for the last-in-first-out principle.

Implications of Results—An important practical aspect of the results of the present paper is apparent in Fig. 2, showing that there may not be a linear relation between release of radioactivity from glycogen breakdown and the actual decrease of the glycogen concentration. This lack of linearity is easily understood as a consequence of the last-in-first-out principle, which we show here applies to muscle glycogen metabolism to a significant degree. Obviously, unawareness of this fact may lead to erroneous interpretation of studies involving incorporation of labeled glucose in glycogen. Similarly, the interpretation of $^{13}$C magnetic resonance spectroscopy results, where global measurements of $^{13}$C content in glycogen is studied after loading with $^{13}$C labeled glucose (19), may be affected.

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