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Proglycogen and macroglycogen: artifacts of glycogen extraction?

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Abstract

Most recent studies on the physiology of proglycogen and macroglycogen in skeletal muscles have adopted a homogenization-free acid extraction protocol to separate these 2 pools of glycogen. The purposes of this study were to determine (*a*) whether this protocol is suitable; (*b*) if the acid-insoluble glycogen fraction corresponds to proglycogen; and (*c*) if this fraction accounts for most of the changes in muscle glycogen content, irrespective of muscle fiber types. Using the rat as our experimental model, this study shows that when the conditions of acid extraction are optimized, 52% to 64% of glycogen in rat muscles is found as acid-soluble glycogen as opposed to approximately 16% when glycogen is extracted using a homogenization-free extraction protocol. Moreover, there is no evidence that the acid-insoluble glycogen corresponds to proglycogen because gel chromatography of the acid-insoluble and acid-soluble glycogen fractions shows similar elution profiles of high–molecular weight glycogen. Finally, irrespective of muscle fiber types, the acid-soluble glycogen accounts for most of the changes in total muscle glycogen levels during the fasting-to-fed transition, whereas the levels of the acid-insoluble glycogen remain stable or increase marginally. In conclusion, this study shows that the homogenization-free acid extraction of muscle glycogen underestimates the proportion of acid-soluble glycogen and that the findings of the studies that have adopted such an extraction protocol to examine the physiology of acid-insoluble and acid-soluble glycogens require reexamination.

1. Introduction

Muscle glycogen homogenized in the presence of acid can be separated into acid-soluble and acid-insoluble pools [1]. Although the mechanism underlying the existence of these pools has remained elusive for several years, this has not prevented a substantial volume of research from being undertaken mainly in the 1950s to elucidate the responses of these glycogen pools to a broad range of physiological conditions [2-7]. It was only in the early 1990s that the basis for the variations in acid solubility of different glycogen fractions was alleged to have been explained at the molecular level. Based on the electrophoretic separation of acid-insoluble glycogen, Lomako and colleagues [8-10] reported that this fraction, referred to as *proglycogen*, was composed mainly of low–molecular weight glycogen particles, each with a molecular mass of approximately 400 kd. The poor solubility of proglycogen was explained on the basis that each of these particles is covalently bound to a 37-kd protein primer, glycogenin, with the resulting high protein-to-carbohydrate ratio being responsible for the low acid solubility of proglycogen. In contrast, the large glycogen particles with masses of up to 10 000 kd, referred to as *macroglycogen*, were claimed to be acid soluble because of their lower glycogenin-to-carbohydrate ratio [8].

In the early 1990s, Lomako and colleagues [9] provided experimental evidence that the acid-insoluble glycogen is an intermediate in the synthesis of acid-soluble glycogen in astrocytes. Given such a possible precursor-product relationship between these 2 pools of glycogen [9], it is hardly surprising that their responses to a wide range of physiological conditions have been the object of renewed interest over the past decade, with most studies attempting to explain the pattern of responses of acid-soluble and acid-insoluble glycogens based on the proglycogen-macroglycogen paradigm [11-23]. What has been reported in general in these studies is that most of the glycogen particles exist as acid-insoluble glycogen, with this glycogen fraction accounting for most of the changes in total glycogen levels, except when

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muscles store above-normal levels of glycogen, under which conditions acid-soluble glycogen plays a role of increasing importance [11,12,15-18,22].

Most of the aforementioned recent studies on acid-insoluble and acid-soluble glycogens share a number of limitations. Firstly, none of these studies has attempted to provide evidence that the acid-insoluble glycogen fraction corresponds to a lower–molecular weight species of glycogen. Another major limitation shared by most recent studies is the high likelihood that the acid-insoluble glycogen is contaminated with acid-soluble glycogen. This is because muscle glycogen in those studies has been extracted using the homogenization-free protocol of Adamo and Graham [13]. In this technique, small pieces of freeze-dried muscles are placed in a glass tube containing acid and then pressed against the wall of the tube with a plastic rod before centrifugation to separate acid-soluble from acid-insoluble glycogen. The problem here is that because most of muscle glycogen particles are closely associated with the sarcoplasmic reticulum and myofibrils [24], it is possible that, in the absence of a homogenization step, a fraction of the glycogen reportedly acid insoluble is precipitated not because of its poor acid solubility per se, but simply because it is trapped by the dense mesh of partially undisrupted muscle myofibrils that coprecipitate glycogen during centrifugation. Finally, another important limiting factor overlooked by all but a few studies [16,20] is the possibility that the proportion of acid-insoluble and acid-soluble glycogens and their patterns of response to changes in glycogen levels might vary according to muscle fiber type.

Against this background, our goals were (*a*) to optimize muscle homogenization conditions to determine whether the proportions of acid-soluble and acid-insoluble glycogens obtained are similar to those using a homogenization-free extraction protocol; (*b*) to determine by gel chromatography the molecular weight profiles of the acid-soluble and acid-insoluble glycogens to ascertain whether the average molecular weight of these 2 fractions of glycogen is different; and (*c*) to examine whether, under extraction conditions involving a homogenization step, the acid-insoluble glycogen fraction across all muscle fiber types accounts for most of the changes in muscle glycogen content. This latter goal was addressed by examining the response of muscle glycogen to the fasting-to-fed transition, a condition resulting in a significant increase in skeletal muscle glycogen content [25,26]. The rat was chosen as our experimental model because it provides a ready means of assessing the effect of muscle fiber types on acid-soluble and acid-insoluble glycogen fractions.

2. Experiment

2.1. Materials

All biochemical reagents and enzymes were obtained from Boehringer Mannheim (Sydney, New South Wales, Australia), except for σ-dianisidine hydrochloride that was purchased from Sigma (St Louis, MO). All chemicals were of analytical grade.

2.2. Animals

Adult male Wistar rats (300 g) were supplied by the Animal Resource Centre (Murdoch University, Western Australia, Australia). The rats were kept at 20°C on a 12-hour light/12-hour dark cycle (light from 7:30 AM) and had unlimited access to water and standard laboratory rat chow (Glen Forrest Stockfeeders, Glen Forrest, Western Australia, Australia; 55% digestible carbohydrate, 19% protein, 5% lipid, and 21% nondigestible residue by weight). Some rats were used for the optimization of glycogen extraction by acid precipitation experiments, and others were used to examine the molecular weight distribution profiles of glycogen fractions using gel filtration chromatography. Finally, 81 rats were used for the starved-to-fed transition experiment, with 9 rats per time point. These rats were fasted in grid-bottomed cages for 48 hours to deplete part of their muscle glycogen before refeeding, which was commenced at 8:00 AM by providing access to standard laboratory chow [27]. At time intervals after the initiation of refeeding (0, 1, 2, 4, 6, 8, 12, and 24 hours), rats were anesthetized and their tissues were sampled. A control group of fed animals was anesthetized and sampled at 8:00 AM, at which time muscle glycogen reaches peak level of its diurnal cycle [28].

2.3. Tissue sampling

Tissues were sampled under halothane anesthesia so as to prevent postmortem muscle glycogen breakdown [29] and because this anesthetic is without effect on muscle glycogen levels [30]. Halothane anesthesia was induced with 4% halothane–96% oxygen (vol/vol), the dosage being reduced to 1.5% halothane–98.5% oxygen (vol/vol) once the animals were anesthetized. While under anesthesia, the following muscles were sampled within 2 minutes: soleus; diaphragm; and the white, red, and mixed portions of the quadriceps muscles, the latter of which corresponding to the superficial, deep, and intermediary regions of the quadriceps muscle, respectively. Immediately after sampling, the muscles were freeze-clamped in liquid nitrogen; and all samples were kept at −80°C before analysis. The muscles selected for analysis were chosen on the basis of their fiber composition. The red, white, and mixed quadriceps are rich in type IIa, type IIb, and type IIab, respectively; the soleus muscle is rich in type I fibers; and the diaphragm contains predominantly type I and type IIa muscle fibers [31,32].

2.4. Determination of acid-soluble and acid-insoluble glycogens

Each muscle was ground to a powder using a mortar and pestle under liquid nitrogen before being transferred to precooled vials for weight determination. Ten volumes of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) was added, and

the muscle was homogenized using a Janke and Kunkel Ultra-Turrax T-25 homogenizer (IKA, Willington, NC) for 1 minute. Acid-soluble and acid-insoluble fractions were separated by centrifugation at 5000*g* for 10 minutes, the supernatant was removed, and the pellet was extracted in 5 vol of 10% (wt/vol) TCA and recentrifuged. This step was then repeated twice to further remove any acid-soluble material from the pellet, after which all the supernatants were pooled.

To determine the levels of acid-insoluble glycogen, the pellet remaining after repeated acid extraction was completely digested by boiling in 3 vol of 30% (wt/vol) KOH for 20 minutes with regular mixing. After cooling to room temperature, glycogen was precipitated by the addition of absolute ethanol to a final concentration of 66% (vol/vol). Two drops of saturated Na_2SO_4 were added to act as a coprecipitant, and the glycogen was left to precipitate overnight at 4°C. The glycogen precipitate was then reextracted in 66% (vol/vol) ethanol and once in acetone before being dissolved in water and assayed for glycogen. The supernatant containing the acid-soluble glycogen was neutralized using 2 mol/L K_2CO_3 before being assayed for glycogen. The concentration of glycogen was determined using a modification [33] of the method of Chan and Exton [34].

To confirm that the methods used to measure acid-soluble and acid-insoluble glycogen levels were quantitatively determining all the glycogen present in the muscle, we also measured glycogen recoveries in some muscles (n = 9) by determining total muscle glycogen levels. This was achieved by using KOH extraction of the frozen powdered muscle by boiling it in 3 vol of 30% (wt/vol) KOH followed by ethanol precipitation as described above for the determination of acid-insoluble glycogen. We found that the summation of acid-soluble and acid-insoluble glycogen was on average 94% ± 2% of the total glycogen determined after KOH extraction and statistically not different from this value ($P = .275$).

2.5. Optimization of the homogenization time and number of extractions for the separation of acid-soluble and acid-insoluble glycogens

The effect of varying the homogenization time on acid-soluble and acid-insoluble glycogen fractions was examined after grinding a muscle sample to a fine powder as described above. After the addition of 10 vol of 10% (wt/vol) TCA, muscle was homogenized for 0.5, 1, 2, 4, or 8 minutes while keeping the solution cold by regular immersion in liquid nitrogen. After homogenization, acid-soluble and acid-insoluble materials were separated by centrifugation at 5000*g* for 10 minutes; and the pellet was treated as described above before measuring the levels of acid-insoluble and acid-soluble glycogens. To examine the effect of repeated extraction on the separation of acid-soluble and acid-insoluble glycogens, a muscle extract was prepared as described above, with the difference that the extraction step with 5 vol of 10% (wt/vol) TCA was repeated up to 6 times, after which the amount of glycogen in each individual supernatant and in the pellet was determined as described above. Finally, in a separate experiment, acid-soluble and acid-insoluble glycogen fractions were prepared using the homogenization-free extraction protocol of Adamo and Graham [13]. Briefly, a portion of the mixed quadriceps muscle was placed in a mortar kept in liquid nitrogen, then broken into small pieces, freeze-dried, and broken into small 2- to 3-mg pieces. Each piece was then placed in a tube containing 10% (wt/vol) TCA, pressed against the wall of the tube with a glass rod, and left to stand for 20 minutes before being centrifuged at 5000*g* for 10 minutes to separate the acid-soluble and acid-insoluble glycogen fractions.

2.6. Gel filtration chromatography of acid-soluble and acid-insoluble glycogens

To determine the molecular weight of acid-soluble and acid-insoluble glycogens, gel filtration chromatography was adopted because this approach has been used successfully in the past to analyze the molecular weight distribution of isolated glycogen, with a sensitivity sufficient to resolve differences in the molecular weight of the 2 glycogen forms [35]. The analysis of the molecular weight profiles of acid-soluble and acid-insoluble glycogens was performed using gel filtration chromatography with Sepharose CL-6B or Sephacryl S-400 HR (Amersham, Little Chalfont, United Kingdom) as chromatography medium. According to the manufacturers, Sepharose CL-6B resolves dextrans with molecular weights ranging from 1×10^4 to 1×10^6 d and for this reason was expected to resolve proglycogen (400 kd) and macroglycogen (10 000 kd) based on the reported differences in their molecular weights. For this purpose, a column containing Sepharose CL-6B medium (100 cm × 1.5 cm) was equilibrated with 1% (wt/vol) NaCl containing 0.02% sodium azide at a constant flow rate of 0.5 mL/min. Sephacryl S-400 HR was also used to examine the molecular weight profiles of the acid-soluble and acid-insoluble glycogens. Sephacryl S-400 HR is reportedly suited for the separation of dextrans of molecular weights 1×10^4 to 2×10^6 d. The Sephacryl S-400 HR column (100 cm × 1.5 cm) was equilibrated with buffer (50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 2 mmol/L 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], pH 7.5) at a constant flow rate of 0.5 mL/min.

Approximately 5 to 6 g of quadriceps muscle from fed male Wistar rats (~300 g) was used for the preparation of acid-soluble and acid-insoluble glycogens as described above. As a means of concentrating the acid-soluble glycogen before application to the column, the pooled supernatants were first dialyzed to remove TCA. Dialysis was performed using 10-kd molecular weight cutoff cellulose dialysis tubing (this size exclusion <10 kd is 40-fold smaller than the reported size of proglycogen) against 3 changes (1 L) of double-distilled water per 30 mL of sample over 24 hours. After

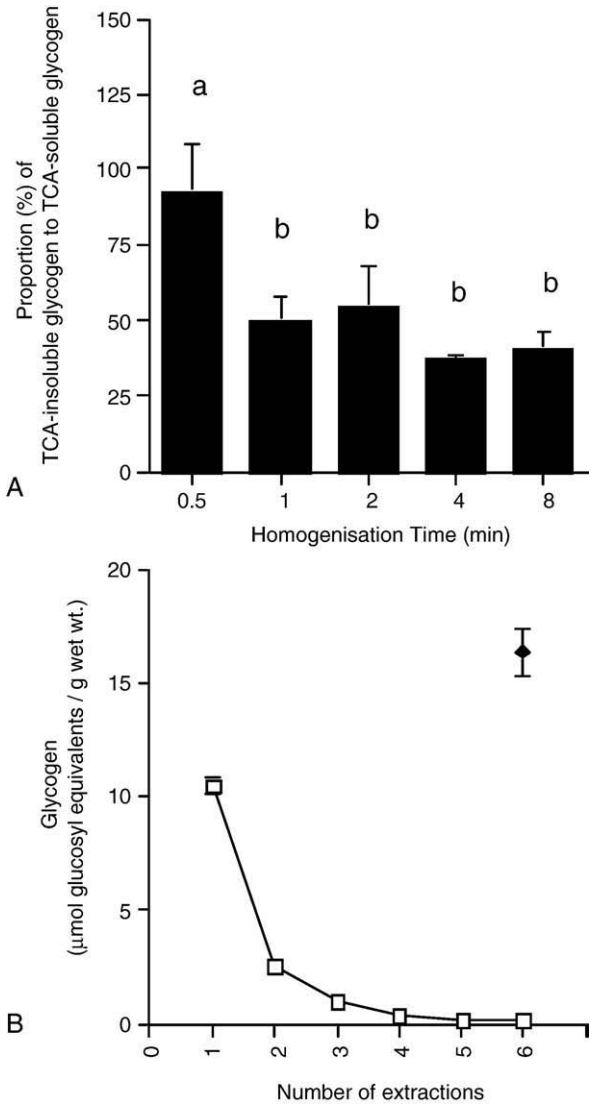


Fig. 1. Establishment of reproducible extraction conditions for the isolation of acid-soluble and acid-insoluble glycogens. A, The effect of homogenization time on the proportion of acid-insoluble glycogen to acid-soluble glycogen. Shown is the ratio of glycogen in the acid-insoluble pool to that in the acid-soluble pool expressed as a percentage at varying times of homogenization. Values shown are means ± SEM of 3 separate measurements. Identical superscripts on different bars indicate the absence of significant difference ($P < .05$). B, The effect of repeated extractions on the separation of acid-soluble and acid-insoluble glycogens. After homogenization as described in the text, the levels of glycogen present in the supernatant after the initial centrifugation and each subsequent extraction of the pellet in acid are indicated by open boxes. The amount of glycogen left in the pellet after 6 extractions is indicated by the closed diamond. Values shown are means ± SEM.

dialysis, the samples were freeze-dried and then dissolved in 1 mL of chromatography buffer. The acid-insoluble glycogen obtained after alkali digestion and ethanol precipitation was also dialyzed and dissolved in 1 mL of chromatography buffer. The 2 samples were applied separately to the columns at a flow rate of 0.5 mL/min, and fractions of approximately 5 mL were collected and assayed for glycogen.

To determine if repeated homogenization together with alkali digestion alters the molecular weight of glycogen, approximately 10 g of quadriceps muscle from fed male Wistar rats was homogenized at low speeds for 30 seconds in the presence of 10 vol of ice-cold 10% (wt/vol) TCA before being centrifuged at 5000*g* for 10 minutes. Half of the supernatant was then homogenized at high speeds (3 × 1 minute) as described earlier for the extraction of acid-soluble glycogen, whereas the other half remained untreated. Afterward, these supernatants were dialyzed for 24 hours against double distilled water before being freeze-dried to concentrate acid-soluble glycogen. The glycogen thus obtained from the supernatant previously homogenized was KOH digested, ethanol precipitated, and resuspended in 1 mL of column buffer (50 mmol/L HEPES, 2 mmol/L CHAPS, pH 7.5) as described above, whereas the glycogen from the other supernatant was resuspended in 1 mL of column buffer. Both glycogen fractions were then applied to a column of Sephacryl S-400 HR under conditions identical to those described earlier.

2.7. Statistics

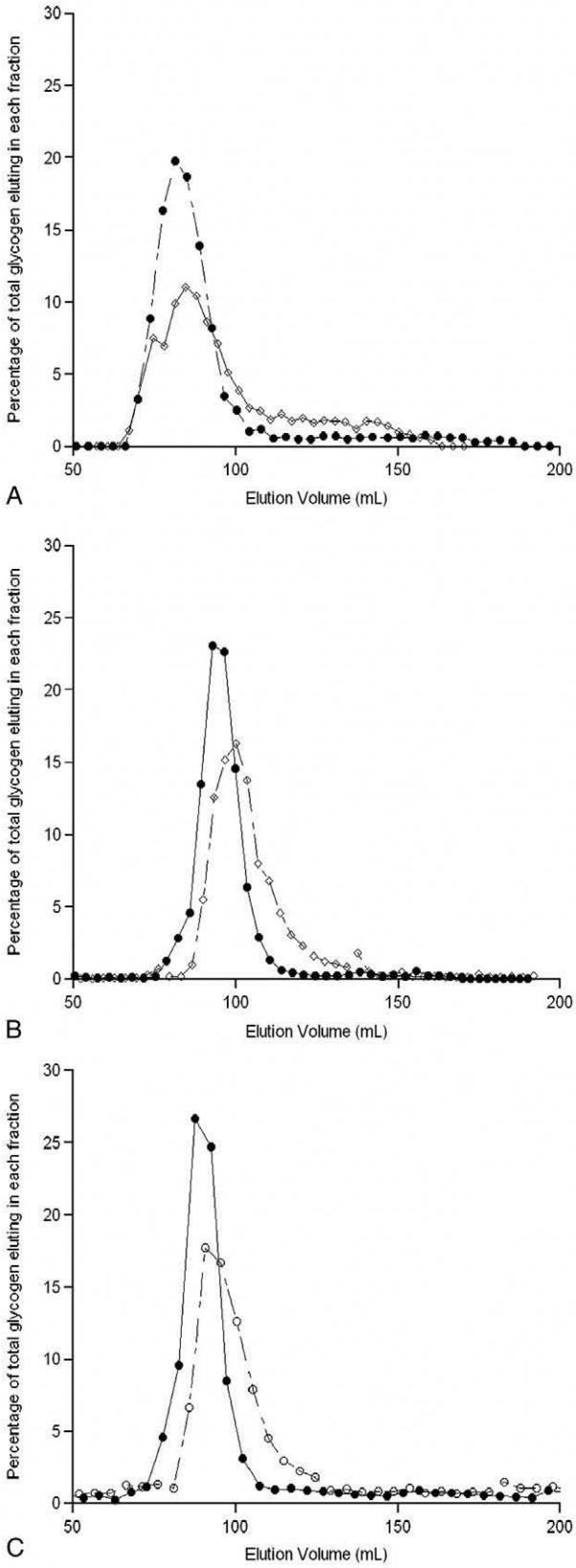
The effects of fasting and subsequent refeeding on the levels of acid-soluble and acid-insoluble glycogens as well as the effect of the time of homogenization on the proportions of acid-insoluble and acid-soluble glycogens were analyzed using a 1-factor analysis of variance followed by Dunnet post hoc tests using SPSS version 11.0 (SPSS, Chicago, IL).

3. Results

3.1. Optimization of the extraction of acid-soluble and acid-insoluble glycogens

To optimize the homogenization conditions for the isolation of acid-soluble and acid-insoluble glycogens, the effect of varying the duration of homogenization time on the proportion of glycogen in the 2 pools was examined. The percentage of acid-soluble glycogen increased significantly as the homogenization time was increased from 0.5 to 1 minute (Fig. 1A, $P < .05$). However, homogenization for in excess of 1 minute did not result in the extraction of a higher proportion of acid-soluble glycogen (Fig. 1A). For this reason, a homogenization period of 1 minute was adopted for the extraction of acid-soluble glycogen in subsequent experiments.

Because a high proportion of the acid-insoluble glycogen remained in the pellet even after 1 minute of homogenization, we tested whether an increased release of acid-soluble glycogen into solution could be achieved by repeated acid extractions of the pellet. Additional extractions of the acid-insoluble glycogen pellet resulted in the further extraction of acid-soluble glycogen (Fig. 1B). However, after 5 such treatments, there was almost no more acid-soluble glycogen extracted, at which point close to 54% of glycogen content was still precipitated by acid (Fig. 1B). Because approximately 97% of the acid-soluble glycogen was extracted after 3 acid



extraction steps, this number of steps was adopted for the separation of acid-soluble and acid-insoluble glycogens in subsequent experiments. Under these conditions, close to half of the total muscle glycogen was extracted as acid-soluble glycogen. It is noteworthy that when acid-soluble glycogen was prepared as described in Adamo and Graham [13], only 15.8% ± 0.8% of glycogen was acid soluble.

3.2. Molecular weight profiles of acid-soluble and acid-insoluble glycogens

The elution profiles of acid-soluble and acid-insoluble glycogens using Sepharose CL-6B as the gel filtration medium were similar in that the elution peak for both species occurred at approximately the same volume, but differed in that the acid-insoluble glycogen showed a small rightward shift and trailing edge of material of lower molecular weight (Fig. 2A). Similar findings were also obtained using Sephacryl S-400 HR (Fig. 2B). To determine whether the intensive homogenization and KOH treatment of the acid-insoluble glycogen could account for the small difference in the elution profile between acid-soluble and acid-insoluble glycogens, the elution profile of KOH-treated and untreated acid-soluble glycogens was compared. There was a small shift in the molecular weight distribution of KOH-treated acid-soluble glycogen in comparison with untreated acid-soluble glycogen (Fig. 2C). This small difference in the elution profile of KOH-treated glycogen relative to untreated glycogen was comparable with the small difference in the elution profile between acid-insoluble and acid-soluble glycogens (Fig. 2B, C).

3.3. Effects of fasting and refeeding on the levels of acid-soluble and acid-insoluble glycogens

In muscle from fed rats, the proportion of total glycogen that is present as acid-soluble glycogen ranged from 52% to 64% of total glycogen. In response to a 48-hour fast, a significant decrease of 23% to 47% in the level of acid-soluble glycogen was observed in the white, red, and mixed portions of the quadriceps muscle as well as in the diaphragm muscle ($P < .05$). There was no statistically significant change in acid-soluble glycogen levels in response to fasting in soleus muscle. The levels of acid-insoluble glycogen were significantly ($P < .05$) reduced by 29% and 31% after fasting in the red quadriceps and diaphragm muscles, respectively. Overall, the proportion of acid-soluble glycogen remained the highest in the soleus in response to fasting in comparison with the other muscles.

Fig. 2. Elution of acid-soluble and acid-insoluble glycogens by gel filtration chromatography. Shown are the elution profiles of acid-soluble (closed circles) and acid-insoluble (open diamonds) glycogen from rat muscle on Sepharose CL-6B (A) and Sephacryl S-400 HR (B) and the elution profiles of KOH-treated acid-soluble glycogen (open circles) and untreated acid-soluble glycogen (closed circles) from rat muscle on Sephacryl S-400 HR (C) as described in the text. Values shown represent the percentage of the total glycogen applied to the column eluting in each fraction.

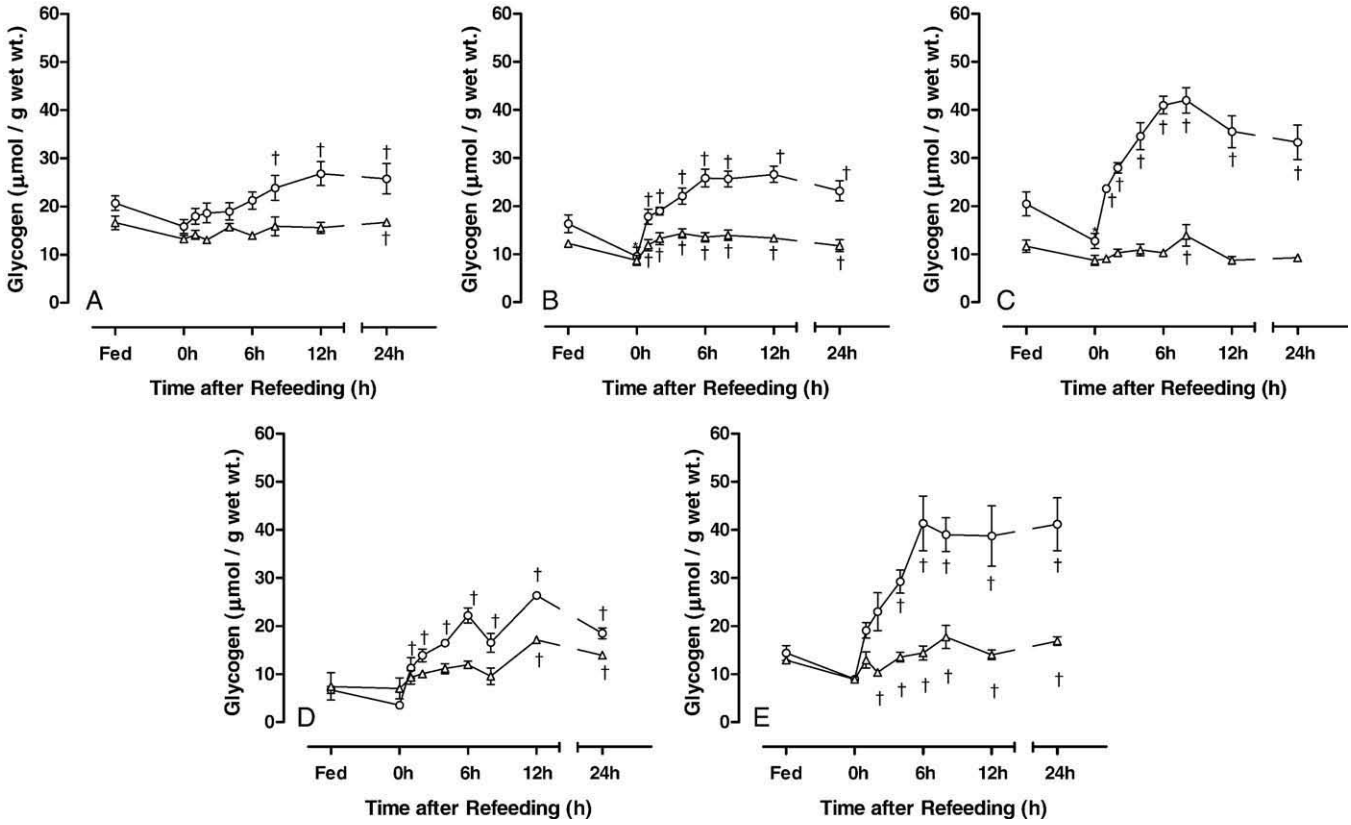


Fig. 3. Changes in acid-soluble and acid-insoluble glycogen levels in different muscles in response to 48 hours of fasting and subsequent chow refeeding. Shown are levels (expressed as micromoles of glucose equivalents per gram wet weight) of acid-soluble (open circles) and acid-insoluble (open triangles) glycogen in white quadriceps (A), red quadriceps (B), mixed quadriceps (C), soleus (D), and diaphragm (E) muscles. The values shown are means ± SEM for 9 rats. Statistically significant differences between values at 48-hour fasting (0-hour refeeding) and those at different times of refeeding are indicated by † ($P < .05$).

In all muscles examined, refeeding was associated with a rapid increase in acid-soluble glycogen to levels that peaked between 8 and 24 hours after the commencement of refeeding (Fig. 3). The acid-insoluble pool of glycogen remained stable or increased marginally in response to refeeding after fasting (Fig. 3). This change in the distribution of glycogen between pools during refeeding was confirmed by the observation that in all muscles examined, except the white quadriceps, the proportion of total glycogen that is found in the acid-soluble pool was significantly increased ($P < .001$, Table 1).

4. Discussion

Since the late 1990s, most of the research on the physiological significance and interrelationship between the acid-insoluble and acid-soluble pools of muscle glycogen have adopted the homogenization-free glycogen extraction protocol of Adamo and Graham [13]; but no attempt has been made to determine whether this protocol achieves its goal of separating these 2 forms of glycogen and whether the acid-insoluble fraction obtained is composed predominantly of low–molecular weight glycogen. Here we show that when

Table 1
Effect of fasting and subsequent refeeding after fasting on the proportion (percentage) of total glycogen that is present as acid-soluble glycogen

	Fed	Fasting	Hours of refeeding						
			1	2	4	6	8	12	24
White quadriceps	55.4 ± 3.2	54.0 ± 1.9	55.7 ± 2.5	57.9 ± 3.0	54.5 ± 2.0	59.6 ± 2.7	59.8 ± 3.9	61.6 ± 1.9	59.7 ± 3.7
Red quadriceps	55.2 ± 3.6	51.1 ± 2.8	59.9 ± 2.0*	59.4 ± 2.7*	60.6 ± 1.4*	65.6 ± 1.0***	64.8 ± 2.4***	66.4 ± 1.3***	66.1 ± 2.4***
Mixed quadriceps	64.0 ± 4.3	60.5 ± 2.6	72.4 ± 1.6**	73.0 ± 2.1**	74.8 ± 3.4***	79.7 ± 1.2***	74.5 ± 2.7**	79.4 ± 2.3***	78.7 ± 1.2***
Soleus	54.3 ± 12.5	35.2 ± 5.6	54.2 ±2.5**	57.5 ± 1.9***	60.2 ± 2.4***	65.0 ± 2.7***	64.3 ± 3.3***	62.9 ± 6.2***	58.3 ± 3.0**
Diaphragm	52.2 ± 1.8	50.2 ± 2.7	59.9 ± 3.5**	67.8 ± 4.3***	68.0 ± 1.6***	73.4 ± 2.0***	68.5 ± 2.9***	72.0 ± 3.9***	69.6 ± 3.7**

Values shown are the percentage of total glycogen that is present as acid-soluble glycogen in each muscle. Statistically significant differences between values at 48-hour fasting and those at different times of refeeding are indicated by * ($P < .05$), ** ($P < .01$), and *** ($P < .001$).

conditions of acid extraction are optimized, (*a*) the proportion of acid-soluble glycogen in rat muscles is much higher than when glycogen is extracted using a homogenization-free extraction protocol; (*b*) the acid-insoluble fraction of glycogen obtained under optimal extraction conditions does not correspond to low–molecular weight species of glycogen; and (*c*) irrespective of muscle fiber type, the acid-soluble glycogen accounts for most of the changes in total glycogen levels in response to refeeding.

The duration of tissue homogenization and number of acid extractions are 2 variables affecting the extent of glycogen extraction by acid. Our results show that for the optimal extraction of acid-soluble glycogen, 1 minute of homogenization is required using a Janke and Kunkel Ultra-Turrax T-25 homogenizer, and the acid-insoluble fraction must be reextracted at least 3 times to recover 97% of acid-soluble glycogen. Under these circumstances, the proportion of acid-soluble glycogen in muscles of fed rats ranges between 52% and 64%, a finding similar to those of the recent [14,20] and earlier studies that have included a homogenization step in their extraction protocols [2-7]. In contrast, when glycogen is extracted without a homogenization step, as described by Adamo and Graham [13], our results show that acid-insoluble glycogen is heavily contaminated with acid-soluble glycogen, with only 16% of glycogen found in the acid-soluble fraction. This result is comparable with those reported in studies using a similar homogenization-free extraction protocol in rats [16] and other species, including humans [12,13,15,17-19,21,22]. Because low levels of acid-soluble glycogen are consistently extracted using the homogenization-free protocol of Adamo and Graham [13] and considering that repeated homogenization is recommended for the optimal extraction of even small metabolites [36], this protocol is most probably too gentle to free the acid-soluble glycogen from the sarcoplasmic reticulum and the tight mesh of muscle myofibrils where most glycogen particles are found [24]. Thus, our findings bring into question the physiological significance and meaningfulness of the findings of the many studies that have extracted muscle glycogen without a homogenization step [12,13,15-19,21,22].

What still remains to be explained is our finding that up to half of muscle glycogen is acid insoluble even using our extraction protocol. This is unlikely to be explained on the grounds that the acid-insoluble glycogen might be composed predominantly of low–molecular weight glycogen. Indeed, the similarity in the elution profiles of the acid-soluble and acid-insoluble glycogen fractions strongly suggests that these 2 fractions do not correspond to glycogen differing significantly in their molecular weight. Although a close inspection of our elution profiles suggests a higher proportion of lower–molecular weight glycogen species in the acid-insoluble glycogen fraction, this is most likely the result of partial glycogen breakdown caused by the KOH treatment adopted in this study to prepare acid-insoluble glycogen for gel chromatography because this treatment results in a difference in glycogen elution profiles similar to that between acid-soluble and acid-insoluble glycogens (Fig. 2). Altogether, our findings thus provide further evidence against the existence of proglycogen in skeletal muscle, an interpretation supported not only by recent findings that the discrete 400-kd proglycogen species [8,9] might be an artifact [23], but also by the work of Skurat and colleagues [23] and that of Marchand and colleagues [24] who showed that the glycogen separated by electrophoresis or examined by transmission electron microscopy exists as a continuum of molecules covering a broad range of molecular weights, with no discrete species corresponding to proglycogen. Factors other than the molecular weight of glycogen must therefore explain the presence of acid-insoluble glycogen in skeletal muscles. Because the glycogen particles are closely bound to the proteins involved in their synthesis and degradation to form a complex referred to by some as a *glycosome* [37], the denaturation of these glycogen-associated proteins by acid treatment might result in the formation of glycogen-protein complexes whose acid insolubility depends on the protein/glycogen ratio present in the glycosome. If this latter explanation were to hold, the acid-soluble and acid-insoluble glycogen fractions could thus represent physiologically distinct pools of glycogen; but until the difference in the structure of these glycogen species is elucidated, this possibility will remain to be examined.

One indirect approach to determine whether acid-soluble and acid-insoluble glycogens represent physiologically distinct glycogen pools is to examine their patterns of response to a physiological challenge that causes a marked change in muscle glycogen levels [2-7,15-18,11,12,22], such as the fasting-to-fed transition. Because muscle fiber composition has the potential to affect our results, the patterns of change in acid-soluble and acid-insoluble glycogen species to refeeding after a fast were examined in muscles of different fiber compositions. Our results show that during the fasting-to-fed transition, changes in acid-soluble glycogen levels account for most of the increase in total glycogen, irrespective of muscle fiber type, whereas the absolute levels of acid-insoluble glycogen remain stable or increase only marginally during refeeding (Fig. 3). These findings corroborate those of the many studies performed mainly in the 1950s that have reported that in response to conditions affecting muscle glycogen levels such as insulin administration, electrical stimulation, adrenaline injection, hyperthyroidism, or death, there is a preferential or even exclusive change in the level of acid-soluble glycogen [2-5,20,38]. In contrast, studies using the homogenization-free glycogen extraction protocol of Adamo and Graham [13] have reported that it is the acid-insoluble glycogen that accounts for most of the changes in total glycogen concentration when the levels of total glycogen are low, whereas the absolute contribution of the acid-soluble glycogen is relatively more important when total glycogen levels are elevated [13,15-18,22]. This is probably explained

on the basis that when total muscle glycogen levels are low, the homogenization-free extraction protocol results in the release of a fixed but only small proportion of acid-soluble glycogen from undisrupted muscle myofilaments, whereas when total glycogen increases to levels that exceed the capacity of the mesh of poorly disrupted muscle cells to trap glycogen effectively, this results in a disproportionate increase in the release of glycogen with any rise in total glycogen levels [13,15-18,22].

In summary, our results challenge the physiological significance and meaningfulness of the findings of most of the recent studies that have examined the pattern of response of acid-soluble and acid-insoluble species of glycogen to physiological changes. This is because these studies have adopted a homogenization-free protocol of glycogen extraction where acid-insoluble glycogen is heavily contaminated by acid-soluble glycogen. We also provide for the first time evidence that the pool of acid-insoluble glycogen in skeletal muscle does not correspond to a fraction rich in low–molecular weight glycogen, a finding that not only challenges further the existence of proglycogen as a distinct species of glycogen, but also questions the meaningfulness of the proglycogen-macroglycogen paradigm. Clearly, more work is required to elucidate the structures of acid-soluble and acid-insoluble glycogens and their responses to physiological changes.

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