Improvement of the classical assay method for liver glycogen fractions: ASG is the main and metabolic active fraction

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Abstract. – OBJECTIVE: Acid digestion of animal tissues yields two fractions of glycogen, acid soluble (ASG) and insoluble (AIG). The current study was performed to improve the assay method for glycogen fractions in rat liver in different physiological states.

MATERIALS AND METHODS: All steps of the assay were manipulated and optimized to measure the content of ASG and AIG in fed and starved rat liver.

RESULTS: In postmortem liver tissue, total glycogen was decreased slowly at 4°C and rapidly at 25°C but was well stabilized at −20°C and −70°C. At room temperature, ASG underwent autolysis at the rate of 1.3% and decreased by half at 35 min, while AIG increased slightly. The yield of the recovery of ASG during four successive extractions depends on the tissue concentration, and at the ratio of 50 mg tissue per 2 mL perchloric acid (PCA) was about 93.2%, 6.3%, 0.3% and 0.05% respectively. The increase in the time and extent of homogenization of the tissue with cold PCA and using ultrasonication had not any significant effect on the extraction yield of ASG. The time of centrifugation of the tissue extract could be reduced from 15 to 7.5 minutes with no significant decrease in the recovery of ASG. On extraction with ethanol, the yield of recovery of ASG reached the maximal level of 97.5% at a final ethanol concentration of 60%. The recovery of ASG was not improved in the presence of KCl. During 24 starvation, total glycogen depleted completely and the change occurred entirely in ASG, while AIG did not change significantly.

CONCLUSIONS: The CV% was less than 5% for the optimized assays of glycogen fractions. ASG is the main and metabolically active portion of glycogen in rat liver.

Key Words: Glycogen, Proglycogen, Macroglycogen, Phenolsulfuric, Liver.

Introduction

Glycogen is immediate storage of energy in the most tissues especially liver, skeletal muscles and central nervous system. The glycogen content of the liver depends on different nutritional, hormonal-metabolic and physiological states. The content of glycogen in the liver of fasted rat is about 1-5 mg/g wet weight (or 0.1-0.5%) and increases rapidly at the rate of about 10 mg/g.h postprandial. Several methods have been described to measure glycogen in animal tissues. In all methods, the tissue is digested by acid- or alkaline-heating, or subjected to cold acid-grinding. The glycosides bonds are sensitive to hot acid, so that glycogen is hydrolyzed to glucose and assayed directly without ethanol extraction. In two other methods, glycogen is extracted from the (supernatant of) tissue homogenate with ethanol. Chemical or enzymatic methods are applied to hydrolyze the glycogen to glucose and subsequent assay of glucose. In the previous report, we optimized the microassay for glucose-based glycogen in small tube or microplate.

There are several difficulties in glycogen measurement as a result of autolysis, binding to proteins, precipitation and contamination of different fractions. Cellular glycogen undergoes rapid autolysis in postmortem tissues and also in the assay medium. Therefore, the condition of tissue preservation and sampling is important to achieve reproducible results. In addition, the pattern of glycogen fractions in postmortem is interesting as a metabolic model. Since the previous reports limited to the changes of total glycogen, it needs to follow the pattern of glycogen fractions postmortem. The early researches showed that tissue glycogen could not be extracted completely by cold acid-grinding. Roe et al.
observed that difficulty of extraction is a result of inadequate homogenization. They reported that total glycogen could be extracted completely in cold acid by using high-speed homogenizer, but they did not examine ultrasonication. Other authors\(^{13-14}\) showed that the insolubility of some glycogen particles in cold acid is attributed to binding to proteins, glycogen phosphorylase or/and glycogenin. Lomako et al\(^{14}\) indicated that acid insoluble glycogen (AIG) was composed mainly of low MW particles with high protein content, while acid soluble fraction (ASG) are large particles with low protein content.

Recently, a new era of research has been conducted to study the physiological importance of glycogen fractions\(^{15-19}\). Many of these works are on the basis of the method of Adamo and Graham\(^{18,19}\). This protocol has been done on a very low weight of tissue and without homogenization. There is a marked difference between the results of this protocol and homogenization methods achieved by the earlier\(^2,9,10\) and more recently studies\(^{21,22}\). The current study performed to improve measurement of liver glycogen fractions in fed and starved state.

**Materials and Methods**

**Liver Sampling**

Albino (Wistar) male rats, weighing 200-220 g were free access to *ad libitum* and water. To study the effect of starvation, the rats were starved 12 and 24 h before sampling. The rat was anesthetized with diethyl ether and the liver was isolated and washed rapidly three times with cold isotonic saline. The lobs incised longitudinally into several parts and put between aluminum plates pre-cooled in liquid nitrogen and preserved at −20°C or −70°C for a longer period.

**Tissue Acid Digestion and Separation of ASG and AIG**

The liver tissue was dried by putting and turning rapidly on a filter paper and weighed (50 or 100 mg) at precision of ± 0.0001 g by an analytical balance (Sartorius), transferred into a 12-mL glass tube and ground 20 sec with 1mL of cold 10% perchloric acid (PCA) using a tissue grinder (IKA Works Ins., Wilmington, NC, USA). To do tissue extraction quantitatively, the homogenizer probe was immersed and washed with 1 mL fresh PCA for 5 sec and was added to the tissue homogenate. The ground sample was centrifuged 7.5 min at 280 × g at 4°C. The supernatant containing ASG in suspension was decanted into another tube, and the pellet was re-extracted for further two steps with 1 mL fresh PCA to extract any acid soluble glycogen. To extract AIG, 200 µL of 30% KOH was added to the last pellet and heated in boiling water bath for 10 min with regular mixing.

**Tissue Alkali Digestion for Total Glycogen**

Fifty mg of tissue directly was transferred to 200 µL 30% KOH and heated in boiling water bath for 10 min with regular mixing.

**Extraction With Ethanol**

Ethanol at a final concentration of 55% was added to the supernatant of tissue ground for ASG and alkali-tissue ground for AIG and total glycogen, vortexed and centrifuged 10 min at 1500 × g at 4°C. The supernatant was decanted off, and the pellet re-suspended in 2 mL of ice cold-distilled water and 10 µL analyzed for glycogen in duplicate. When the content of glycogen is high due to fed state, the size of the sample was decreased to 50 mg or one-half (1 mL) of the supernatant was extracted with ethanol. By this means the absorbance of the final assay mixture will be kept around 0.420, i.e. the best colorimetric values.

**Assay of Glycogen**

The suspension of glycogen was mixed by vortex 1 sec just before the sampling. A short vortex step is essential to have uniform suspension of glycogen and reproducible results. 10 µL of sample was used for the measurement of glycogen fractions as described previously\(^7\). The curve of glucose standard was used to calculate glycogen concentration. The factor is multiplied by 0.927 to convert the results to glycogen that corresponds to 97% hydrolysis of glycogen\(^4\).

**Statistical Analysis**

The results are presented as the means ± SD of three inter-assays performed at least in three samples. The significant differences between samples and corresponding control were accessed by Student t-test. \(p < 0.05\) was considered statistically significant.
Results

Tissue Digestion With Hot, Cold Acid or Alkaline

Fifty mg of liver tissue was weighed and digested by different methods (Figure 1). In the hot acid method, 2 mL of 10% PCA was added to liver tissue and put in boiling water bath for 10 minutes. Then, 10 µL of tissue homogenate was analyzed directly (without ethanol extraction) by the phenol-sulfuric acid reagent. The procedures of tissue digestion with hot alkaline and cold acid were as described in the methods section. The difference between hot acid and hot alkaline methods indicates carbohydrates except glycogen. The procedure of hot acid digestion was not used in the present study. AIG is taken as the difference in the amounts of glycogen determined by the methods of hot alkaline and cold acid.

The Effect of Sample Weight on Assay Accuracy

The content of glycogen varies widely in the liver according to the nutritional status of the rats. So the optimum weight of the sample is different in fed and 24 h starved rats. Different weights of the liver tissue were used to address the effect of sample size on the accuracy of the method. The weights of 25, 50 and 100 mg of fed rat liver and 100, 200, 300 and 400 mg of starved rat liver tissue were weighed and analyzed for ASG, AIG and total glycogen. Figure 2 shows that the absorbance of equal volumes (10 µL) of the final ASG suspension with phenol-sulfuric acid reagent increases with the sample size linearly. Also, the glycogen content of the samples calculated as mg/g wet weight of liver was the same for all sample preparations. A similar pattern was seen for total glycogen (results not shown). The weight of 50 mg of liver was adopted for analysis in subsequent experiments.

The Effect of Temperature of Tissue Preservation on Glycogen Stability

The liver was isolated from the rat and washed quickly three times with cooled isotonic saline. The lobs were incised longitudinally into several parts on a filter paper and preserved at different temperatures of −20°C, 4°C and 25°C. At the time intervals of 0, 10, 20, 30, 60 and 90 minutes, 50 mg of pieces were weighed and analyzed for total glycogen. Figure 3 shows that glycogen was well stabilized at −20°C, as the glycogen content was the same during of 90 min and also after three months (result not shown). But, the content of total glycogen in the postmortem liver tissue was decreased slowly at 4°C and rapidly at 25°C. The content of glycogen fractions, ASG and AIG were also followed in postmortem liver.

Figure 1. The method of tissue digestion. Fifty mg portions of liver tissue were weighed and digested by different methods. The results are presented as the means ± SD of three inter-assays (n) performed in duplicate.

Figure 2. The effect of sample weight on the accuracy of measurement. Different weights of the liver of fed and 24 h starved rats were analyzed for ASG, AIG and total glycogen. The opened circles (*) indicate the absorbance of 10 µL of the final suspension of ASG with the phenol-sulfuric acid reagent. The filled circles (•) show the glycogen content of the samples calculated as mg/g wet weights of liver. All measurements were done on three samples in duplicate.

Figure 3. The effect of temperature of tissue preservation on glycogen stability.
Homogenization of hepatic glycogen fractions

Figure 3. The effect of temperature on glycogen stability. The pieces of liver tissue were kept at different temperatures and at the time intervals of 0, 10, 20, 30, 60, and 90 minutes, 50 mg of tissues were weighed and analyzed for total glycogen (curves A, B, C). ASG and AIG were also measured at 25°C. The results are presented as the means ± SD of three inter-assays (n) performed in triplicate. * And ** indicate significance at a confidence levels of $p < 0.01$ and 0.001 respectively.

Figure 4. The effect of homogenization extent on recovery of ASG. Fifty mg portions of the liver tissue were weighed, homogenized with ice cold PCA at different duration and extent, centrifuged, extracted with ethanol and analyzed for glycogen. The measurements were done on three samples in duplicate.

Figure 5. The effect of centrifugation time on recovery of ASG. Fifty mg pieces of the liver tissue were weighed, homogenized with cold PCA and centrifuged at 280 × g for different times (Figure 5). The supernatants were extracted with ethanol and analyzed for ASG. The appearance of supernatant and amount of ASG had no any significant change when the duration of centrifugation was reduced from 15 to 5 minutes.

The Duration and Extent of Homogenization

The extent of homogenization was changed to optimize the homogenization condition for extraction of ASG (Figure 4). Fifty mg portions of the liver tissue were weighed, homogenized at 15,000 rpm with different duration, centrifuged, extracted with ethanol and analyzed for glycogen. The amount of ASG and also the ratio of ASG/AIG had no any significant change when the duration of grinding was changed from 15 up to 180 sec. We also examined 30 sec grinding followed by 30 sec ultrasonication, but it failed to liberate all glycogen. The extent of homogenization was also increased into 30 sec at 20,000 rpm without any progress (results not shown). Therefore, 20 sec at 15,000 rpm was adopted for the extraction of ASG in subsequent experiments.

The Extent of Centrifugation

To shorten the time of centrifugation and contaminations of the fractions, 50 mg portions of the liver tissue were weighed, homogenized with ice cold PCA and centrifuged at 280Xg for different times. The supernatants were extracted with ethanol and analyzed for glycogen. The results are presented as the means ± SD of three samples performed in triplicate. * indicates significance at a confidence levels of $p < 0.001$. 

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But at the shorter time, the supernatant was slightly turbid and less glycogen was recovered. So, ten minutes centrifugation at 280×g was adopted for the extraction of ASG in subsequent experiments.

**The Efficiency of Tissue Digestion with Perchloric Acid-Grinding**

To address the efficiency of ASG extraction, the liver tissue was ground four times with cold PCA (Figure 6). Different weights of the sample were homogenized 20 sec at 15,000 rpm, centrifuged 10 min at 280 × g and the supernatant was collected and the pellet was reground in 1 mL of PCA. The extraction was repeated four times. The supernatants were extracted separately with ethanol and analyzed for glycogen. The yield of the recovery of ASG during four successive extractions depends on the sample size, and at the ratio of 50 mg tissue per 2 mL PCA was about 93.2%, 6.3%, 0.3% and 0.05% respectively. Figure 6 shows that higher amount of ASG will be transferred to the subsequent extractions when the sample size increases. It is concluded that at least two steps of acid extractions are required to measure ASG quantitatively.

**The Effect of Ethanol and Electrolyte Concentration on Glycogen Recovery**

To determine the effect of ethanol concentration on glycogen recovery during extraction, different volumes of ethanol used to precipitate glycogen from the same solution. The 500 mg of liver tissue was homogenized in a total volume of 5 mL of cold PCA and centrifuged 10 min at 280 × g. The supernatant was decanted and divided into nine 0.5 mL portions, and different volumes of ethanol were added. The samples were mixed and centrifuged 10 min at 1700 × g, and the supernatant was decanted out. The pellet re-suspended in 2 mL of distilled water and 10 µL analyzed for glycogen. Glycogen was precipitated considerably at ethanol concentration more than 40% and reached a plateau at 50% (Figure 7). The optimum ethanol concentration was deduced to be 55-60% in final solution. Direct analysis of glycogen in the pellet and supernatant of ethanol extract indicates that the efficiency of extraction is about 97% at 60% final concentration of ethanol. There was not any improvement in recovery of glycogen in the presence of KCl (results not shown).

**The Inter- and Intra-Assay Precision of Glycogen Measurement**

Three 50 mg pieces of the same lob of the liver of a single rat were weighed separately and
analyzed for ASG, AIG and total glycogen. The final assay for glycogen on any sample was also done in triplicate. The results indicate that % CV of both the inter- and intra-assays of total glycogen and ASG were less than 5%, but for AIG were higher. The low level of AIG in liver tissue is responsible for high % CV.

### The Changes of Glycogen Fractions During 24 h Starvation

The levels of ASG, AIG and total glycogen were measured in fed and 12 and 24 h starved rat liver (Table I). There were not any significant changes during 12 h fasting (result not shown). The data shows that during 24 h fasting, total glycogen depleted completely (70.5 ± 7.7 vs. 5.9 ± 3.3, \( p < 0.001 \)) and the change occurred entirely in ASG (64.5 ± 6.4 vs. 2.5 ± 1.6, \( p < 0.001 \)), while AIG did not change significantly (3.6 ± 1.0 vs. 3.7 ± 0.3).

### Discussion

#### The Method of Tissue Digestion; Hot, Cold Acid or Alkaline

The glycoside bonds are labile to hot acid and undergo hydrolysis. Tissue digestion with hot acid produces mono- and oligosaccharides not only from glycogen but also by other glycoconjugates. To this was added phosphorylated monosaccharides that still exist in the cell as metabolites\(^5,6\). Since mono- and oligosaccharides could not be extracted with ethanol, the assay of glycogen was done directly on tissue extract as glucose units. On the other hand, glycosidic linkages of glycogen are more stable with alkaline\(^4,5\), and the glycogen or its large clusters could be extracted with ethanol. Thus, the difference between hot acid and hot alkaline methods indicates other carbohydrates\(^11\). The hot acid digestion was not applied in the present study because phenol-sulfuric reagent is not specific for glucose\(^7\). Nevertheless, if glucose has been measured by the enzymatic method, the metabolites of glucose would interfere\(^18,19\). Total glycogen is extractable with hot alkaline, but only ASG could be recovered with cold acid. Therefore, the difference in the amounts of glycogen determined by the methods of hot alkaline and cold acid is taken as AIG\(^11\).

#### ASG but not AIG Undergoes Enzymatic Autolysis in Postmortem

On death, metabolism continues anaerobic postmortem, so glycogen mobilizes\(^8\). Glycogen is labile and undergoes hydrolysis in tissue extract and also in the assay medium. Johnson and Fusaro\(^9\) showed that in postmortem liver tissue of rat, the levels of glucose and oligoglycosides increased gradually with time, but glycogen decreased. In order to measure the level of glycogen accurately, postmortem autolysis must be stopped as rapidly as possible. As the slope of the curve-3C shows the content of glycogen decreased at the rate of 1.3% per minute at the ambient temperature, and lost by half during 35 min. The rate of glycogen autolysis measured here is slightly less than that reported elsewhere\(^8\). The liver glycogen is well stabilized when the tissue preserved at −20°C not only for 90 minutes but also for three months (results not shown).

ASG accounts for more than 90% of total glycogen on liver tissue and is the most responsive portion to changes in total glycogen. The current data shows for the first time that, ASG was diminished while AIG was changed slightly in postmortem liver tissue at room temperature. This finding implies a possible role for AIG in glycogen degradation as well as in biosynthesis. It is assumed that the outer clusters of ASG are more metabolically active and removed during autolysis. This causes AIG to remain statistically unchanged or slightly increased. The signal controls of the degradation of ASG and shifting autolysis from AIG to another ASG is an interesting aspect to investigate.

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**Table I.** The effect of 24 h starvation on the glycogen fractions.

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<tr>
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<th>Fed</th>
<th>24 h starved rat</th>
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<tbody>
<tr>
<td>ASG (sum of three extractions)</td>
<td>64.5 ± 6.4</td>
<td>2.5 ± 1.6*</td>
</tr>
<tr>
<td>AIG</td>
<td>3.6 ± 1.0</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Total glycogen (measured)</td>
<td>70.5 ± 7.7</td>
<td>5.9 ± 3.3*</td>
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The 50 mg portions of liver tissue of fed and 24 h starved rats (n = 3) was analyzed for ASG, AIG and total glycogen. All measurements were done on three samples in triplicate. *Indicates significance at a confidence levels of \( p < 0.001 \).
ing issue remained to be studied. Fournier et al.
reported a similar pattern of changing in AIG and
ASG during excessive exercise and re-feeding in
human muscle if a tissue homogenization proto-
col was applied.

**Extraction with Cold Acid: Acid Soluble and Insoluble Glycogen**

The early studies showed that glycogen of
animal tissues could not be extracted completely
with cold water or trichloroacetic acid even with
several extractions, and some portions remain in
denatured protein fraction. All glycogen was
achieved if only the tissue is digested with hot al-
aline. Two possibilities were proposed for the
difficulty of glycogen extraction with cold acid,
inadequate homogenization or binding to protein.
Roe et al. claimed that total glycogen could be ex-
tracted completely with cold acid by making use of
high-speed homogenizer (we used the same de-
vice) and homogenizing beads. The results of the
current work indicate that increasing the time and
extent of homogenization followed by sonication
failed to extract total glycogen (Figure 4). Former-
ly, it was supposed that binding of glycogen to
phosphorylase causes the extraction to be diffi-
cult, but later it was attributed to glycogenin,
the core protein primer of glycogen synthesis.
Whelan et al reported that AIG, referred as to desmo-
or pro-glycogen was composed mainly of low MW
particles (~400 kD). The high protein-to-
carbohydrate ratio of AIG is responsible for its
poor solubility in acid. The ASG, named as lyo-
or macro-glycogen are large glycogen particles
(10000 kD) with low protein content. The Whelan
research group also provided evidence that AIG
is an intermediate in ASG synthesis. This model
has not been supported by some researches.
Skurat et al studied glycogen biogenesis in rat fi-
broblasts expressing glycogenine. Their results
showed a continuum of glycogen particle sizes by
using two-dimensional gel electrophoresis. Gra-
ham et al. assessed the size and number of glyco-
gen granules with transmission electron mi-
croscopy in human muscles during recovery of ex-
cise. They observed that distribution of particle
diameter was continua throughout the recovery pe-
riod. Fournier et al. failed to separate ASG and
AIG on the basis of molecular weight in human
muscles by using gel filtration chromatography.

Although ASG fraction is acid soluble, it does
not mean that it is a true solution but a suspen-
sion, so its particles precipitate gently. The need
for several extraction steps to recover ASG com-
pletely may attribute to inadequate homogeniza-
tion, low volume of solvent and precipitation
during centrifugation. The present findings show
that the increase of the extent of homogenization
by using both homogenizer and ultrasonicator
could not extract all glycogen. But the time of
centrifugation could be reduced from 15 to 5
minutes with no any significant effect on the re-
covery of ASG. Five up to 10 minutes centrifug-
ation of the initial tissue extract is essential to
precipitate denatured proteins and nucleic acids
but causes to precipitate some ASG again. There-
fore, it seems that ASG is dissolved in acid and
extracted by the first grind completely, but pre-
cipitates slightly during subsequent centrifuga-
tion. The sample size or the tissue concentration
i.e. mg of tissue per mL of solvent affects the rel-
ative recovery of ASG in successive extractions
significantly. Figure 6 shows that the yield of the
recovery of ASG in successive extractions corre-
lates inversely with the tissue concentration.
The yield of the recovery of ASG during four suc-
cessive extractions at the ratio of 50 mg tissue per 2
mL PCA was about 93.2%, 63.3%, 0.3% and
0.05% respectively. The effect of decreasing the
sample weight on the recovery of ASG in the
first grind is counterbalanced by the increasing
the error in sample weighing and quantitative ho-
logenization. The present data show that the
glycogen values deduced from the first extraction
are reproducible with CV% less than 5%. Hence,
the first extraction is adequate in compara-
tive studies. But, if it is desired to determine and
compare ASG and AIG the second acid extrac-
tion is necessary.

**ASG is the Main and Metabolically Active Portion of Glycogen**

Most of the recent researches on glycogen
fractions have been done on the basis of the ho-
rogenization free protocols. In these reports, AIG
is the higher fraction and more responsive
to the changes of glycogen content in different
metabolic conditions. The homogenization free
procedure encountered three main criticisms.
Firstly, the very low weight of tissue sample, 3
mg taken by biopsy is too low, so that the rela-
tive error of the weighting by analytical balance
will be high. The authors incorrectly calculated
the coefficient of mean error instead of variance
(CV%) and reported it as less than 5%. It is read-
ily evident from their results that the CV% of
their method is more than 20%. Secondly, the
The Efficiency of Glycogen Extraction with Ethanol

The solubility of glycogen has been studied in aqueous alcohol in the presence of different electrolytes. The concentration of ethanol used to precipitate glycogen has varied considerably in different studies. Pfluger and Cori in separate reports offered evidence that 2 volumes of alcohol are fully adequate for quantitative precipitation of glycogen. The current results in accordance with the findings of Good et al. and Kerly indicates that 55-60% of ethanol in the final liquor is not less effective for complete precipitation of glycogen than any of the higher concentration. Figure 7 indicates that it is useless to employ a higher concentration of ethanol than 60%, since the loss of glycogen due to increased final volume counterbalances the gain from decreased solubility. The recovery of glycogen was over 97% that is acceptable in comparative studies (results not shown).

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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