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Glycogen Determination in Post-Mortem Beef Muscles

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ABSTRACT

Different opinions are held regarding the stoichiometric conversion of glycogen into the intermediate metabolites of glycolysis and into lactate in post-mortem muscle. Results suggest that this could be due to difficulties in assessing glycogen content. In the present study it was found that glycogen was particularly difficult to analyse in beef of a high ultimate pH, but no apparent explanation for this can be given. Four different methods involving enzymatic hydrolysis and acid hydrolysis were compared for measurement of glycogen. It was found that enzymatic hydrolysis was preferable and that the glycogen method should include boiling, particularly if the samples had been frozen for several months. Prolonged freezer storage decreased the glycogen values obtained by about 15–25%. Stoichiometry in the conversion of glycogen into intermediates of the Embden–Meyerhof pathway and lactate was found if glycerol-3-phosphate was taken into consideration and the results were adjusted for the effect of freezer storage.

INTRODUCTION

In post-mortem muscle part of the glycogen is degraded, lactate is formed and muscle pH is subsequently lowered. There is a general belief that glycogen is stoichiometrically converted into the intermediate metabolites of glycolysis and lactate in the post-mortem beef muscle, but large

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variations have been found between carcasses (Bodwell *et al.*, 1965; Bendall, 1973; Dalrymple & Hamm, 1975). There are also reports of a non-stoichiometric relationship (Follet & Ratcliff, 1969; Hamm *et al.*, 1973; Laser Reuterswärd *et al.*, 1981). Losses of glycogen in slow glycolyzing musculature and gains of lactate in fast glycolyzing musculature have been reported (Kastenschmidt *et al.*, 1968; Hamm & van Hoof, 1970). Contrary to these reports Beecher *et al.* (1965) noted that a fast breakdown of glycogen early *post mortem* was not reflected in a corresponding increase in lactate concentrations, presumably due to a build-up of glycolytic intermediates or the distribution of intermediates into pathways other than normal post-mortem glycolysis.

The conflicting results reported could be due to difficulties involved in assessing glycogen. Methods for glycogen analyses were, for many years, based on the isolation of glycogen from interfering substances by boiling the tissue in KOH and precipitating glycogen with ethanol, after which the glycogen was measured by the anthrone reaction (Roe & Dailey, 1966). Alternatively, the isolated glycogen was hydrolyzed and the resulting glucose measured by a variety of methods (Passonneau *et al.*, 1967; Lo *et al.*, 1970). Some of the methods used have been criticized for incomplete glycogen precipitation or lack of specificity (Passonneau *et al.*, 1967; Bartley & Dean, 1968).

The use of specific enzymatic hydrolysis has been shown to be advantageous as an accurate and quantitative measurement of muscle glycogen. Several methods have been proposed using diazyme (Bartley & Dean, 1968), phosphorylase in combination with debranching enzyme (Passonneau *et al.*, 1967) or amyloglucosidase (Johnson *et al.*, 1963; Krebs *et al.*, 1963; Keppler & Decker, 1974). Preparation of samples includes either homogenization in 0·6M $HClO_4$ (Dalrymple & Hamm, 1973), homogenization and boiling in 0·03M HCl (Passonneau *et al.*, 1967) or heating in 1·0M KOH (Harris *et al.*, 1974). Determinations have also been performed after extraction of the glycogen into trichloroacetic acid or perchloric acid (Hultman, 1967; Laser Reuterswärd *et al.*, 1981), or on a precipitate after perchloric acid extraction (Essén *et al.*, 1977; Hermansen & Vaage, 1977).

Dalrymple & Hamm (1973) compared KOH boiling and ethanol precipitation with perchloric acid homogenization for the determination of glycogen and found higher values of glycogen with the latter method. Passonneau & Lauderdale (1974) compared two enzymatic procedures with acid hydrolysis and found that they yielded equivalent results when

examining muscle tissue. Keppler & Decker (1974) pointed out that acid hydrolysis generally yielded values about 5% lower than enzymatic hydrolysis. Jansson (1981) found no differences between acid and enzymatic hydrolyses, but when comparing acid hydrolysis of the untreated sample with acid hydrolysis of the residue after a brief perchloric acid extraction of the sample the latter method showed 15% lower values.

When analyzing glycogen in the perchloric acid extract of muscles from carcasses with a high ultimate pH we found (Laser Reuterswärd *et al.*, 1981) that the glycogen lost, between 1 and 24 h *post mortem*, only accounted for 35% of the lactate formed. This conclusion is critically dependent on the reliability of the method used for quantifying glycogen. In the light of the conflicting reports mentioned above and the many different methods used, we found it necessary to check our method and to re-evaluate our previous findings.

The aims of the present investigation were:

(1) To check the extractability of glycogen.
(2) To evaluate the importance of the conditions of homogenization.
(3) To compare different methods for estimating glycogen.
(4) To re-assess earlier findings of the quantitative conversion of glycogen into lactate in post-mortem muscle.

MATERIALS AND METHODS

Preparation of samples and pH measurements

Young bulls were stunned, exsanguinated and electrically stimulated within 5 min of stunning using the Swedish low-voltage system (MITAB, Simrishamn) as described previously (Fabiansson & Buchter, 1984). *M. longissimus dorsi* was removed from seven carcasses immediately after electrical stimulation, placed in polyethylene bags and stored for 3 h at 22 °C and a further 21 h at 10 °C. All other carcasses were dressed using conventional methods and chilled for 24 h at an air temperature of 3 °C according to normal practice at the abattoir. Samples were removed from the intact or hot-boned *M. longissimus dorsi* adjacent to the 11th rib using a stainless steel cylinder (8 mm diameter) at 1 and 24 h after slaughter. Visible fat and connective tissue were trimmed off, and the samples were

immediately frozen in liquid nitrogen. Samples were stored at −85 °C and usually analyzed within 2 months.

Measurements of pH were carried out 24 h *post mortem*. After homogenization of the frozen samples in iodoacetate–KCl, as described by Bendall (1978), pH was measured using an Ingold type 401 electrode (Ingold, Switzerland) and an Orion 601A pH meter (Orion Research Inc., Massachusetts). The carcasses were classified as normal, intermediate or DFD (dark, firm, dry) using the classification system of Fabiansson *et al.* (1984).

Determination of glycogen

Initial method

The method of Dalrymple & Hamm (1973) was used, with slight modifications. The frozen samples were pulverized in liquid nitrogen using a mortar and pestle, diluted with about 5 parts by weight of frozen pulverized 0·6M perchloric acid and homogenized in an Ultra Turrax (Janke & Kunkel, GFR) for 100 s. Glycogen was hydrolyzed enzymatically using amyloglucosidase according to Dalrymple & Hamm (1973) and the resulting glucose was determined using hexokinase and glucose-6-phosphate dehydrogenase as described by Bergmeyer *et al.* (1974). After subtraction of the originally free glucose, the glycogen content was expressed in μmol glycosyl units per g of wet weight of the tissue.

Importance of homogenization conditions

Samples from each of three normal carcasses were analyzed using different homogenization conditions. The samples were homogenized using either an Ultra Turrax for 15, 30, 90 or 180 s or using an Omni Mixer (Ivan Sorval Inc., Connecticut) with and without a micro-attachment for 30, 90 or 180 s. The samples were chilled in ice-water during homogenization. The perchloric acid homogenates were analyzed as described above but the homogenate was transferred volumetrically using a micro-pipette where the plastic tip was cut in order to allow the passage of larger particles. The weight of the homogenate was recorded and used for calculation of the glycogen content.

Comparison of different methods of estimating glycogen

One sample from a normal carcass was powdered in liquid nitrogen and used for analyses of glycogen. Five different methods were tested:

(1) homogenization in 0·03M HCl for 30 s in an Ultra Turrax and boiling for 10 min according to McVeigh (1980); (2) heating at 60 °C for 10 min in 1·0M KOH according to Harris *et al.* (1974); (3) homogenization in 0·6M $HClO_4$ for 30 s in an Ultra Turrax; (4) boiling of the perchloric acid homogenates at 100 °C for 10 min; and (5) dilution of the samples with 9 volumes by weight of 1·0M HCl and boiling for 2 h according to Jansson (1981). After treatment with amyloglucosidase as above for methods 1 to 4, but none for method 5, glucose was determined as described by Bergmeyer *et al.* (1974). The powdered sample was stored at −85 °C and reanalyzed after 2 months using three of the methods employed earlier (1, 3 and 4).

Ten samples, earlier analyzed within 2 months of storage using method 3, were stored for a further 4 months at −85 °C and reanalyzed using methods 1, 2 and 3.

Modified method

The methodology for glycogen determination was modified according to the results in the preceding sections. The sample was homogenized in perchloric acid for 30 s using an Ultra Turrax. The homogenate was transferred using the modified micro-pipette and the weight was used for calculations. If samples are kept frozen for prolonged times, boiling of the homogenate should preferably be included.

Determination of metabolites

The frozen, pulverized samples were diluted with perchloric acid as described above, homogenized for 30 s using an Ultra Turrax and centrifuged at 40 000*g* for 20 min at 0 °C. The supernatant was neutralized with solid $KHCO_3$ and the resulting precipitate was centrifuged down. Analyses for free glucose, glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate were carried out in the same cuvette by combining the procedures of Bergmeyer *et al.* (1974), Bergmeyer & Michal (1974) and Lang & Michal (1974). Fructose-1,6-diphosphate, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were determined according to the procedure described by Michal & Beutler (1974). Analyses of pyruvate, phosphoenol-pyruvate, glycerate-2-phosphate and glycerate-3-phosphate were performed as outlined by Czok & Lamprecht (1974) with the addition of phosphoglycerate mutase as described by Czok & Eckert (1962). Lactate was determined using the test-combination Cat.

No. 139084 from Boehringer & Mannheim (GFR) while glycerol-3-phosphate was measured according to Michal & Lang (1974). Alanine was determined by the method of Williamson (1974). In some samples glycogen concentration was also determined in the supernatant. When calculating absolute concentrations the water content of the muscle samples was assumed to be 75%.

RESULTS AND DISCUSSION

Initially we wanted to repeat earlier findings (Laser Reuterswärd *et al.*, 1981) of the non-stoichiometric relationship between glycogen, intermediates and lactate. Using the initial method (homogenization in cold perchloric acid and enzymatic hydrolysis of the homogenate) glycogen was analyzed in 38 beef carcasses at 1 and 24 h after slaughter. Glucose plus hexosemonophosphates and lactate were analyzed simultaneously. Results were grouped according to the ultimate muscle pH as previously described (Fabiansson *et al.*, 1984).

Table 1 shows that increases in the total amount of glucose equivalents of 15% occurred in normal as well as in intermediate carcasses and of 33% in DFD carcasses at 24 h compared to 1 h, indicating a non-stoichiometric conversion of glycogen into the metabolites examined. These metabolites are believed to constitute more than 95% of all metabolites involved in glycolysis (Hamm *et al.*, 1973). The present results were much in line with the findings of Laser Reuterswärd *et al.* (1981), although in that investigation glycogen was determined in the perchloric acid extract after previous centrifugation at 10000*g*. Determination of glycogen directly in the homogenate, as in the present study, thus did not improve the overall stoichiometric relationship.

Recovery of glycogen

When extracting glycogen with water or dilute acids, only part of the glycogen is easily extractable (Russell & Bloom, 1955; Jansson, 1981). The amount of acid-soluble glycogen seems to vary with species, the type of tissue, the total glycogen concentration and particularly according to the method of preparation of the samples (Roe *et al.*, 1961; Bartley & Dean, 1968; Nahorski & Rogers, 1972; Hermansen & Vaage, 1977).

TABLE 1

Glycogen, Glycolytic Intermediates and Lactate in Carcasses of Different Ultimate pH at 1 and 24 h after Slaughter

	Normal carcasses[a] *mean (μmol/g)*		*Intermediate carcasses*[a] *mean (μmol/g)*		*DFD carcasses*[a] *mean (μmol/g)*	
	1 h	*24 h*	*1 h*	*24 h*	*1 h*	*24 h*
Glycogen	48·4[b]	18·5	7·16	0·37	3·93	0·30
Glucose + hexosemonophosphates	6·02	17·8	2·46	1·32	0·72	0·21
Lactate	33·2	91	36·5	60·8	21·0	39·3
Sum of glucose equivalents	71	81·9	27·9	32·1	15·2	20·2

[a] Normal carcasses—$pH_{24} \leq 5{\cdot}8$ ($n = 20$); intermediate carcasses—$5{\cdot}8 < pH_{24} < 6{\cdot}2$ ($n = 3$); DFD carcasses—$pH_{24} \geq 6{\cdot}2$ ($n = 15$).
[b] Glycogen content is expressed in glucosyl units.

However, Roe *et al.* (1961) have suggested that glycogen can be extracted completely from different tissues using thorough homogenization.

In order to estimate the recovery of glycogen using the initial method the following experiment was performed on samples from normal and DFD carcasses. Glycogen was analyzed directly in the perchloric acid homogenate (Hom 1). Glycogen was also determined in the supernatant (Sup 1) after centrifugation of the homogenate. The residue was resuspended twice in perchloric acid, centrifuged and the resulting two supernatants (Sups 2 and 3) as well as the last resuspension (Hom 3) were analyzed for glycogen enzymatically (Table 2). After three extractions (Sups 1, 2 and 3) 70 % of the total amount of glycogen (Hom 1) in normal carcasses could be recovered. The corresponding value for DFD carcasses was 75 %. Thus, it was not possible to extract all of the glycogen into perchloric acid extracts despite three successive homogenizations, at least not in the Ultra Turrax.

Glycogen was also determined in the third homogenate (Table 2). The sum of the glycogen content found in the third homogenate and the preceding two supernatants was, for normal carcasses, very close to the value found in the first homogenate, as would be expected. However, in samples from DFD carcasses at 1 h the highest values were found in the third homogenate. No apparent explanation for this finding can be given. It is possible that inhibiting concentrations of other metabolites, later extracted into the supernatant, could have been influencing the results of the glycogen analyses, but this question needs further study. No

TABLE 2

Recovery of Glycogen using Repeated Extraction of Muscle Samples from Normal ($n = 10$) and DFD ($n = 12$) Carcasses

Treatment[a]	*Glycogen (μmol glucosyl units/g)*			
	Normal		*DFD*	
	1 h	*24 h*	*1 h*	*24 h*
Hom 1	47·3	18·3	4·54	0·33
Sup 1	18·3	9·81	2·60	0·20
Hom 2	Not analyzed	Not analyzed	Not analyzed	Not analyzed
Sup 2	8·42	3·20	0·47	0·05
Hom 3	18·8	8·95	8·58	0
Sup 3	6·48	1·81	0·26	0·01

[a] Hom = homogenate, Sup = supernatant after centrifugation at 40 000*g* at 0 °C for 20 min.

anomalies were found at 24 h. The sum of the glycogen found in the third homogenate and the two preceding supernatants was, for DFD carcasses, 11·7 μmol/g at 1 h. A correction of the 1 h value accordingly gives a stoichiometric relationship between the metabolites studied at 1 and 24 h for DFD carcasses. However, a non-stoichiometric relationship still remains for normal carcasses.

Importance of homogenization

An attempt was made to locate methodological errors involved in the analysis of glycogen. The original method of Dalrymple & Hamm (1973) involves homogenization for 100 s using a Bühler homogenizer and an aliquot of the homogenate is thereafter volumetrically transferred to the test-tube. In the present study the effects of high-speed (Ultra Turrax) and low-speed (Omni Mixer) homogenizers were tested for different homogenization times (Fig. 1). More glycogen was recovered with increasing homogenization time when using the Omni Mixer, with and without the micro-attachment. The opposite was true when using the Ultra Turrax. Owing to the high speed of the Ultra Turrax, the muscle tissue was thoroughly homogenized within a few seconds. In the Omni Mixer complete homogenization was not accomplished even after 180 s.

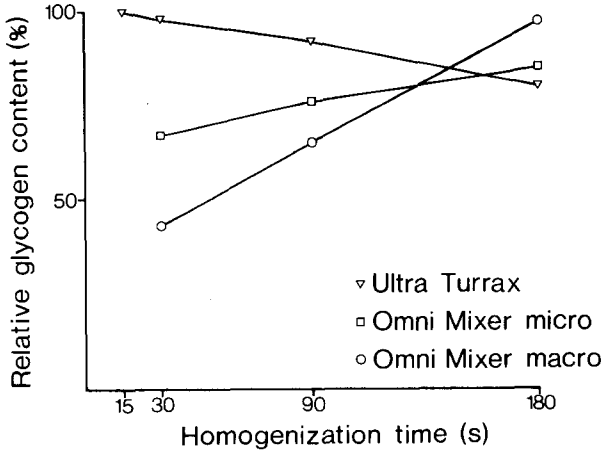

Fig. 1. Effects of a high-speed (Ultra Turrax) and a low-speed (Omni Mixer with and without micro-attachment) homogenizer on the recovery of glycogen ($n = 6$). The highest value was found using homogenization in the Ultra Turrax for 15 s (100%).

Despite attempts to cool the samples during homogenization, the heat produced by the Ultra Turrax might have influenced results. Homogenization for 15–30 s in the Ultra Turrax seemed to be the preferable method and 30 s was therefore used further on in the present investigation.

Furthermore it was difficult to transfer an aliquot of the homogenate volumetrically as described by Dalrymple & Hamm (1973). Errors of about $\pm 7\%$, as determined by weighing, were easily introduced. Therefore, the method was changed so that the homogenate was weighed directly into the test-tube, and weights were used for all calculations.

Comparison of five different methods for estimating glycogen

With the above improvements the results of the perchloric acid method were compared to the results of the four other methods of analyzing glycogen. Results are given in Table 3. The highest values of glycogen were found when the samples were boiled (100 °C) for 10 min in 0·03M HCl. The values from this method were significantly different ($P < 0{\cdot}05$) from the values of all other methods except for boiling in 0·6M $HClO_4$. The reason for this is not clear. The presence of other glucosylated compounds could possibly have influenced the results. Since all values decreased after 2 months of freezer storage (Table 3) and the difference

TABLE 3
Comparison of Different Methods of Analyzing Glycogen
(The samples were analyzed within 1 week or after 2 months of storage at −85 °C)

Methods used	*Glycogen (μmol glucosyl units/g)**			
	Immediate analyses		*Analyses after storage*	
	Mean	*SD*	*Mean*	*SD*
1 0·03M HCl, 100 °C	$58{\cdot}3^{b}$	1·09	$53{\cdot}9^{e}$	1·26
2 1·0M KOH, 60 °C	$55{\cdot}1^{ac}$	1·97	—	
3 0·6M $HClO_4$	$54{\cdot}4^{a}$	1·69	$47{\cdot}6^{d}$	1·86
4 0·6M $HClO_4$, 100 °C	$56{\cdot}9^{bc}$	1·56	$52{\cdot}7^{e}$	0·84
5 1·0M HCl	$54{\cdot}8^{ac}$	2·65	—	

* Values with different superscripts in the same row or column are significantly different ($P < 0{\cdot}05$); $n = 5$.

between method 3 (non-boiling) and methods 1 and 4 (boiling) increased from 6 to 12%, it was assumed that boiling did influence the recovery of glycogen.

Similar results were obtained for ten samples with different levels of glycogen analyzed after storage for 2 months at −85 °C using method 3 and reanalyzed after an additional storage for about 4 months at −85 °C (Fig. 2). Compared to the values at 2 months of storage, the values of method 3 were 9% lower and the values of methods 1 and 2 were 13 and 15% higher after the additional storage. The difference was significantly higher ($P < 0{\cdot}01$) for method 2, close to significant ($P = 0{\cdot}08$) for method 1 but was not significant for method 3.

It has previously been reported that the configuration of the glycogen molecule was changed during prolonged freezer storage but restoration occurred after boiling for a few minutes (Lowry & Passonneau, 1972). However, Keppler & Decker (1974) pointed out that the difficulty in hydrolyzing old glycogen enzymatically was true only when phosphorylase and debranching enzyme were used and not when amyloglucosidase was used. This is not in accordance with our results. We also found a decrease in the values after freezer storage when amyloglucosidase was used. Assuming that the results of methods 1 and 4

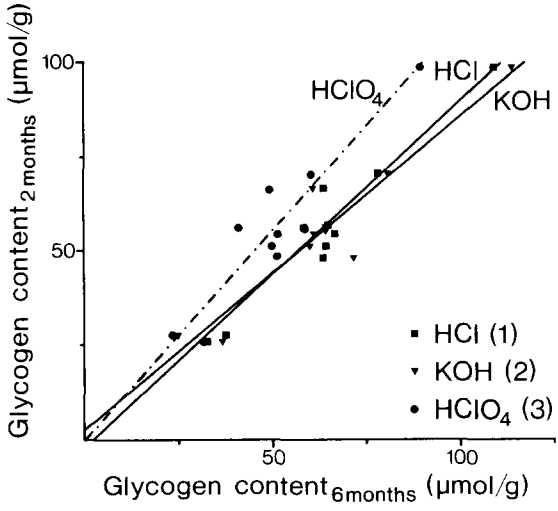

Fig. 2. Recovery of glycogen after storage for an additional 4 months at −85 °C (analyzed by methods 1, 2 and 3) in relation to the initial values after 2 months of storage (analyzed by method 3).

(boiling) are correct, a correction factor when using method 3 (non-boiling) of about 1·2 after 2 months and 1·3 after a further 4 months of storage can be calculated from the above results. However, since no systematic study of the effects of freezer storage was undertaken, the results may not be applicable to other conditions of freezer storage.

Reassessment of the stoichiometric relationship

In seven normal carcasses glycogen (analyzed using the modified perchloric acid method without boiling), lactate and intermediate metabolites (glucose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, glycerate-3-phosphate, glycerate-2-phosphate, phosphoenol-pyruvate and pyruvate) were determined as well as glycerol-3-phosphate and alanine (Table 4). Despite the use of the improved perchloric acid method, there was a 10% significant ($P < 0.05$) increase in total glucose equivalents between 1 and 24 h when including glycogen, glucose, hexosemonophosphates and lactate. Inclusion of the remaining intermediates did not significantly influence the result, since they

TABLE 4
Glycogen, Intermediates, Lactate and Related Substances in Normal Carcasses at 1 and 24 h after Slaughter

	Mean (μmol/g)		*Accumulated difference between 1 and 24 h in glucose equivalents*[a] *Mean (SD) (μmol/g)*
	1 h	*24 h*	
Glycogen (glucosyl units)	51·6	17·8	
Glucose + hexosemonophosphates	7·47	17·3	
Lactate	37·5	101	7·78 (6·30)*
Remaining intermediates	0·65	0·27	7·40 (6·37)*
Glycerol-3-phosphate	4·78	1·40	4·02 (6·72)NS
Alanine	2·50	2·87	4·39 (6·71)NS
Glycogen corrected	62·0	21·4	−2·37 (7·77)NS

[a] Mean and mean of paired difference between 1 and 24 h with standard deviation.
* Denotes significant difference ($P < 0.05$); NS denotes non-significant difference ($P > 0.05$); $n = 7$.

accounted for less than 1% of the total amount of glycolytic substances. However, appreciable amounts of glycerol-3-phosphate were degraded between 1 and 24 h and, when including this substance into the glucose equivalents, the difference between 1 and 24 h, although still 5%, became insignificant. The incorporation of alanine into the values did not influence the results substantially (Table 4).

Since the samples had been stored in the freezer before being analyzed it would be appropriate to adjust the results of the glycogen analyses accordingly. When applying the calculated correction factor of 1·2 for the values given in Table 4 there was almost 100% recovery of glucose equivalents at 24 h compared to 1 h for the normal carcasses.

CONCLUSIONS

Some methods of analyzing glycogen can easily introduce erroneous results. Too vigorous homogenization can lower the recovery of glycogen as can prolonged freezer storage, even at −85 °C. Methods involving heating or boiling of the samples seemed to be advantageous in minimizing the effect of freezer storage. A general stoichiometric conversion of glycogen to glycolytic metabolites was found between 1 and 24 h post mortem, if glycerol-3-phosphate was included.

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