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Journal of Nutritional Biochemistry 14 (2003) 139–146

THE JOURNAL OF Nutritional Biochemistry

Modification of analytical procedures for determining vitamin C enzyme (L-gulonolactone oxidase) activity in swine liver

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Received 8 April 2002; received in revised form 23 October 2002; accepted 25 November 2002

Abstract

Modifications of the analytical method to determine L-gulono-γ-lactone oxidase (EC 1.1.8) enzyme activity were conducted in pig liver by evaluating the concentration of added substrate (L-gulono-γ-lactone), glutathione, and various tissue sample-to-buffer ratios in the incubation mixture. Sampling different liver sites (lobes), the effect of different cooling temperatures of the liver immediately after collection, and the effect of tissue storage length on subsequent enzyme activity were evaluated. Our results demonstrated that 10 mM of substrate added to the reaction media maximized L-gulono-γ-lactone oxidase enzyme activity, whereas increasing levels of glutathione did not greatly affect enzyme activity. High sample-to-buffer ratios resulted in higher L-gulono-γ-lactone oxidase activities but sample analytical variations and background interferences were greater. A 1:4 tissue sample to buffer ratio (weight:weight) resulted in repeatable values, but the importance of maintaining the same ratio of the two components seems to be critical within an experiment. Expressing L-gulono-γ-lactone oxidase enzyme activity on a liver protein rather than on a liver weight basis also resulted in more consistent results. No difference in liver L-gulono-γ-lactone oxidase enzyme activities or ascorbic acid concentrations occurred between liver lobes. L-gulono-γ-lactone oxidase enzyme activity from 0 to 90 day of storage was not affected when tissue samples were immediately frozen in liquid nitrogen, or placed on crushed ice. During a 90-day storage the oxidized form of ascorbic acid (dehydroascorbic acid) decreased ($P < 0.01$), the reduced (ascorbic acid) form increased ($P < 0.01$), while total ascorbic acid concentration remained constant.

Keywords: L-gulono-γ-lactone oxidase; Vitamin C; Ascorbic acid; Pigs

1. Introduction

L-gulono-γ-lactone oxidase, [EC 1.1.3.8], (GLO) is involved in the biosynthesis of ascorbic acid and is the enzyme missing in those animal species that cannot synthesize ascorbic acid [1]. Glucose and galactose participate in the glucuronic acid pathway forming L-gulono-γ-lactone, whereupon the GLO enzyme converts the substrate to L-ascorbic acid. Synthesis occurs in the liver of most mammals [1], but in the kidney of lower vertebrates [2]. Recent evidence in the pig has demonstrated that GLO enzyme activity is high during early pig fetal development, but seems to be lowered toward the end of gestation and when pigs nurse the sow [3,4].

Tissue ascorbate concentrations and its excretion in urine had been previously used to indirectly characterize the synthesis of this vitamin in young swine [5], but its supply in the urine may originate from exogenous sources not just tissue synthesis. Liver GLO enzyme activity should be another indicator to measure the animal's ability to synthesize vitamin C.

The GLO enzyme activity is measured by determining the rate of total ascorbic acid (reduced and oxidized form) synthesized in liver tissue by adding the substrate (L-gulono-γ-lactone) and the antioxidants that prevent its oxida-

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Salaries and research support were provided by State and Federal funds appropriated to the Ohio Agric. Res. and Dev. Center and The Ohio State Univ.

The experimental use of animals and procedures followed were approved by the University Animal Care Committee.

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0955-2863/03/$ – see front matter
doi:10.1016/PII S0955-2863(02)00276-0

tion upon synthesis. The analytical procedures for determining GLO activity seems to differ between species. For example, potassium cyanide (KCN) added at 50 mM protects the lactone ring in D-glucurono-γ-lactone from degradation in the livers of rats [6]. Others [7] have demonstrated a small decrease in the conversion of D-glucurono-γ-lactone to L-ascorbic acid in goat liver when 5 mM KCN was added, whereas in rat liver the conversion was greater with added KCN.

Maximum GLO synthesis in both rat and goat liver and in chick kidney is attained after the addition of 5 mM of the substrate L-gulono-γ-lactone [8,9]. Others have demonstrated that 10 mM L-gulono-γ-lactone was necessary to maximize GLO enzyme activity in the kidney of the sea lamprey, in beef and rat livers, and in the kidneys from pigeons and frogs [2,10].

Glutathione (GSH) protects synthesized ascorbic acid from oxidation in mouse liver [11]. These latter researchers suggested that GSH was needed in approximately equimolar amounts to the ascorbic acid produced.

A 0.3 to 0.5 g tissue sample and a phosphate buffer volume of 2 to 3 ml have previously been used for determining GLO activity [8]. The phosphate buffer maintains the reaction mixture at pH 7.4, but the buffer solution also contains 0.2% sodium deoxycholate, which solubilizes the microsomal membranes where the GLO enzyme is located. A high buffer to tissue ratio may allow for more of the GLO enzyme to be released because of the higher supply of sodium deoxycholate.

The effect of storage length and the homogeneity of the GLO enzyme in various liver sites have not been extensively studied. Rat livers stored at 0°C in sealed vacuum containers have shown similar GLO enzyme activities for 1 year [8]. Others have reported that GLO enzyme activities were similar between the different lobes in rat or rabbit livers, suggesting that the enzyme was uniformily distributed in this tissue [12].

Our studies were conducted to evaluate the effect of various concentrations of substrate and other chemicals used in the analytical procedures for measuring GLO enzyme activity in swine liver. We also evaluated the effect of storage duration, uniformity of the GLO enzyme in different liver locations (i.e., lobes), and the effect of rapid or slow cooling procedures following tissue collection on GLO activity and ascorbic acid concentrations in swine liver.

2. Materials and Methods

2.1. General

The livers used in these trials were obtained from 30 crossbred ([Yorkshire × Landrace] × Duroc) pigs that had been weaned at least 4 weeks and averaged approximately 20 kg body weight. The diet fed during the experiment was devoid of supplemental vitamin C, but met or exceeded the other nutrient recommendations of the NRC [13]. The diet was formulated to contain supplemental levels of vitamin E (45 IU/kg) as dl α-tocopheryl acetate, vitamin A (4,000 IU/kg) as retinyl acetate, copper (8 ppm) as copper sulfate, zinc (100 ppm) as zinc sulfate, manganese (20 ppm) as manganese oxide, selenium (0.30 ppm) as sodium selenite, and iron (90 ppm) as ferrous sulfate. At the designated time, pigs were stunned by electrocution and killed by exsanguination. Livers were removed within 2 min of killing with samples collected from the same site and lobe for all experiments except in the fourth experiment where sample location was the treatment variable. Unless noted otherwise collected liver samples (<5 g) were frozen immediately in liquid nitrogen, transported to the laboratory and stored at −80°C in sealed Eppendorf test tubes for the later determination of GLO enzyme activity. Treatment analyses within each replicate were conducted at the same time with each experiment being conducted in five replicates. A Randomized Complete Block design was used in experiments 1 to 4 with a Split Plot design used in experiment 5.

2.2. Experiment 1

The first experiment evaluated the level of substrate (L-gulono-γ-lactone) added to the media during the incubation of liver tissue for determining maximum GLO enzyme activity. Five levels of L-gulono-γ-lactone (0, 35, 70, 100, and 130 mM) were evaluated resulting in 0, 3.5, 7, 10, 13 mM of the substrate in the final reaction media.

2.3. Experiment 2

The second experiment evaluated various levels of reduced GSH on subsequent GLO enzyme activity. Levels of 0, 0.8, 1.6, 2.4, and 3.2 mM GSH in the final reaction mixture was used in one trial, and 1.6, 3.2, 4.8, 6.4, and 8.0 mM GSH was evaluated in a second trial. A concentration of 2.5 mM GSH had been previously used where tissues from different animal species were evaluated [10].

2.4. Experiment 3

The third experiment evaluated the quantity of liver sample to the added phosphate buffer on subsequent GLO enzyme activity. Dabrowski [10] used a range of 0.3 to 0.5 g liver and 2 to 3 ml of the phosphate buffer for his study. Our experiment evaluated specific sample (g) to buffer (mL) ratios of 1:1, 1:2, 1:4, 1:8, or 1:16.

2.5. Experiment 4

The fourth experiment evaluated GLO activity and liver ascorbic acid concentrations from different locations in the pig's liver. Samples were from the mid portion of four different liver lobes while the fifth sample was collected at

the extremity of one of the lobes. Each sample was collected at the same location within each lobe for each replicate.

2.6. Experiment 5

Storage length and collection procedures at the time of tissue sample collection on subsequent liver GLO enzyme activities and ascorbic acid concentrations were studied in a fifth experiment**.** It is not known if different temperatures at the time of collection can affect GLO enzyme activity or tissue ascorbic acid concentration. Therefore, we evaluated the effect of immediately freezing the liver samples (<5 g) in liquid nitrogen, placing the samples on crushed ice, or allowing the sample to remain at room temperature (25°C) for a 2 hr period, whereupon all samples were stored at −80°C until analyzed. Because the ratio of the reduced (ascorbic acid) to the oxidized (dehydroascorbic acid) ascorbic acid may change during collection and upon storage, this was measured at each period. Samples were stored in sealed Eppendorf test tubes for 0, 15, 30, 45, 60, or 90 days at −80°C, whereupon GLO enzyme activity was determined. Samples for determining liver ascorbic acid concentration were stored at −20°C. The experiment was a Split Plot design with collection method being the main plot and length of storage the sub plot.

2.7. Analytical procedures

The GLO enzyme activity procedure that we used was derived from Dabrowski [14], but modified as we progressed through our studies. Liver samples were homogenized with the buffer solution (50 mM sodium phosphate at pH 7.4, 1 mM EDTA, and 0.2% sodium deoxycholate) in a ratio of 1 g liver to 4 ml buffer. This mixture was maintained on crushed ice during the homogenization process. Homogenization was conducted using a spin homogenizer (OMNI international 5000) at 4,500 × g for 20 s, and then centrifuged for 30 min at 30,000 × g at 4°C (Beckman J2-21). The supernatant, which contained the enzyme extract, was subsequently used for determining both GLO enzyme activity and the soluble protein concentration by the Bradford procedure (Coomassie Protein Assay Reagent, Pierce, Rockford, IL). In a glass tube, 400 μl of the supernatant (maintained at 4°C) was mixed with 650 μl of 50 mM sodium phosphate buffer (pH 7.4) and maintained at room temperature. The addition of 650 μl of 50 mM sodium phosphate buffer at 37°C, 100 μl GSH (4°C), and 200 μl 100 mM L-gulono-γ-lactone (room temperature) was used to bring the total volume to 2,000 μl. The tube was incubated in a water bath (Lauda water bath) at 37°C. Aliquots were transferred to Eppendorf test tubes at 0, 12, 24, and 48 min after incubation, whereupon the reaction was stopped by adding equal volumes of a 5% trichloroacetic acid (TCA) solution, which also contained 250 mM perchloric acid and 3 mM disodium ethylenediamine tetraacetate (EDTA, ice-cold). The reaction was stopped at 0, 12, 24, and 48 min to determine the linearity of GLO activity. A correlation coefficient (r) > 0.95 was obtained for these measurements. The tubes were centrifuged for 30 min at 15,000 × g at 4°C (Beckman GS-15R) with the supernatant stored at -20°C for subsequent ascorbic acid concentration analysis.

Ascorbic acid concentration from each reaction mixture from each liver sample was determined by the dinitrophenyl-hydrazine (DNPH) method [14]. Ascorbic acid was measured at 524 nm by multi-wavelength spectrophotometer (Beckman DU-70) and subtracting background interference at 440 nm. Dichlorophenol indophenol (DCIP) was used as an oxidizing agent for total ascorbic acid or was omitted when determining dehydroascorbate by parallel analysis. Total ascorbic acid for each sample was defined as the oxidized (dehydroascorbic acid) plus the reduced (ascorbic acid) form of the vitamin with the total and oxidized forms being measured at the same time. Ascorbic acid (reduced) concentration was determined by subtracting dehydroascorbic acid concentration from the total ascorbic acid concentration. The GLO activity was determined by measuring the increasing rate of ascorbic acid formation by the linear equation of $Y = a + bX$ (X = time, Y = total ascorbic acid concentration). The slope (b) was considered the GLO enzyme activity. The data were evaluated either as μmol total ascorbic acid/g liver/h or μmol total ascorbic acid/g liver protein/h.

2.8. Statistical analysis

Four experiments were conducted as a randomized complete block design with experiment 5 as a Split Plot [15]. Each experiment contained five replicates and analyzed by the Mixed Model procedure of SAS [16]. Individual pig liver or the individual sampling site from the same liver in experiment 4 was the experimental unit. Treatment means were contrasted in experiment 1, 2, 3, and 5 by regression analysis. The means of GLO enzyme activity and liver ascorbic acid concentrations of liver samples from experiment 4 were compared by lsd. Repeat measure analysis was used for the contrasting day effects in experiment 5.

3. Results and Discussion

3.1. Experiment 1

Measuring GLO enzyme activity and expressing the values as either μmol total ascorbic acid/g liver protein/h or μmol total ascorbic acid/g liver/h produced similar results, but tissue dehydration that occurred upon storage affected the outcome over time. Consequently, the GLO enzyme activity when expressed on a liver protein basis was found to be more consistent.

The addition of 10 mM L-gulono-γ-lactone to the reaction medium was necessary to obtain maximum GLO activity in pig liver (Fig. 1). The GLO activity would be

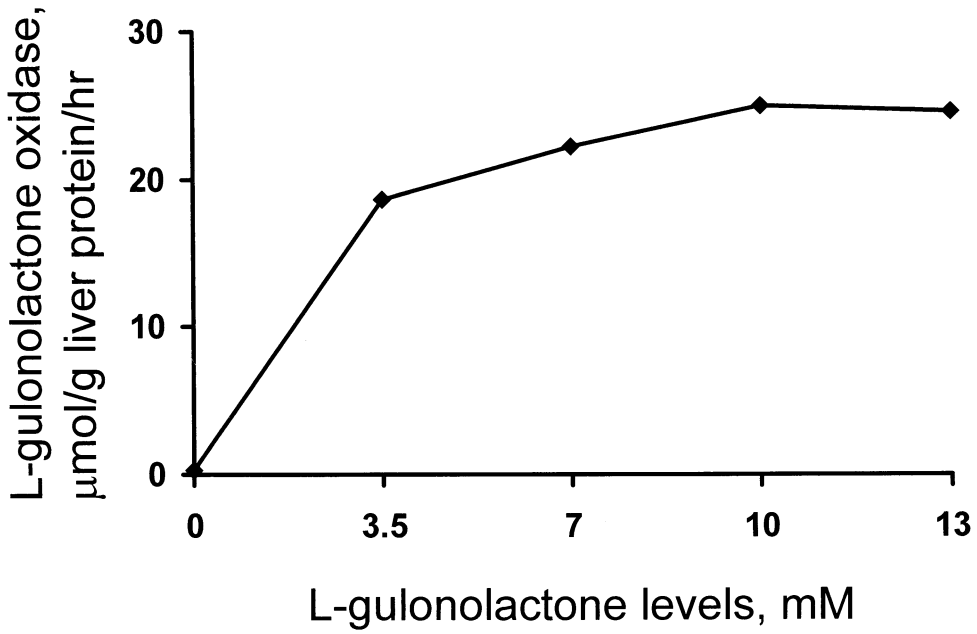

Fig. 1. Effect of added substrate (L-gulono-γ-lactone) on L-gulonolactone oxidase (GLO) activity in pig liver. The experiment evaluated the livers from five pigs killed at 19.5 kg body weight. The response was linear ($P < 0.01$) with an overall SEM of 3.8.

expected to increase until enzyme sites become saturated with the substrate, whereupon enzyme activity reaches maximum velocity (V_{max}). These results are in agreement with others [10] who determined that 10mM of substrate resulted in maximum GLO activity in beef liver, fish, pigeon and frog kidney. A plateau occurred when 5 mM L-gulono-γ-lactone was added, when evaluating the livers of the rat and the goat, and the kidney of the chick [8]. Maximum GLO enzyme activity in rats is reported to occur with the addition of 0.4 mM L-gulono-γ-lactone [12]. It is of interest to note that when no substrate (L-gulono-γ-lactone) was added to the incubation media GLO enzyme activity was absent in pig liver samples. This may be due to metabolism of the substrate during sample preparation, storage loss of the substrate, or to a low level of the substrate (L-gulono-γ-lactone) initially in the liver of the pigs at the time of collection. Stress during the slaughter process may also have increased the animal's metabolism of L-gulono-γ-lactone.

3.2. Experiment 2

The effect of GSH levels on subsequent GLO enzyme activity presented in Fig. 2 suggested that enzyme activity tended to increase numerically as GSH levels increased but the effect was not significant ($P > 0.15$). The GLO enzyme activity of trial 1 was lower, however, than the enzyme activity in the second trial. Pigs used in the second trial were older and heavier than in trial 1. Our previous results indicated that vitamin C synthesis was higher for older and heavier pigs postweaning and was the probable reason for the discrepancy between the two trials. Because many factors can oxidize ascorbic acid during the analysis procedure, the addition of 2.5 mM GSH was used in our subsequent studies but a higher level (i.e. 10 mM) may be needed for older swine. Others [9] had previously demonstrated that adding GSH at 10 mM resulted in higher L-ascorbic acid concentrations in rat liver.

3.3. Experiment 3

The effect of liver sample weight to the amount of buffer used on GLO activity (Fig. 3) demonstrated that GLO enzyme activity increased ($P < 0.01$) as the ratio (weight: weight) of these two components changed from 1:1 to 1:16. The higher relative amounts of added buffer resulted in a higher GLO enzyme activity with more protein also extracted into the supernatant after centrifugation. Others [8] have demonstrated that ascorbic acid synthesis increased as the amount of rat and goat liver and chick kidney increased in the incubation mixture.

Although GLO enzyme activities increased as the ratio increased, there were difficulties in obtaining repeatable results at the high ratios at the multi-wavelength spectrum. At the higher ratios the background interference at 440 nm was higher than the scan of the ascorbate complex at 524 nm. Clearly the ratio of sample to buffer would influence the reported GLO enzyme activity. Although the GLO enzyme activity is lower at the 1:4 ratio, the activities would be replicated better than at the higher ratios.

Others [12] have used the ratio from 1:10 to 1:100 and their resulting GLO enzyme activity values may have been different had a lower ratio been used.

Increasing the sodium deoxycholate concentration in the

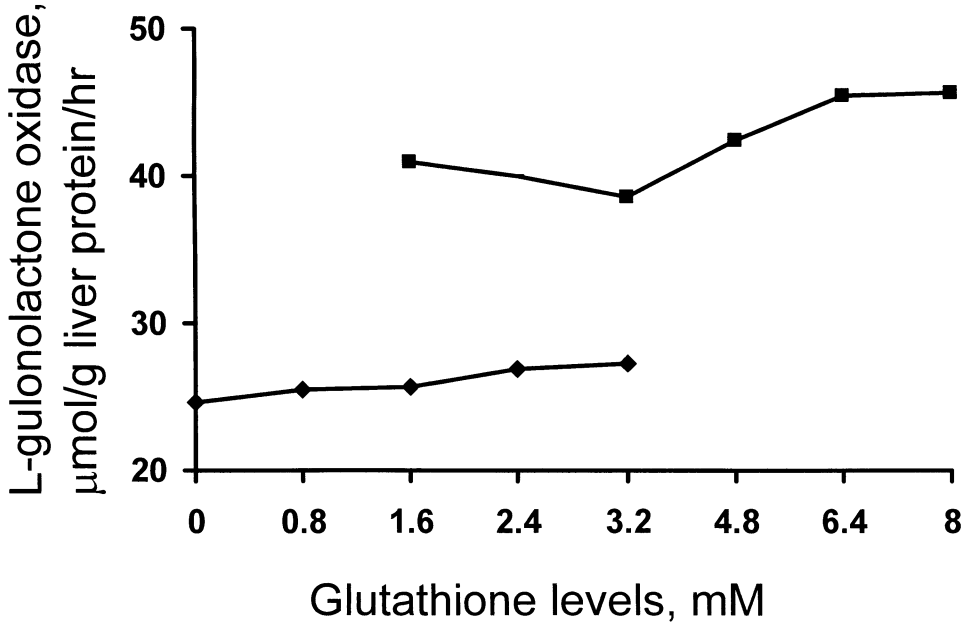

Fig. 2. Effect of added glutathione (GSH) levels on L-gulonolactone oxidase (GLO) activity in pig liver. Trial 1 evaluated the livers from five pigs with an average 19.0 kg body weight. The second trial contained five pigs per treatment group with an average 23.0 kg body weight. The SEM of trial 1 was 1.4 and in trial 2 the SEM was 1.0. The regression response was not significant ($P > 0.15$).

phosphate buffer may increase GLO enzyme activity because of the enzyme's greater release from the microsomal fraction. However, when a 0.5% sodium deoxycholate concentration in the sodium phosphate buffer was used, no increase in GLO activity occurred [9].

3.4. Experiment 4

The effect of sample location in swine liver presented in Table 1 showed no difference in GLO enzyme activity from the five sampling sites. However, GLO enzyme activity tended to be higher when the sample was

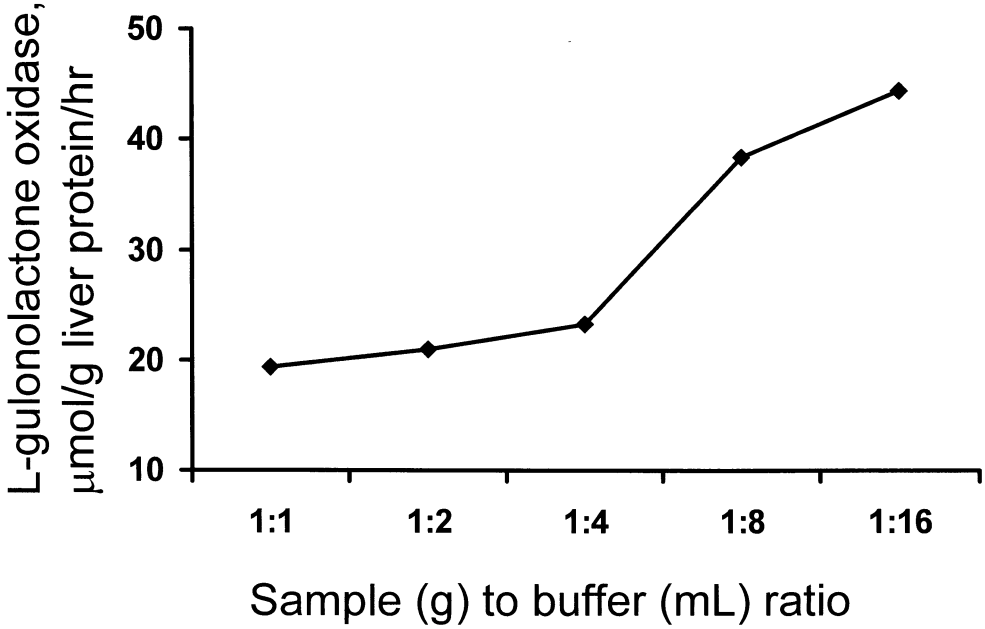

Fig. 3. Effect of liver sample to buffer ratio (weight:weight) on L-gulonolactone oxidase (GLO) activity in pig liver. The experiment evaluated the livers five pigs killed at 19.0 kg body weight. The response was linear ($P < 0.01$) with a SEM of 5.7.

Table 1
Pig liver L-gulono-γ-lactone oxidase (GLO) enzyme activity and ascorbic acid concentration at different location sites[a,b]

	Liver location					
Item	1	2	3	4	5	SEM
GLO enzyme activity[c]	27.2	25.5	25.2	24.8	25.2	0.9
Total ascorbic acid, μmol/g	1.33	1.30	1.30	1.29	1.33	0.11
Dehydroascorbic acid, μmol/g	0.29	0.26	0.24	0.25	0.28	0.02
Ascorbic acid, μmol/g	1.04	1.04	1.06	1.04	1.05	0.11
AA to dehydro AA ratio[d]	3.70	3.91	4.41	4.06	3.75	0.50

[a] A total of 5 pigs were killed at an average body weight of 20.5 kg.
[b] Livers were frozen in liquid nitrogen and stored at −80°C for GLO enzyme activity assay and additional samples stored at −20°C for ascorbic acid analysis.
[c] Enzyme activity: μmol total ascorbic acid/g liver protein/h
[d] AA = ascorbic acid

collected from the edge of the liver lobe (i.e. location 1). At this site the liver tissue was thin and may have had less blood flow. Others have reported that GLO enzyme activity was equally distributed throughout the livers of rats and rabbits [12]. Our results also indicate that the liver lobes of swine also have similar GLO enzyme activities.

No differences were found for either the oxidized (dehydroascorbic acid) or reduced (ascorbic acid) form or for the total ascorbic acid concentrations from the five locations in the liver (Table 1). About 80% of the total ascorbic acid was in the reduced (ascorbic acid) form while 20% was in the oxidized (dehydroascorbic acid) form.

3.5. Experiment 5

When collected liver samples were maintained at room temperature (25°C) for 2 hr the GLO enzyme activity tended to be somewhat higher ($P < 0.07$) at day 0 and 15 than those frozen immediately in liquid nitrogen or placed on crushed ice, whereas from day 30 to 90 of storage the GLO values were similar for all treatment groups. (Fig. 4) The reason for the higher values for the livers maintained at the room temperature for 2 hr is unclear. The GLO enzyme activity values were similar from 0 to 90 day when samples were frozen in liquid nitrogen or placed on crushed ice. Others [12] have analyzed rat liver GLO enzyme activity when samples were stored in wrapped

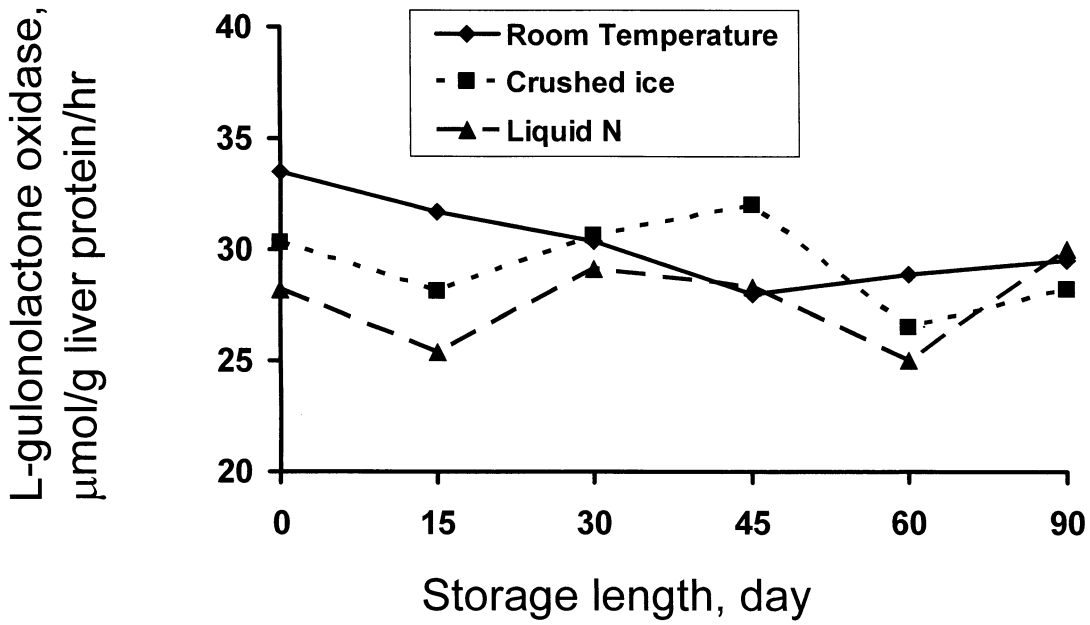

Fig. 4. Effect of collection process and storage duration (-80°C) on L-gulonolactone oxidase (GLO) activity in pig liver. The experiment evaluated the livers from five pigs with an average 24.3 kg body weight. When samples were maintained at room temperature GLO enzyme activity was higher at day 0 and 15, but was similar to the other groups from 30 to 90-day. Samples frozen in liquid nitrogen or placed on crushed ice had similar values for the 0 to 90 day period. The SEM was 1.9.

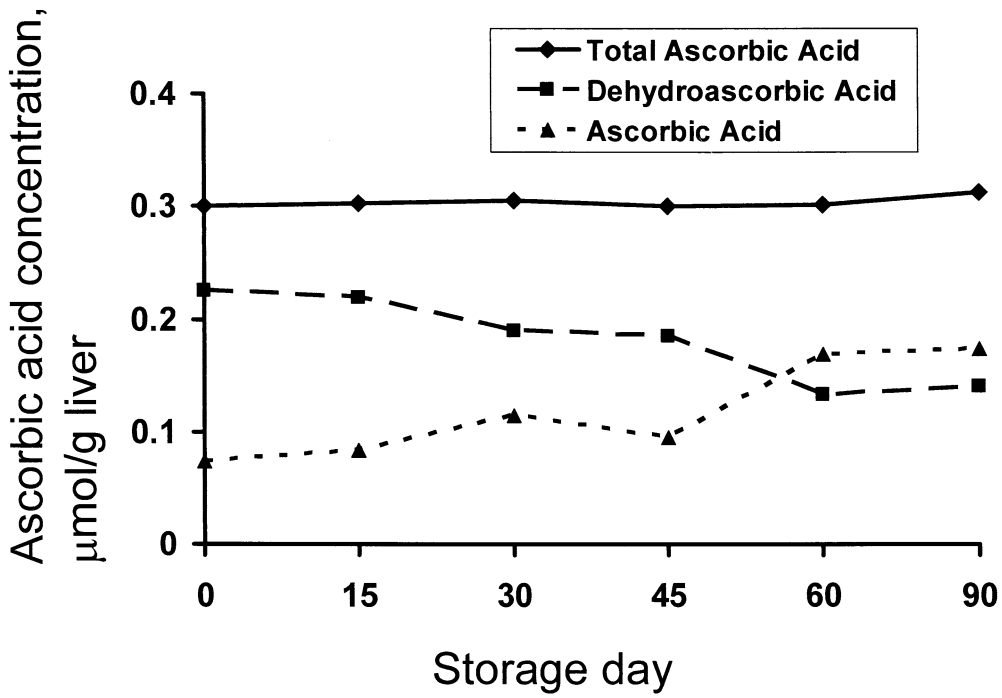

Fig. 5. Effect of storage time on total ascorbic acid concentration and on the various forms of ascorbic acid in swine liver. Total ascorbic acid concentration was similar for each of the 90 day measurement period. There was a linear decline ($P < 0.01$) in the reduced form (ascorbic acid) and a linear increase ($P < 0.01$) n the oxidized form (dehydroascorbic acid) over the 90 day storage period. The SEM for the two forms were 0.01 and it was 0.02 for the total ascorbic acid.

parafilm and frozen at −20°C. They demonstrated that GLO enzyme activity increased 125% during the first week of storage but then decreased after 22 weeks. The GLO activity in the kidney of chickens was found to be similar during a 2 weeks storage period at −20°C but was reduced when stored for 4 weeks [17]. In another study rat liver stored at −15°C for 21 days had no loss of GLO enzyme activity [18].

Total ascorbic acid concentration in liver tissue remained at a constant concentration for the 90-day storage period (Fig. 5). However, there was a linear increase ($P < 0.01$) in the reduced (ascorbic acid) form and a linear decrease ($P < 0.01$) in the oxidized (dehydroascorbic acid) form during the storage period. Consequently, the ratio of the oxidized to the reduced form increased linearly ($P < 0.01$) during the 90 storage period (Fig. 6). It has been demonstrated that ascorbic acid can be oxidized to dehydroascorbic acid by free radicals such as singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals generated during storage [19,20,21]. It is possible that liver minerals may have caused this oxidation. Although dehydroascorbic acid can be oxidized irreversibly to other compounds such as 2,3-diketogulonic acid [22], this would result in lowered total ascorbic acid content. Our results indicate that the ratio of the reduced form (ascorbic acid) and the dehydroascorbic acid changed during storage but there was no effect on the total concentration of ascorbic acid in the liver. Our results further suggest that tissue samples can be stored at −20°C for 90 days without losing its total ascorbic acid concentration, but the ratio of the two ascorbic acid forms can change.

In conclusion, the addition of 10 mM L-gulono-γ-lactone and 10 mM GSH to the final incubation medium seems necessary to obtain maximum GLO enzyme activity in swine liver. The sample-to-buffer ratio of 1:4 resulted in a lower GLO enzyme activity but replications were more satisfactory. It is clear that the ratio should be constant within an experiment to obtain repeatable results and to better evaluate treatment effects. Liver samples collected at the same location of the liver and analyzed at the same time post collection for both GLO enzyme activity and ascorbic acid concentration is preferred. It does not appear that tissue temperature from collection to the initial analysis had any effect on subsequent GLO activity. Consequently, the immediate freezing of liver samples in liquid nitrogen or allowing the liver to remain at room temperature for a 2 hr period post collection resulted in similar GLO enzyme activities and total ascorbic acid concentrations. To maintain greater uniformity for a longer storage period, GLO enzyme activity unit expressed as μmol total ascorbic acid/g liver protein/h would be more reliable.

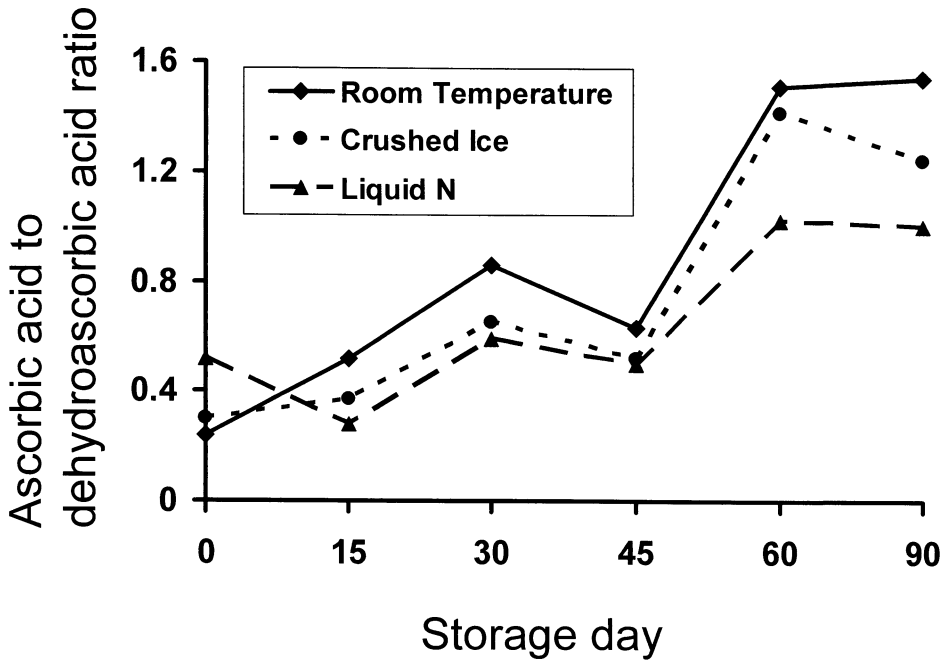

Fig. 6. Effect of collection procedure and storage time on the ratio of the reduced (ascorbic acid) and oxidized (dehydroascorbic acid) forms over the 90 day storage period. The response was linear ($P < 0.01$) with a SEM of 0.08.

Acknowledgments

Appreciation is expressed to K. Mays for animal care and data collection, F. Cihla and M. Watts for laboratory assistance, and B. Bishop for statistical analysis.

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