

## ANALYSIS OF A CHARACTERISTIC ALTERATION OF GLYCOGEN IN THE LIVER OF ALLOXAN DIABETIC RAT BY FASTING

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### ABSTRACT

A characteristic alteration of hepatic glycogen content by fasting was analyzed about alloxan diabetic rats.

1) In the fed state, hepatic glycogen content was suppressed in alloxan diabetic rats. However, when rats were fasted for 48 hours hepatic glycogen content was higher in alloxan diabetic rats than in the normal ones. There was a positive correlation between the blood glucose level and the liver glycogen level in fasted diabetic condition.

2) When rats were fasted, the incorporation of the blood glucose into both hepatic glycogen and glyceride-glycerol was more in the alloxan diabetic rats when compared with the intact rats. This was calculated from the values of the incorporated [ $U\text{-}^{14}\text{C}$ ] glucose.

3) On the other hand, under the same condition as 2), the incorporation from [ $2\text{-}^{14}\text{C}$ ] lactate into liver glycogen was less in alloxan-treated rats. Although  $^{14}\text{C}$ -glucose in the blood converted from [ $2\text{-}^{14}\text{C}$ ] lactate was more in alloxan-treated rats, it was attributed to delayed disappearance of glucose from the blood stream.

### INTRODUCTION

Alloxan diabetic rats fasted for 48 hours have relatively high liver glycogen content, whereas in normal ones liver glycogen quickly decreases by fasting and becomes negligible after 24 hours of fasting. Such a phenomenon has already been pointed out by some investigators.<sup>1)-5)</sup> However, the relationship between the carbohydrate metabolism and the liver glycogen content has not yet been analyzed clearly. If gluconeogenesis is stimulated more in alloxan diabetic rats as suggested previously in the diabetic and fasted condition,<sup>6)-13)</sup> the retarded decrease of liver glycogen in fasted diabetic rats might be attributed to an increased formation of glucose. When alloxan diabetic rats were fasted, the level of the remaining liver glycogen was affected by the blood glucose level. No evidence has been obtained showing that, in the fasted condition, enhanced gluconeogenesis occurs in the diabetic rat compared with the control rat. Thus, it will be suggested and discussed that the high liver glycogen content in fasted diabetic rats is dependent upon their hyperglycemia.

### METHODS AND MATERIALS

*Chemicals:* [ $2\text{-}^{14}\text{C}$ ] lactate (29 mCi/m mole) was purchased from Radiochemical Centre and [ $U\text{-}^{14}\text{C}$ ] glucose (5 mCi/m mole) was from Daiichi Pure Chemicals Co., Ltd. Glucose oxidase, peroxidase, lactate dehydrogenase and NAD were from Boehringer Corp. Other

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chemicals were from Katayama Chemicals Co., Ltd.

*Treatment of animals:* Female Sprague-Dawley rats, weighing 200–250 g, were used. Alloxan diabetic rats were prepared by an intraperitoneal injection of alloxan at a dosage of 150 or 175 mg/kg body weight, after they were fasted for 48 hours. After treatment with alloxan, rats were fed with a standard pellet diet. Seven days after administration of alloxan, experiments were performed. When experiments were done, body weights were about equal between diabetic and the control rats. 5  $\mu$ Ci of [2-<sup>14</sup>C] lactate or 4  $\mu$ Ci of [U-<sup>14</sup>C] glucose dissolved in 1 ml of distilled water was injected intraperitoneally after 48 hours of fasting. In each experiments, rats were decapitated at the indicated time and blood was collected in heparinized containers. The livers were rapidly excised and used for each analysis.

*Determination of glucose, lactate and <sup>14</sup>C-glucose in the blood*

After deproteinization by the method of Somogyi<sup>14)</sup>, <sup>14</sup>C-glucose in the blood was separated from other labeled intermediates by column chromatography according to the method described previously.<sup>15),16)</sup> Blood glucose was determined by the glucose oxidase method.<sup>17)</sup> Blood lactate was determined by the method of Hohorst.<sup>18)</sup>

*Determination of the liver glycogen and its radioactivity*

The livers were rapidly excised and digested in hot 30 % KOH. After precipitation of glycogen with alcohol, the precipitate was dissolved with 10 % trichloroacetic acid. Protein was sedimented centrifugally and the supernatant was precipitated again with alcohol. This purification procedure was repeated. After suitable dilution of the sediment with water, glycogen was determined by the anthrone method,<sup>19)</sup> and the same solution, which involved 1 mg of glycogen, was mixed with 18 ml of diotol\* and radioactivity in the liver glycogen was counted by the liquid scintillation counter.

*Determination of <sup>14</sup>C in the liver lipid*

The livers were homogenized in 20 vol of chloroform-methanol (2 : 1, V/V) and the homogenate was filtered 3 hours later. This filtrate was purified according to the method of Folch *et al.*<sup>20)</sup> Analytical methods were the same as described previously.<sup>21)</sup> One portion of the extract was taken to dryness, and the lipid was dissolved in toluene\*\* and counted to determine the total <sup>14</sup>C incorporated into lipid fraction. Another portion of the same sample was hydrolyzed with 4 N KOH for 6 hours. After the pH was acidified with 6 N H<sub>2</sub>SO<sub>4</sub>, fatty acids were extracted with petroleum ether. The solvent was evaporated and the radioactivity incorporated into fatty acids was counted by the same method used for the total lipids. Radioactivity incorporated into glyceride-glycerol was calculated as follows: radioactivity in total lipid fraction minus that in fatty acids.

## RESULTS

Liver glycogen contents and blood glucose levels in both the normal and alloxan diabetic rats are shown in Table 1. Whereas liver glycogen levels were lower in the diabetic when compared with the normal animals fed adequately, significantly higher levels were found in the diabetic group when compared in the fasted condition. When normal rats were fasted, the liver glycogen promptly decreased in accord with the decrease of the blood

\* Dioxane 500 ml, Methanol 300 ml, Naphtalene 104 g, Dotite DPO (2,5-diphenyloxazole) 6.5 g, POPOP (1,4-Bis-2-(5-phenyloxazolyl)benzene) 130 mg.

\*\* Toluene 500 ml, DPO 2.0 g, POPOP 40 mg.

Table 1. Effects of fasting on blood glucose and liver glycogen in alloxan diabetic rats

Treatment		Blood Glucose mg/dl	Liver Glycogen mg/g liver
(A)	Fed		
	Alloxan (43)	451 ± 172 <sup>a,b</sup>	23.9 ± 7.9 <sup>b</sup>
	Control (21)	144 ± 16	52.7 ± 10.4
(B)	Fasted <sup>c</sup>		
	Alloxan (50)	230 ± 103 <sup>b</sup>	13.5 ± 9.2 <sup>b</sup>
	Control (35)	82 ± 10	2.8 ± 1.1

a: Each value is the mean ± SD for the number of animals shown in parenthesis.

b: Significantly different between the two groups. ( $P < 0.01$ )

c: Animals were fasted for 48 h. Other experimental details are described in text.

glucose and after 24 hours of fasting, almost all the liver glycogen was exhausted. On the other hand, in alloxan diabetic rats the decrease of the liver glycogen content by fasting was less and a considerable amount of liver glycogen was reserved even after 48 hours of fasting. Furthermore, rats which had higher blood glucose levels after fasting tend to have more liver glycogen content after fasting. As shown in Figure 1, there was a positive correlation between the blood glucose level and the liver glycogen content when they were fasted for 48 hours. Although there are some disaccords among other reports,<sup>4),22)</sup> the present results show a close correlation between the both. On this matter, this author tried to confirm the result with more elaboration. The obtained evidence indicated that despite marked suppression of glucokinase in alloxan diabetic rats,<sup>23)-27)</sup> the liver glycogen level appeared to be affected by the blood glucose level.

To get further understanding on the interrelationships between the liver glycogen and the carbohydrate metabolism in alloxan diabetic rats, conversion of [ $2\text{-}^{14}\text{C}$ ] lactate and [ $\text{U-}^{14}\text{C}$ ] glucose was further investigated. The results are shown in Tables 2–5. In the

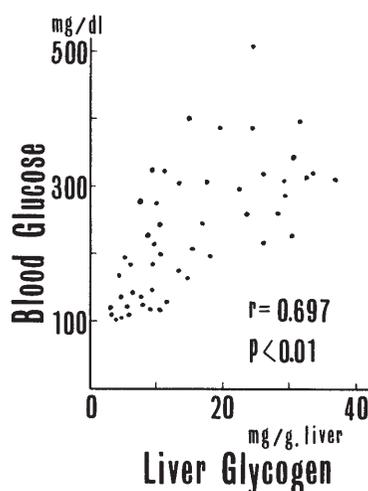


Fig. 1. Correlation between blood glucose levels and liver glycogen concentrations of alloxan diabetic rats fasted for 48 hours.

Table 2. Effects of alloxan on the incorporation of [2-<sup>14</sup>C] lactate into blood glucose and liver glycogen

Treatment <sup>a</sup>	Blood Glucose	<sup>14</sup> C-Glucose in Blood	Liver Glycogen	<sup>14</sup> C in Liver Glycogen
	mg/dl	cpm/ml	mg/g liver	cpm/g liver
Control (4)	80 ± 3 <sup>b,c</sup>	17700 ± 2800 <sup>c</sup>	2.4 ± 0.9 <sup>c</sup>	9641 ± 4143 <sup>c</sup>
Alloxan (4)	324 ± 91	33600 ± 2100	20.6 ± 3.4	3296 ± 1490

a: 7 days after administration of alloxan, 5  $\mu$ Ci of [2-<sup>14</sup>C] lactate was injected intraperitoneally to the rats and one hour later the rats were killed. Experimental animals were fasted for 48 h prior to injection of the isotopic tracer.

b: Each value is the mean  $\pm$  SD for the number of animals shown in parenthesis.

c: Significantly different between the two groups. ( $P < 0.01$ )

experiments shown in Table 2, 5  $\mu$ Ci of [2-<sup>14</sup>C] lactate was injected intraperitoneally after rats were fasted for 48 hours, and one hour later the incorporation into blood glucose and liver glycogen was determined. The incorporation of [2-<sup>14</sup>C] lactate into liver glycogen was less in alloxan diabetic rats despite the higher liver glycogen contents. Since plasma lactate did not increase in alloxan diabetic rats, (Alloxan;  $1.35 \pm 0.21$ , Control;  $1.36 \pm 0.13 \mu$  moles/ml), the present results suggest that formation of glycogen from lactate would not be increased. On the other hand, radioactivity incorporated into blood glucose was greater in alloxan diabetic rats, and another possibility was presented that glucose formation from lactate might be stimulated but glycogen synthesis would be suppressed by some other mechanism. However, the results presented hereafter suggest that formation of glucose or glycolytic intermediates from lactate may not be stimulated in alloxan diabetic rats. First, the ratio, <sup>14</sup>C-glucose in the blood/blood glucose, was markedly lower in alloxan diabetic rats. Furthermore, as shown in Figure 2, the disappearance rate of [U-<sup>14</sup>C] glucose from the blood was significantly slower in alloxan diabetic rats compared with the control rats. Therefore, the higher radioactivity in blood glucose of alloxanized rats was presumed to be due to the slower disappearance rate of <sup>14</sup>C-glucose from the blood stream. In addition to a suppressed incorporation of [2-<sup>14</sup>C] lactate into liver glycogen, the incorporation into liver lipid also decreased in alloxan diabetic rats. As shown in Table 3, in alloxan diabetic rats, the incorporation into both liver lipid and glyceride-glycerol fractions was suppressed one hour after administration of [2-<sup>14</sup>C] lactate.

Table 3. Effects of alloxan on the incorporation of [2-<sup>14</sup>C] lactate into liver lipid

Treatment <sup>a</sup>	Blood Glucose	Liver Lipid	
		<sup>14</sup> C in Total Lipid	<sup>14</sup> C in Glyceride-Glycerol
	mg/dl	cpm/g liver	cpm/g liver
Control (4)	78 ± 10 <sup>b,c</sup>	13700 ± 2600 <sup>c</sup>	13500 ± 2600 <sup>c</sup>
Alloxan (4)	391 ± 41	8400 ± 1600	8300 ± 1700

a: Experimental procedures were the same as in Table 2.

b: Each value is the mean  $\pm$  SD for the number of animals shown in parenthesis.

c: Significantly different between the two groups. ( $P < 0.01$ )

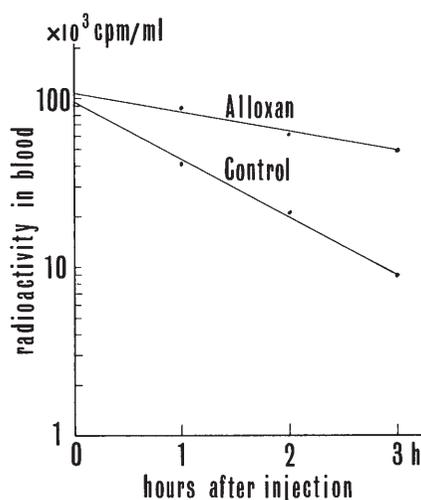


Fig. 2. Disappearance of [U-<sup>14</sup>C] Glucose from blood  
4  $\mu$ Ci of [U-<sup>14</sup>C] glucose was injected intraperitoneally to both alloxan diabetic and control rats fasted for 48 hours, and the blood was collected from tail vein 1, 2 and 3 hours later. Before administration of tracer, blood glucose level of alloxan diabetic rat was 376 mg/dl and that of control rat was 78 mg/dl.

As to the utilization of glucose, Table 4 shows the incorporation of [U-<sup>14</sup>C] glucose into liver glycogen. When the blood glucose level was high, the disappearance rate of [U-<sup>14</sup>C] glucose from the blood was slow and the incorporation of [U-<sup>14</sup>C] glucose into liver glycogen was apparently suppressed in alloxan diabetic rats. However, since blood glucose level was different between the two groups, the amount of blood glucose incorporated into liver glycogen was determined as follows: i.e., radioactivity in the blood soon after administration of [U-<sup>14</sup>C] glucose was determined from Figure 2.<sup>28)</sup> Then the following calculation was made.

Table 4. Effects of alloxan on the disappearance of [U-<sup>14</sup>C] glucose from the blood and its incorporation into liver glycogen

Treatment <sup>a</sup>	Time after injection of [U- <sup>14</sup> C] Glucose	Blood Glucose	<sup>14</sup> C-Glucose	<sup>14</sup> C in Liver
			Remaining in Blood	Glycogen
	min	mg/dl	cpm/ml	cpm/g liver
Control (4)	30	92 ± 7 <sup>b,c</sup>	62900 ± 4700	1560 ± 900
Alloxan (4)	30	241 ± 70	99400 ± 14800	930 ± 510
Control (4)	180	87 ± 5 <sup>c</sup>	9000 ± 1100 <sup>c</sup>	9230 ± 3090
Alloxan (4)	180	416 ± 2	66300 ± 6700	5520 ± 2930

a: Pretreatments of experimental animals were the same as in Table 2. 4  $\mu$ Ci of [U-<sup>14</sup>C] glucose was injected intraperitoneally and at the designated time animals were killed.

b: Each value is the mean ± SD for the number of animals shown in parenthesis.

c: Significantly different between the two groups. (P < 0.01)

$\frac{\text{blood glucose}}{\text{cpm in blood glucose at zero time}} \times \text{cpm in liver glycogen after 30 min or 3 h}$   
 respectively.  
 = amount of blood glucose incorporated into liver glycogen at 30 min or 3 h afterwards.

As a result, 30 minute after the administration, the ratio of the diabetic to the normal rats was about 3 : 2 and after 3 hours about 5 : 2. In accord with high liver glycogen level, the amount of glucose incorporated into liver glycogen was larger in the diabetic rat compared with the control rat. Thus, the much higher blood glucose level in the diabetic rat may contribute to maintain a higher liver glycogen level. Table 5 shows the incorporation of [U-<sup>14</sup>C] glucose into liver lipid. The radioactivity incorporated into glyceride-glycerol in the diabetic rat liver was slightly lower than that of the control. However, correction in regard to the blood glucose level was also made according to the similar calculation used for the incorporation into liver glycogen, and the actual amount of glucose incorporated into glyceride-glycerol was found to be larger in the diabetic rat compared with the control.

Table 5. Effects of alloxan on the incorporation of [U-<sup>14</sup>C] glucose into liver lipid

Treatment <sup>a</sup>	Time after injection of [U- <sup>14</sup> C] glucose min	Blood Glucose mg/dl	Radioactivity incorporated into	
			Total Lipid	Glyceride-Glycerol
			cpm/g liver	
Control (4)	30	80 ± 6 <sup>b,c</sup>	4700 ± 400 <sup>c</sup>	4100 ± 300 <sup>c</sup>
Alloxan (4)	30	270 ± 40	2000 ± 300	1900 ± 250
Control (4)	180	77 ± 5 <sup>c</sup>	8800 ± 600	8200 ± 740 <sup>c</sup>
Alloxan (4)	180	380 ± 60	4500 ± 450	4100 ± 280

a: Experimental procedures were the same as in Table 4.

b: Each value is the mean ± SD for the number of animals shown in parenthesis.

c: Significantly different between the two groups. (P < 0.01)

## DISCUSSION

Although almost all the liver glycogen of the normal rats disappeared after only 24 hours of fasting, rather large amount of liver glycogen was reserved in alloxan diabetic rats even when they were fasted for 48 hours. The amount of the remaining liver glycogen was affected by the blood glucose level. The present results suggest that the slower disappearance of liver glycogen in fasted alloxan diabetic rats may be attributed to their hyperglycemia. On the problem of gluconeogenesis of the 48 hours-fasted diabetic rats, as described in the 'result' section of this paper, no evidence of enhanced gluconeogenesis compared to the normal rats was obtained.

Several factors which suppress liver glycogen by fasting may be pointed out; First one of them is the decrease of the blood glucose which may be followed by marked suppression of glucokinase. Secondly, the suppression of glycogen synthetase together with the stimulation of phosphorylase may be related to the suppression of liver glycogen. As glucokinase activity in alloxan diabetic rats has been reported to be suppressed markedly,<sup>23)-27)</sup> the supply of the precursors to form glycogen through conversion of glucose to glucose-6-phosphate by glucokinase is presumed to be negligibly small in alloxan-treated rats. However, fasted alloxan diabetic rats had considerable amounts of the liver glycogen when their blood glucose remained higher. (See Figure 1 and Table 1) Then such a

relatively high liver glycogen must be derived from other ways than glucokinase. The enhanced rate of gluconeogenesis in alloxan diabetic rats has been suggested by some investigators.<sup>6), 7), 10), 11)</sup> Also according to our unpublished data, more glucose was formed from lactate in the diabetic fed rats than in the normal fed rats. These results may indicate that the high liver glycogen of fasted alloxan diabetic rats might be dependent upon enhanced gluconeogenesis. However, the present results suggest that gluconeogenesis in alloxan diabetic rats is not stimulated under the fasted state compared with that in the control. As has already been mentioned in the 'result' section, when [2-<sup>14</sup>C] lactate was administered, higher radioactivity was found in the blood of alloxan diabetic rats, whereas less radioactivity was found in the liver glycogen. Although these results might suggest stimulated formation of glucose in fasted alloxan diabetic rats, this seemed unlikely, because of the following results. Namely, as seen in Figure 2 and Table 4, the disappearance rate of [U-<sup>14</sup>C] glucose from the blood stream was slower in alloxan-treated rats which showed hyperglycemia. This may well lead to the following conclusion. Namely, from comparison between [2-<sup>14</sup>C] lactate incorporation into blood glucose and disappearance rate of [U-<sup>14</sup>C] glucose from the blood, the amount of <sup>14</sup>C-glucose formed from <sup>14</sup>C-lactate would not be larger than in the control rats, while radioactivity in the blood was higher. The ratio, <sup>14</sup>C-glucose/blood glucose, was lower in the alloxan group than in the control. Since blood lactate was not increased in diabetic rats under this experimental condition, dilution of [2-<sup>14</sup>C] lactate with endogenous lactate may be discounted. Moreover, radioactivity incorporated into liver glyceride-glycerol from [2-<sup>14</sup>C] lactate was lower in diabetic rats (Table 3). In addition to the results of <sup>14</sup>C-lactate administration, gluconeogenesis from amino acids would not be increased under this experimental condition, since plasma insulin level was not so different between the two groups that it affected the supply of amino acids as gluconeogenic precursor. (unpublished data)

On the other hand the amount of blood glucose incorporated into liver glycogen and glyceride-glycerol may be larger in alloxanized rats, when incorporation of [U-<sup>14</sup>C] glucose was corrected by values of blood glucose. (Table 4 and 5) These results suggest that the glycolytic flux in the liver may be relatively larger in alloxan-treated rats after long terms of fasting, if they had hyperglycemia. However, even in such alloxan diabetic rats, blood glucose sometimes decreased to as low as intact rats after 48 hours of fasting. In such rats the liver glycogen also decreased to almost control values. In most fasted alloxan-treated rats used here, hyperglycemia was still maintained after long terms of fasting and the amount of blood glucose incorporated into liver glycogen was larger as compared with the control rats.

Concerning the correlation between the blood glucose level and the liver glycogen content in the fasted alloxan diabetic rats, some differences were found between the present result and an other report which was performed after 24 hours fasting<sup>4)</sup>. However, this may be prescribed to the differences in the experimental condition; namely, in this study rats were fasted for 48 hours to completely remove the effect of the feeding before experiments. Since Friedmann *et al.*<sup>4)</sup> already reported that the injection of glucose to the fasted diabetic rats led to a prompt synthesis of liver glycogen, their result was not essentially contradictory to our experiment.

Nordlie *et al.*<sup>29)-31)</sup> reported that glucose-6-phosphatase is a multifunctional enzyme capable not only of degrading glucose-6-phosphate, but also synthesizing this important metabolic intermediate via inorganic pyrophosphate(PPi)-glucose phosphotransferase activity. Furthermore, this activity increases significantly in diabetes and is further favored

by hyperglycemia. Weinhouse and coworkers<sup>32)</sup> suggested that the glucokinase activity is insufficient to account for the phosphorylation of administered glucose in the liver of glucagon-treated alloxan diabetic rat and that PPI-glucose phosphotransferase activity may be involved in this process.

One aspect of the functions of glucose-6-phosphatase favourably presents a key to understand such a tightly interrelated causality between hyperglycemia and slower disappearance of liver glycogen in fasted alloxan diabetic rats. Findings and interpretation obtained so far may well lead to the conclusion that the slower disappearance of liver glycogen by fasting in alloxan diabetic rats would be dependent upon their hyperglycemia, however, not dependent upon enhanced gluconeogenesis.

### CONCLUSION

In an attempt to determine the origin of high liver glycogen level in fasted diabetic rats, this study was made, using [2-<sup>14</sup>C] lactate and [U-<sup>14</sup>C] glucose. It may be concluded that the high liver glycogen level in fasted diabetic rats is due to their hyperglycemia, and not to their enhanced gluconeogenesis.

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