GLUCURONIC ACID PATHWAY IN ALLOXAN DIABETIC RABBITS

(I) URINARY EXCRETION OF METABOLITES RELATED TO THE GLUCURONIC ACID PATHWAY

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Abstract—Studies on the activity of the glucuronic acid pathway in alloxan diabetic rabbits were carried out. Amount of D-glucaric acid, L-ascorbic acid, and D-glucuronic acid in urine increased in the case of the alloxan diabetic rabbits. The transformation from D-glucuronolactone to D-glucaric acid was higher than normal in the diabetic animals. The expired 14CO2 decreased and urinary excretion of labeled L-gulonic acid increased after administration of 6-14C-glucuronolactone in the diabetic rabbits. L-Gulonic acid dehydrogenase, lactonase II, and β-glucuronidase activities were reduced, and UDPGA-pyrophosphatase, D-glucuronic acid-l-phosphatase, and UDPGA-transferase activities increased in the diabetic rabbit liver. From these results, it may be concluded that an increase of endogenous D-glucuronic acid in the diabetic states could be attributed to a metabolic defect in the step of L-gulonic acid dehydrogenation and to the enhancement of UDPGA-pyrophosphatase and D-glucuronic acid-l-phosphate phosphatase activities.

It has been reported that the serum level of L-xylulose (1) and D-glucaric acid (2) and the urinary excretion of D-glucuronic acid (3, 4) and D-glucaric acid (5) were markedly increased in diabetic patients, and that the relative contribution of the glucuronic acid pathway to D-glucose catabolism as shown in Fig. 2 was greater in adipose tissue from alloxan diabetic rats than from normal ones (6, 7). These results suggest that the activity of the D-glucuronic acid–L-xylulose route is enhanced in diabetes. However these results are contrary to the findings that the activity of the glucuronic acid pathway was reduced in the diabetic liver in the two enzymatic steps, dehydrogenation of UDPG (Uridine diphosphate glucose) and the pathway from D-glucuronic acid to L-xylulose (8, 9). Thus the activity of this pathway in diabetes is still uncertain. In the present study, the authors attempted to elucidate the dynamic aspects of the glucuronic acid pathway in alloxan diabetic rabbits.

MATERIALS AND METHODS

D-Glucaric acid, D-glucuronic acid and D-glucuronolactone were synthesized by the authors. NAD, NADH, NADP, NADPH, UDPG, and UDPGA (Uridine diphosphate glucuronic acid) were obtained from Sigma Chemical Co. Alloxan monohydrate was obtained from Wako Pure Chemical Co. 6-14C-Glucuronic acid was obtained from Radio
Chemical Center and 6-^{14}C-glucurononolactone was prepared according to the following method. 6-^{14}C-Potassium D-glucuronate (284 μmole, 15.5 μCi/mg) was diluted with 100 μmole of sodium D-glucuronate and dissolved in water. The solution was passed through a column of Amberlite CG-120 to convert to free acid and the column was washed with 3 ml of water containing 100 μmole of sodium D-glucuronate. The eluted solution was evaporated to dryness in vacuo at 50°C. Glacial acetic acid and unlabeled D-glucurononolactone were added. The acidic solution was boiled at 100°C for 30 min. After cooling, the crystals obtained had a specific activity of 0.74 μCi/mg.

Experimental animals

Healthy male albino rabbits bred in our laboratories, weighing 2.8 to 3.2 kg, were injected i.v. with alloxan monohydrate (100 mg/kg body weight) dissolved in Ringer’s solution. These animals were fed 150 g of RC-5 (Oriental Yest Co., Ltd., Tokyo) and 100 g of carrots a day. The onset of diabetes was determined by measuring the increase in blood glucose level and urinary glucose contents, and the loss in body weight and by checking for polyuria and polydipsia. The animals were not employed until at least 4 weeks after the injection of alloxan and only if the fasting blood glucose level exceeded 250 mg per 100 ml of blood.

Protamine-Zinc-Insulin was injected s.c. in a dosage of 5 unit per 1.0 g urine glucose per day, and the animal was used for studies at least 16 hours later.

Collection of expired ^{14}CO_{2}

Each animal was immediately placed in a metabolic chamber which was designed to permit periodic collection of expired carbon dioxide in monoethanolamine.

Measurement of radioactivity

The expired ^{14}CO_{2} was assayed for radioactivity in the form of ethanolamine carbonate. The scintillator solution contained 4 g PPO, 0.2 g POPOP, 600 ml 2-methoxyethanol, and 300 ml of toluene. Two ml of ethanolamine carbonate, 0.1 ml of urine, or 0.1 ml of the elute from Dowex 1 × 8 (borate type) column were added respectively to each vial containing 12 ml scintillator solution and the radioactivity was measured in Nuclear Chicago Mark I liquid scintillation counter.

Separation of urinary metabolites

The metabolites were applied on a 1.0 × 4.5 cm column of Dowex 1 × 8 resin. Dowex 1 × 8 (borate type) was prepared from Dowex 1 × 8 (chloride type) by the method of Ishidate (10). The metabolites of D-glucuronic acid were separated by the method of Fujita (11).

Determination of the urinary metabolites

Separated D-glucuronic acid was determined by naphtoresolcinol reaction (12). D-Glucaric acid was determined by the method of Ishidate (10). Ketose was isolated and determined by cysteine-carbazole-H_{2}SO_{4} reaction (13) and the method of Kulka (14).

Preparation of tissue for enzyme measurements

For the measurement of enzymatic activity 25% homogenate was prepared from rabbit liver. The homogenizing medium was ice-cold 0.154 M KCl solution. The homogenate was centrifuged for 20 min at 6,000 × g to remove all nuclei and cell debris. The
supernatant thus obtained was subjected to ultracentrifugation at 105,000 × g for 90 min. The supernatant fluid was used for assay of D-glucuronolactone-, L-gulonic acid-, NADP-L-hexonate-, and UDPG-dehydrogenase and lactonase II activities. The sediment was washed with 0.154 M KCl and centrifuged again as above. The microsomal fractions finally were suspended in 0.154 M KCl and used for assay of UDPGA-transferase.

The supernatant fraction collected after centrifugation at 6,000 × g from 25% homogenate in 0.154 M KCl and 1.0% homogenate in distilled water assayed for UDPGA-pyrophosphatase and β-glucuronidase activities, respectively. All subsequent steps were carried out at 0-4°C.

Enzyme assays

The following enzyme activities were measured by the methods as indicated in the references: D-Glucuronolactone dehydrogenase (15), L-gulonic acid dehydrogenase (16), UDPG-dehydrogenase (17), NADP-L-hexonate dehydrogenase (18), UDPGA-pyrophosphatase (19), D-glucuronic acid-1-phosphate phosphatase (20), β-glucuronidase (21), and lactonase II (22). UDPGA-transferase was assayed by a modified method of Isselbacher (23). In the UDPGA-transferase reaction mixture, 5 μmole of D-glucaro-1, 4-lactone was added as the inhibitor of β-glucuronidase contained in this enzyme. Serum β-glucuronidase was assayed according to Shioya (24). All enzymes were assayed at 37°C.

Definition of unit and specific activity

D-Glucuronolactone-, L-gulonic acid-, UDPG-, and NADP-L-hexonate dehydrogenase activities were expressed as μmole of produced NADH or NADPH per min per mg of protein. UDPGA-transferase activity was expressed as μmole of conjugated p-nitrophenol per 30 min per mg of protein. D-Glucuronic acid-1-phosphate phosphatase activity was expressed as μmole of released inorganic phosphate per hr per mg of protein.

UDPGA-pyrophosphatase activity was expressed as μmole of released D-glucuronic acid per hr per mg of protein. β-Glucuronidase activity was expressed as μg of released p-nitrophenol per hr per mg of protein. Lactonase II activity was expressed as μmole of decreased L-gulonolactone per 10 min per mg of protein.

Blood and urinary sugar

Blood glucose level was determined by the method of Hagedorn and Jenson (25), and glucose oxidase method (26). Urinary glucose content was determined by the method of Fujii (27).

Determination of protein

Protein was determined by the method of Warburg and Christian (28), and by the biuret method (29).

RESULTS

Urinary excretion of D-glucuronic acid, D-glucaric acid, and L-ascorbic acid

The results in normal and diabetic rabbits are summarized in Table 1. The amount of urinary excretion of D-glucuronic acid, D-glucaric acid, and L-ascorbic acid was higher in the diabetic animals than in the normal ones, respectively in the 24-hr urine.
Conversion to D-glucaric acid from D-glucuronolactone

The transformation from D-glucuronolactone to D-glucaric acid in normal and diabetic rabbits was studied following both oral and intravenous administration of the lactone. Table 2 shows that the conversion rate of administered D-glucuronolactone to D-glucaric acid was higher in the diabetic animals than normal.

Table 2. Conversion percentage to D-glucaric acid after the administration of 500 mg/kg D-glucuronolactone in normal and alloxan diabetic rabbits.

<table>
<thead>
<tr>
<th>Conversion percentage (%)</th>
<th>Normal</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally</td>
<td>4.4±1.9 (9)</td>
<td>7.9±1.7 (12)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Intravenously</td>
<td>1.4±0.5 (9)</td>
<td>3.7±0.3 (7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results are given as means±S.D.
Figures in parentheses indicate number of expts.

a) Calculated as follows:
conversion (%) = treated value−control value × 100
              dose (mg) of D-glucuronolactone 1.2

b) molecular weight of D-glucaric acid
molecular weight of D-glucuronolactone ×1.2

Table 3. Urinary and respiratory excretion of radioactivity after oral administration of 5 μCi/500 mg/kg 6-14C-glucuronolactone in normal and alloxan diabetic rabbits.

<table>
<thead>
<tr>
<th>Excretion percentage (%)</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Diabetic Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expired 14CO₂</td>
<td>62.3±3.6 (5)</td>
<td>32.1±3.1 (5)*</td>
<td>50.4±2.7 (4)**</td>
</tr>
<tr>
<td>Urine</td>
<td>28.7±3.3 (5)</td>
<td>46.1±5.6 (5)</td>
<td>27.5±3.3 (4)**</td>
</tr>
</tbody>
</table>

Results are given as means±S.D.
Figures in parentheses indicate number of expts.

a) Samples were collected 24-hr following the administration. Values are expressed as % of dose of lactone.

b) Diabetic rabbits were given 5 units of protamine-Zn insulin per 1.0 g urine sugar per day s.c. 16-hr prior to experiment.

* Differs significantly from normal; P<0.001

** Differs significantly from diabetic; P<0.001
Fig. 1 Fractionation of metabolites in normal (A) and alloxan diabetic (B) rabbit urine after oral administration of 5 µCi,500 mg/kg 6-14C-glucuronolactone by Dowex 1×8 (borate) column.

Urine samples were collected 24-hr following the administration. The effluent was collected in 10 ml fractions and analyzed by measuring radioactivity (-----), naphthoresorcinol reaction (---), periodate and phenylhydrazine reaction (----), cysteine-carbazole reaction (-----), and glucose oxidase reaction (-----), respectively. I, II, III, IV and V correspond to D-glucose and D-fructose, L-gulonic acid, D-glucuronic acid, D-glucaric acid, and D-gluconic acid, respectively.
Excretion of radioactivity after the administration of 6-[^14]C-glucuronolactone

The excretion pattern (urine and expired air) has been studied following both oral and intravenous administration of 6-[^14]C-glucuronolactone. In Table 3, it is shown that the expired 14C02 was significantly decreased in the diabetic rabbits, and the decrease was nearly returned to normal level as a result of insulin treatment.

The radioactivity of the urine was increased in the diabetic animals, and it was returned to the normal level by insulin treatment.

Fractionation of the urine by Dowex 1 × 8 resin column

Analyses of the radioactivity excreted in the urine after the oral administration of 6-[^14]C-glucuronolactone are shown in Fig. 1.

In Fig. 1, peak (I), (II), (III), (IV) and (V) correspond to D-glucose and D-fructose, L-gulonic acid, D-glucuronic acid, D-glucaric acid, and D-gluconic acid, respectively. In the normal and diabetic rabbit, radioactivity was observed in the peak of (III) and (IV), and (II), (III) and (IV), respectively.

The results of the fractionation by Dowex column are summarized in Table 4. Increased radioactivity was observed in peak (II) in the diabetic animals. Insulin was effective in decreasing the increased L-gulonic acid (II) in the diabetic rabbits. In contrast to L-gulonic acid (II), in the urine from diabetic rabbits D-glucaric acid was increased above the level of normal by treatment of insulin.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Excretion percentage (%)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td>2.8±1.0(5)</td>
<td>22.3±5.6(5)</td>
<td>4.8±0.4(5)</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td>3.2±0.4(5)</td>
<td>8.1±1.0(5)*</td>
<td>24.9±6.1(5)</td>
<td>5.6±0.3(5)**</td>
</tr>
<tr>
<td>Diabetic+Insulin</td>
<td></td>
<td>3.3±0.4(4)</td>
<td>3.0±0.8(4)**</td>
<td>13.8±4.6(4)</td>
<td>7.2±0.6(4)***</td>
</tr>
</tbody>
</table>

Results are given as means±S.D.
Figures in parentheses indicate number of expts.
I, II, III and IV correspond to D-glucose and D-fructose, L-gulonic acid, D-glucuronic acid, and D-glucaric acid, respectively.

* Differs significantly from normal ; P<0.001
** Differs significantly from normal ; P<0.01
*** Differs significantly from diabetic ; P<0.001
**** Differs significantly from diabetic ; P<0.01

Enzyme activity

Tables 5 and 6 show the activities of various hepatic enzymes, in both diabetic and control rabbits. The activity of UDPGA-transferase, UDPGA-pyrophosphatase and D-glucuronic acid-1-phosphate phosphatase was remarkably elevated in the diabetic rab-
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TABLE 5. Activities of several D-glucuronic acid synthesizing enzymes in the liver of diabetic rabbit.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Enzyme activity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Diabetic</td>
</tr>
<tr>
<td>UDPG-dehydrogenase</td>
<td>2.70±0.19 (4)</td>
<td>1.16±0.19 (5)</td>
</tr>
<tr>
<td>UDPGA-transferase</td>
<td>0.28±0.24 (4)</td>
<td>0.71±0.19 (4)</td>
</tr>
<tr>
<td>$\beta$-Glucuronidase</td>
<td>68.84±8.09 (6)</td>
<td>49.35±7.10 (9)</td>
</tr>
<tr>
<td>UDPGA-pyrophosphatase</td>
<td>1.28±0.11 (6)</td>
<td>2.67±0.15 (6)</td>
</tr>
<tr>
<td>D-Glucuronic acid-I-phosphate phosphatase</td>
<td>10.41±0.47 (6)</td>
<td>24.27±0.82 (6)</td>
</tr>
</tbody>
</table>

Results are given as means±S.D.
Figures in parentheses indicate number of expts.

a) pmole/min/mg-protein, b) p-Nitrophenol (mg)/30 min/mg-protein,
c) p-Nitrophenol (mg)/hr/mg-protein, d) pmole/hr/mg-protein.

TABLE 6. Activities of several D-glucuronic acid metabolizing enzymes in the liver of diabetic rabbits.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Enzyme activity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Lactonase II</td>
<td>1.56±0.57 (4)</td>
<td>0.57±0.24 (4)</td>
</tr>
<tr>
<td>D-Glucuronolactone dehydrogenase</td>
<td>1.02±0.18 (4)</td>
<td>0.98±0.18 (5)</td>
</tr>
<tr>
<td>NADP-L-Hexonate dehydrogenase</td>
<td>3.01±0.28 (4)</td>
<td>1.96±0.19 (5)</td>
</tr>
<tr>
<td>L-Galonic acid dehydrogenase</td>
<td>3.80±0.04 (4)</td>
<td>1.15±0.19 (5)</td>
</tr>
</tbody>
</table>

Results are given as means±S.D.
Figures in parentheses indicate number of expts.
a) pmole/10 min/mg-protein, b) pmole/min/mg-protein.

bits. Conversely, the activity of L-galonic acid dehydrogenase, lactonase II, $\beta$-glucuronidase, and UDPG-dehydrogenase was remarkably reduced in the diabetic rabbits.

D-Glucuronolactone dehydrogenase activity was unchanged. NADP-L-Hexonate dehydrogenase activity was reduced in the diabetic rabbits.

DISCUSSION
The increase in the excretion of both D-glucaric acid and L-ascorbic acid provides evidence for the accumulation of endogenous D-glucuronic acid in diabetic rabbits. Endogenous D-glucuronic acid could be formed in the living body, via the direct hydrolysis of UDPGA by UDPGA-pyrophosphatase (30), the hydrolysis of glucuronide by $\beta$-glucuronidase (31), the oxidation of myo-inositol (32), and the hydrolysis of mucopolysaccharides (33, 34). The possible role of the $\beta$-glucuronidase route in the release of D-glucuronic acid from UDPGA has been the subject of many discussions (31, 35, 36). In these discussions, the increased excretion of endogenous D-glucuronic acid found in their experiments was explained by increased hepatic $\beta$-glucuronidase activity. However, in our experiments, hepatic $\beta$-glucuronidase activity was markedly reduced in the diabetic animals. The fact that the hepatic $\beta$-glucuronidase activity was significantly reduced
in the diabetic animals does not explain why such a large amount of endogenous D-glucuronic acid was excreted in the urine of the diabetic animals.

On the contrary, the levels of both UDPGA-pyrophosphatase and D-glucuronic acid-1-phosphate phosphatase activities were enhanced in the diabetic animals. Hence the pyrophosphatase route may play the main role in the formation of D-glucuronic acid for the synthesis of D-glucaric acid and L-ascorbic acid in the diabetic rabbits.

On the other hand, a marked reduction of D-glucuronic acid catabolism through L-gulonic acid may result in the accumulation of endogenous D-glucuronic acid in the diabetic rabbits. The increased excretion of both D-glucaric acid and L-ascorbic acid in the diabetic animals may reflect the elevation of biosynthesis of those precursor D-glucuronic acid.

The present study indicates that the urinary excretion of D-glucuronic acid, D-glucaric acid and L-ascorbic acid was considerably increased in the diabetic rabbit. The results in the excretion of D-glucuronic acid and D-glucaric acid are in agreement with the observation of other investigators (3–5), however, the findings in L-ascorbic acid excretion differ from the evidence given by Stirpe (37) for an impaired synthesis of L-ascorbic acid in the liver of alloxan diabetic animals.

Burns (38) reported that the administration of 6-14C-glucuronolactone increased urinary excretion in the normal rat and guinea pig by 2–3% of the dose. In the present investigation, urinary labeled L-gulonic acid was significantly increased when 6-14C-glucuronolactone was administered to the diabetic rabbits. Furthermore, intravenous administration of 6-14C-glucuronolactone resulted in a greater increase of radioactive L-gulonic acid (II) as compared with the oral dose. (data not included). In these experiments, there was a marked decrease in the expired 14CO2 from the diabetic rabbits after oral administration of 6-14C-glucuronolactone. These results indicate that decarboxylation of L-gulonic acid to L-xylulose was depressed as a result of the decreased L-gulonic acid dehydrogenase activity in the diabetic animals.

The enzymatic dehydrogenation of D-glucuronolactone in the diabetic rabbit liver remained unchanged (Table 6), while the conversion rate to D-glucaric acid increased in the diabetic rabbit (Table 2).

This indicates that an impairment in catabolism of D-glucuronic acid may occur in the diabetic liver.

Injection of insulin to the diabetic rabbits elevated above normal the level of radioactivity in D-glucaric acid (IV) after oral administration of 6-14C-glucuronolactone and this level of radioactivity remained elevated in the case of intravenous administration of the lactone also. (data not included) Our experiments do not completely rule out the possibility that D-glucuronolactone is also dehydrogenated by an enzyme in the intestine, or that insulin does effect the dehydrogenase activity. Additional experiments to clarify this point are underway.

It has been shown that the metabolism of D-glucose via the glucuronic acid pathway in diabetes mellitus is more active than that in normal (1–7), whereas our data showed that
the activity of the glucuronic acid pathway decreased in the alloxan diabetic rabbit. It appears that principally the decreased activity of the glucuronic acid pathway in the diabetic rabbits is due to the reduced activity of L-gulonic acid dehydrogenase. Our experiments demonstrated that as a result of reduced L-gulonic acid dehydrogenase activity in the diabetic animals, endogenous D-glucuronic acid increases. This is accompanied by an increase in the synthesis of D-glucaric acid and L-ascorbic acid. Also the reduced L-gulonic acid dehydrogenase activity produces an increase of L-gulonic acid excretion and reduced decarboxylation of 6-14C-glucuronolactone in the diabetic rabbit.

In conclusion, certain metabolic abnormalities in the alloxan diabetic rabbits can be, at least in part, attributed to a defect in the step of L-gulonic acid dehydrogenation and to the enhancement of UDPGA-pyrophosphatase and D-glucuronic acid-1-phosphate phosphatase activity (Fig. 2).

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