

# Effects of Endotoxin on Gluconeogenesis, Glycogen Synthesis, and Liver Glycogen Synthase in Mice

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This study was undertaken to characterize the nature of carbohydrate loss due to endotoxin poisoning in mice and to elucidate mechanisms responsible for the changes. Female ICR mice, fasted overnight, were injected intraperitoneally with a mean lethal dose of endotoxin extracted from *Salmonella typhimurium* strain SR-11. Liver glycogen levels, alanine- $U\text{-}^{14}\text{C}$  and pyruvate- $2\text{-}^{14}\text{C}$  incorporation into blood glucose and liver glycogen, glucose- $U\text{-}^{14}\text{C}$  incorporation into liver glycogen, and liver glycogen synthase activities were measured at intervals after treatment. Liver glycogen in fasted mice given endotoxin was diminished significantly as early as 1 h after treatment. Liver glycogen synthase was significantly decreased in poisoned mice at 17 h. The use of actinomycin D showed that the induction of this enzyme due to fasting or hydrocortisone, or both, was inhibited by endotoxin. The incorporation of the  $^{14}\text{C}$ -label from alanine- $U\text{-}^{14}\text{C}$ , pyruvate- $2\text{-}^{14}\text{C}$ , or glucose- $U\text{-}^{14}\text{C}$  into blood glucose and liver glycogen was substantially impaired in endotoxemic animals at 12 h. Decreases in incorporation occurred as early as 4 h after treatment. The progressive increase in glycogen synthase activity observed in fasted controls was not seen in endotoxin-poisoned mice. The administration of a glucose or pyruvate load to endotoxin-treated mice did not restore gluconeogenesis, glycogen synthesis, or liver glycogen synthase activity to normal levels. The *in vivo* activation of glycogen synthase by glucose was significantly reduced in endotoxemic animals. These changes indicate reduced carbohydrate synthesis as a probable cause for rapid sugar loss during endotoxemia in mice.

Since the pioneering observations of Menten and Mannig (26) and Zeckwer and Goodell (41), bacterial endotoxins have been known to deplete an animal of its carbohydrate reserves. These observations have been confirmed in a number of laboratories but without a valid explanation for their underlying causes. Kun (17, 18) was the first to find that endotoxin prevents the conversion of glucose to glycogen in rats as well as the entry of sugar into the isolated diaphragm. The studies of Berry et al. (4) demonstrated that injection of a lethal dose of killed *Salmonella typhimurium* into mice results in the reduction of liver glycogen and almost complete loss in total body carbohydrate. Cortisone not only protected mice against the lethality of endotoxin but also prevented the total loss of glycogen.

Further studies by Berry and Smythe (2) demonstrated that, when endotoxin was given to mice concurrently with cortisone, the amount of

protein catabolized was not significantly different from that found with cortisone alone, whereas the increase in total body carbohydrate was much less. These data suggested that endotoxin interferes with gluconeogenesis. Utilizing a different approach, Shands et al. (32, 33) clearly demonstrated impaired gluconeogenesis as responsible for the decreased conversion of pyruvate- $2\text{-}^{14}\text{C}$  into blood glucose in BCG-infected mice hyperreactive to endotoxin. Moon (28) has shown that severe hypoglycemia accompanies an injection of tryptophan into mice pretreated with endotoxin and suggests that this may be responsible for the convulsive deaths that follow.

There is evidence to indicate that certain enzyme alterations in poisoned mice are responsible for impaired regulation of body carbohydrate. Inhibition of glucocorticoid induction of phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis, has been demonstrated (3) in endotoxin-poisoned mice. Studies by La Noue et al. (19) and Williamson et al. (39), in which rat liver slices and perfused rat liver, respectively,

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were employed, have shown the overall rate of gluconeogenesis to be impaired by *Pseudomonas aeruginosa* infection as well as by *Escherichia coli* endotoxin. The activity of glucose-6-phosphatase was significantly lower in infected and poisoned animals than in controls. More recently, reduced hepatic fructose-1,6-diphosphatase and glucose-6-phosphatase activity in endotoxin-treated mice have been reported by McCallum and Berry (24), although the changes were slower than the rapid liver carbohydrate depletion seen in poisoned animals. For this reason, the role of the glycogen cycle becomes increasingly important in understanding possible mechanisms.

This investigation was undertaken to further characterize alterations in carbohydrate synthesis in mice treated with a mean lethal dose ( $LD_{50}$ ) of endotoxin. It was thought that measurements of liver glycogen levels, the conversion of certain precursors into blood glucose and liver glycogen, as well as determinations of liver glycogen synthase (uridine 5'-diphosphate [UDP]-glycogen  $\alpha$ -4-glucosyltransferase, EC 2.4.1.11) activity in poisoned animals would contribute to an elucidation of mechanisms responsible for the irreversible carbohydrate depletion caused by endotoxin.

#### MATERIALS AND METHODS

**Animals.** Female random-bred, pathogen-free mice (ICR strain, Texas Inbred Mice Co., Houston, Tex.) weighing 18 to 20 g were employed for these studies. Animals were kept in a room with controlled lighting (6:00 a.m. to 6:00 p.m.) and housed 10 per cage in a Health Guard System cage rack (Research Equipment Co., Inc., Byran, Tex.). They were given food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) and water ad libitum. Mice were periodically screened for *Salmonella* contamination by plating suspensions of macerated fecal samples on SS agar plates (Difco) and incubating at 37 C for 24 h.

**Endotoxin.** The endotoxin employed was extracted and purified from *S. typhimurium* strain SR-11 by the phenol-water method described by Nowotny (29). Batch cultures were grown in aerated Brain Heart Infusion broth (Difco) at 37 C for 24 h and harvested by continuous-flow centrifugation (Ivan Sorvall, Inc., Norwalk, Conn.). The cells were washed three times with sterile saline before lyophilization. After extraction, the lipopolysaccharide was further purified by three absolute methanol precipitations and ultracentrifugation at  $105,000 \times g$ . The mouse  $LD_{50}$  of the purified preparation was determined by the method of Reed and Muench (31) using a 48-h end point, and was found to be approximately 235  $\mu$ g per mouse.

**Fasting, injections, and sacrifice.** Since the purpose of this study was to evaluate carbohydrate synthesis in mice, fasted animals were employed. It is well established that mice poisoned with

endotoxin stop eating (1); therefore, fasting partially balances the experimental and control animals. In the experiments in which liver glycogen levels were surveyed for 24 h and the response of glycogen synthase to actinomycin D, hydrocortisone, and endotoxin was followed, food was withdrawn from all animals at 5:00 p.m. At this time injections were made, and 0-h measurements were performed. In all other experiments mice were allowed to fast overnight (5:00 p.m. to 8:00 a.m.) prior to the initial injections and the 0-h measurements at 8:00 a.m. Fasting was continued throughout the course of each experiment.

Mice were injected intraperitoneally (i.p.) with one  $LD_{50}$  (235  $\mu$ g per mouse) of purified lipopolysaccharide suspended in 0.5 ml of sterile non-pyrogenic saline (Baxter Laboratories, Morton Grove, Ill.) at the start of each experiment (0 h). Control animals received 0.5 ml of sterile saline alone. Actinomycin D (Schwarz/Mann, Orangeburg, N.Y.) was injected i.p. at about the  $LD_{50}$  level (20  $\mu$ g per mouse). Hydrocortisone acetate (Sigma Chemical Co., St. Louis, Mo.) was injected (1 mg per mouse) subcutaneously in 0.1 ml of sterile saline immediately prior to endotoxin injection. In experiments concerned with the effect of glucose and pyruvate loads on gluconeogenesis and glycogenesis, 200  $\mu$ mol of glucose (36 mg) or 400  $\mu$ mol of sodium pyruvate (35.2 mg) was injected i.p. in 0.2 ml of saline 30 min before sacrifice.

At appropriate intervals after treatment, groups of 10 mice each were sacrificed by decapitation. The livers were quickly removed, rinsed, blotted dry, weighed, and used immediately for enzyme or metabolite assays. Blood for blood glucose determinations was obtained by the insertion of a 0.1-ml disposable micro-sampling pipette (Scientific Products, Evanston, Ill.) into the ophthalmic venous plexus.

**Measurement of gluconeogenesis and glycogen synthesis.** Mice were fasted overnight and the initial injections were made at 8:00 a.m. (0 h). Liver and blood samples were obtained at the following intervals after treatment: 2 h (10:00 a.m.), 4 h (12:00 p.m.), 6 h (2:00 p.m.), and 12 h (8:00 p.m.). Gluconeogenesis was evaluated on the basis of the incorporation of the  $^{14}C$ -label from alanine- $U$ - $^{14}C$  (specific activity = 0.05 mCi/mmol) and sodium pyruvate- $2$ - $^{14}C$  (specific activity = 0.025 mCi/mmol) into blood glucose 30 min after the i.p. injection of 1  $\mu$ Ci per animal. Glycogen synthesis was measured by the incorporation of the  $^{14}C$ -label of glucose- $U$ - $^{14}C$  (1  $\mu$ Ci per 20  $\mu$ mol) as well as labeled alanine and pyruvate into liver glycogen 30 min after i.p. injection.

For evaluation of gluconeogenesis, paired blood samples were obtained from each mouse. One of the paired samples was assayed for blood glucose, and the other sample was mixed with an equal volume of 10% (wt/vol) trichloroacetic acid and centrifuged to yield a protein-free supernatant fluid which was used for glucose estimation. With a method similar to that of Shands et al. (33), a 0.05-ml sample of the protein-free supernatant

fluid was placed on two microcolumns (0.4 by 5 cm) connected in series consisting of AG-50W-X8 cation exchange resin (200 to 400 mesh) in the H<sup>+</sup> form and AG-2-X8 anion exchange resin (200 to 400 mesh) in the Cl<sup>-</sup> form (Bio-Rad Laboratories, Richmond, Calif.). A 0.1-ml sample of the eluate was added to 10 ml of Bray's solution (5) and counted in a Packard liquid scintillation spectrometer model 3310 (87% efficiency). It was found that elution of the columns with 2 ml of distilled water allowed 99.2% recovery of 0.1  $\mu$ Ci of <sup>14</sup>C-glucose. The columns were also tested for their ability to retain alanine and pyruvate. When 0.1  $\mu$ Ci of sodium pyruvate-2-<sup>14</sup>C or alanine-U-<sup>14</sup>C was placed on the columns, no counts above background were found in the 2-ml eluate.

Glycogen synthesis was measured by counting the liver glycogen isolated and purified from mice 30 min after injection of 1  $\mu$ Ci of glucose-U-<sup>14</sup>C (specific activity = 0.05  $\mu$ Ci/mmol). Each liver (approximately 1 g) was placed in 6 ml of 30% (wt/vol) KOH in a glass centrifuge tube (40 ml) and heated at 100 C for 45 min. After cooling, 0.4 ml of saturated Na<sub>2</sub>SO<sub>4</sub> and 10 ml of absolute ethanol were added to each tube. The tubes were heated to boiling, cooled in an ice bath for 30 min, and centrifuged. The glycogen pellet was dissolved in 5 ml of distilled water and precipitated with 6.25 ml of absolute ethanol, heated to boiling, cooled, and centrifuged. This procedure was repeated, and the final glycogen pellet was dissolved in 5 ml of distilled water. A 1-ml sample of the glycogen solution was added to 10 ml of Phase Combining System solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and counted. Another 0.05-ml sample of the glycogen solution was assayed for glycogen content.

**Glycogen synthase assay.** Mouse liver glycogen synthase activity was assayed by modification of the method described by Gold and Segal (10). A 1-g amount of liver was homogenized in three volumes of 0.1 M glycylglycine buffer (pH 7.4) at 0 C for 30 s, and the homogenate was centrifuged at 2 C at 8,000  $\times$  g for 10 min. The resultant supernatant solution was held at 0 C, and the first assay for the glycogen synthase activity was made 30 min after removal of the liver from the animal. Enzyme activity was determined by measuring the amount of radioactivity incorporated into glycogen from <sup>14</sup>C-labeled UDP-glucose. The assay mixture contained 20 mg of mouse liver glycogen prepared as follows: 1  $\mu$ mol of UDP-glucose, 0.02  $\mu$ Ci (38,600 counts/min) of UDP-glucose-U-<sup>14</sup>C (final specific activity = 0.02 mCi/mmol), 264  $\mu$ mol of glycylglycine, pH 7.4, were added with 4  $\mu$ mol of glucose-6-phosphate in a volume of 3.8 ml. The reaction was initiated by adding to the assay mixture 0.2 ml of the supernatant solution from the 8,000  $\times$  g centrifugation of the mouse liver homogenate. The assay incubations were made in screw-capped 40-ml glass centrifuge tubes at 37 C for 15 min with gentle shaking.

The reaction was stopped by the addition of 6 ml of hot 30% (wt/vol) KOH to each tube, and

the mixture was heated for 10 min at 100 C. Saturated Na<sub>2</sub>SO<sub>4</sub> (0.5 ml) was added to the mixture, and glycogen was precipitated with 1.1 vol of absolute ethanol. The mixture was again heated to boiling, cooled for 30 min in an ice bath, and centrifuged. The glycogen pellet was dissolved in 5 ml of distilled water and precipitated with 6.25 ml of absolute ethanol. The mixture was heated, cooled, and centrifuged as before. The glycogen pellet was dissolved in distilled water to a final volume of 5 ml. A 1-ml sample of the glycogen solution was added to 10 ml of Phase Combining System solubilizer and counted. Another 0.02-ml sample was assayed for glycogen content. Enzyme activities are expressed as nanomoles of glucose transferred from uridine diphosphate glucose (UDPG) to glycogen per minute per gram of liver (dry weight) under the conditions of the assay corrected for the 2 to 5% loss of glycogen in the isolation procedure. A correction was also made for a small amount of radioactivity accompanying glycogen in tubes to which the enzyme was added after KOH; although background incorporation was negligible. As defined by Lerner and Villar-Palasi (20), independent (I)-form activities are those obtained in the absence of glucose-6-phosphate, and dependent (D)-form activities are the difference between those obtained in the presence (total) and absence of added glucose-6-phosphate, since a "plus-minus" assay for glycogen synthase was used. In a typical enzyme assay, approximately 5% of the <sup>14</sup>C-label from UDP-glucose-U-<sup>14</sup>C was transferred to glycogen during the 15-min incubation. No lag period was observed, and the rate of transfer was linear with time until approximately 20 min. Dry-weight determinations were made on each enzyme preparation in lieu of protein measurements since the enzyme was contained in a crude supernatant fraction.

**Liver glycogen and blood glucose.** In the experiment in which liver glycogen levels were determined in mice over a 24-h period, liver glycogen was assayed by the method of Kemp and Kits van Heyningen (16). In all other experiments, glycogen was assayed by the anthrone method described by Kahan (15). Glycogen standards were prepared from mouse liver glycogen isolated and purified as described above except that dialysis was at 4 C for 24 h against 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4), and a final ethanol precipitation was included. Glucose was determined in 1 to 40 Somogyi blood filtrates by the glucostat micromethod (Worthington Biochemical Corp., Freehold, N.J.). Commercial glucose standards were employed (Sigma Chemical Co., St. Louis, Mo.).

**Radiochemicals.** D-Glucose-U-<sup>14</sup>C (240  $\mu$ Ci/mmol) and sodium pyruvate-2-<sup>14</sup>C (23  $\mu$ Ci/mmol) were obtained from Schwarz/Mann, Orangeburg, N.Y. L-Alanine-U-<sup>14</sup>C (128  $\mu$ Ci/mmol) and uridine diphosphate glucose-U-<sup>14</sup>C (227  $\mu$ Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

**Statistics.** All results were analyzed by the

rank order test (37) and the Student's *t* test (22) for significant differences ( $P \leq 0.05$ ).

## RESULTS

**Liver glycogen levels.** Liver glycogen levels in fasted control and endotoxin-poisoned mice over a 24-h period are seen in Fig. 1. All food was withdrawn from the animals at 5:00 p.m. At this time half of the animals received an LD<sub>50</sub> of endotoxin, 10 mice from each group were sacrificed, and the liver was assayed for glycogen content (0 h). Every 2 h until 6 h post-treatment (11:00 p.m.) and then every 4 h until 24 h after treatment (5:00 p.m. the following day) groups of mice were sacrificed, and liver glycogen was assayed.

Although the control mice were fasting throughout the observation period, a cyclic pattern in liver glycogen levels was seen. Liver glycogen in fed animals usually ranges from 4 to 6%; thus, the levels measured at 5:00 p.m. (0 h) were relatively low when the experiment was started. Glycogen in the controls decreased to approximately 0.4% 6 to 12 h after treatment and then began to increase to a peak of 1.2% at 20 h.

In contrast to the cyclic pattern seen in the controls, liver glycogen was rapidly depleted in mice treated with an LD<sub>50</sub> of endotoxin. A severe drop was observed as early as 1 h after treatment (0.2%). This was followed by a slight but significant increase at 2 and 3 h, most likely in response to the initial rapid depletion. Liver gly-

cogen in poisoned mice, however, remained at near depletion levels (0.2%) for the duration of the observation period.

The period between 12 and 24 h after treatment suggested active glycogen synthesis by fasted control mice and thus established a model for future studies. Animals fasted overnight were "primed" for glycogen synthesis; therefore, in all other experiments described, mice were fasted overnight before initiating the experiments.

### Effect of endotoxin, hydrocortisone, and actinomycin D on liver glycogen synthase.

In order to characterize further glycogen depletion in endotoxin-treated mice, liver glycogen synthase was measured 17 h after treatment with an LD<sub>50</sub> (Table 1). It can be seen that fasting overnight increased the enzyme from 370 to 826 U in normal control animals, thus confirming induction of the enzyme due to fasting. In fasted mice treated with an LD<sub>50</sub> of endotoxin, however, the increase in glycogen synthase was reduced to 59% of the fasted control value. Likewise, the increase in the enzyme due to the administration of hydrocortisone acetate was significantly diminished by endotoxin treatment (from 764 to 570 U). The injection of 20 μg of actinomycin D blocked the induction of the enzyme due to fasting and suggested that the increases observed in glycogen synthase were due to new enzyme synthesis.

**Gluconeogenesis and glyconeogenesis.** Gluconeogenesis and glyconeogenesis from noncarbohydrate precursors as well as glycogen synthesis from glucose were examined in mice 12 h after

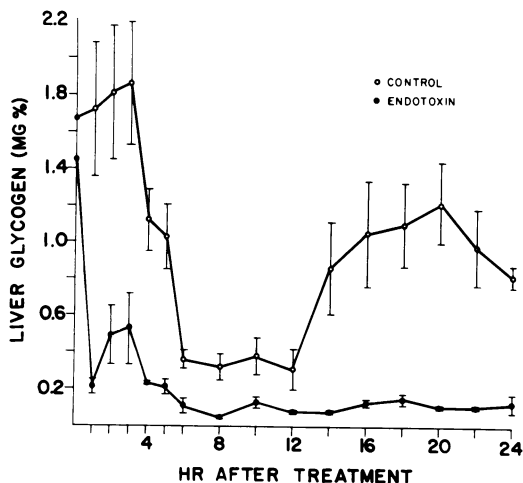


FIG. 1. Liver glycogen levels in fasted control mice (○) and endotoxin-poisoned mice (●) over a 24-h period. The points represent mean values  $\pm$  standard errors obtained from 10 mice at each time interval. Food was withdrawn from all animals at 0 hr (5:00 p.m.), and the mice were fasted for the duration of the experiment.

TABLE 1. Mouse liver glycogen synthase activity measured 17 h after the treatment indicated

| Treatment                              | Glycogen synthase activity <sup>a</sup>        |
|--|--|
| Fed normal (17 h)                      | 370 $\pm$ 31 <sup>b</sup><br>(20) <sup>c</sup> |
| Fasting alone (17 h)                   | 826 $\pm$ 83<br>(20)                           |
| Fasting + 1 LD <sub>50</sub> endotoxin | 490 $\pm$ 54<br>(20)                           |
| Fasting + 1 mg of hydrocortisone       | 764 $\pm$ 106<br>(18)                          |
| Fasting + hydrocortisone + endotoxin   | 570 $\pm$ 39<br>(18)                           |
| Fasting + 20 μg of actinomycin D       | 251 $\pm$ 26<br>(20)                           |

<sup>a</sup> Expressed as nanomoles of product formed per minute per gram of liver.

<sup>b</sup> Mean  $\pm$  standard error.

<sup>c</sup> Numbers in parentheses refer to numbers of animals.

treatment with an LD<sub>50</sub> of endotoxin (Tables 2 and 3). In these experiments groups of 10 mice each were fasted overnight and injected i.p. with an LD<sub>50</sub> of endotoxin the following morning at 8:00 a.m. Therefore at 12 h after endotoxin treatment (8:00 p.m.), all animals had been fasting approximately 27 h. The incorporation of the <sup>14</sup>C-label into either glucose or glycogen is expressed in two ways: (i) the total amount incorporated (counts per minute per milliliter of blood or counts per minute per gram of liver) and (ii) the specific activity or stoichiometric incorporation of label into specific end product (counts per minute per milligram of glucose or counts per minute per milligram of glycogen). The total incorporation of the <sup>14</sup>C-label from both alanine-<sup>14</sup>C and pyruvate-<sup>2-14</sup>C into blood glucose was significantly reduced by endotoxin to approximately 70% of the control values (Table 2). The specific

activity of the isolated blood glucose from poisoned mice was not, however, significantly different from control mice. The data indicate that blood glucose in mice 12 h after poisoning was approximately 65% of the control level. The decrease in total incorporation of the <sup>14</sup>C-label into glucose in poisoned mice paralleled the decrease in total blood sugar. Values for blood glucose in control mice were lower than those usually reported (75 to 125 mg/100 ml), but they no doubt reflect not only the 12 h of fasting but the time of day (8:00 p.m.) when blood sugar in mice is normally low.

Table 3 shows the effect of endotoxin on glyconeogenesis from alanine and pyruvate and glycogenesis from glucose 12 h after treatment. Liver glycogen levels were essentially depleted in poisoned mice (a 40-fold reduction from 0.97 to 0.02%). Likewise, the total incorporation of the

TABLE 2. *Glyconeogenesis 12 h after endotoxin (1 LD<sub>50</sub>) treatment in fasted mice*

| Precursor employed <sup>a</sup> | Blood glucose (mg/100 ml)                    |                                 | Amt incorporated into blood glucose |                                 |                      |                        |
|---------------------------------|--|---------------------------------|-------------------------------------|---------------------------------|----------------------|------------------------|
|                                 | Control                                      | Endotoxin                       | Control <sup>b</sup>                | Endotoxin <sup>b</sup>          | Control <sup>c</sup> | Endotoxin <sup>c</sup> |
| None                            | 65.5 ± 5.7 <sup>d</sup><br>(10) <sup>f</sup> | 33.5 ± 3.5 <sup>e</sup><br>(10) | —                                   | —                               | —                    | —                      |
| Alanine- <sup>14</sup> C        | 62.8 ± 5.8<br>(8)                            | 42.2 ± 4.8 <sup>e</sup><br>(9)  | 1.74 ± 0.15<br>(10)                 | 1.16 ± 0.13 <sup>e</sup><br>(6) | 2.87 ± 0.24<br>(10)  | 2.57 ± 0.21<br>(6)     |
| Pyruvate- <sup>2-14</sup> C     | 65.7 ± 5.9<br>(10)                           | 41.5 ± 4.5 <sup>e</sup><br>(10) | 1.20 ± 0.10<br>(10)                 | 0.92 ± 0.07 <sup>e</sup><br>(8) | 2.10 ± 0.20<br>(9)   | 2.80 ± 0.30<br>(9)     |

<sup>a</sup> One  $\mu$ Ci/20  $\mu$ mol of carrier injected i.p. at 30 min before sacrifice.

<sup>b</sup> Counts/min  $\times 10^{-4}$ /ml of blood.

<sup>c</sup> Counts/min  $\times 10^{-4}$ /mg of glucose.

<sup>d</sup> Mean  $\pm$  standard error.

<sup>e</sup> Significantly different from control values ( $P \leq 0.05$ ).

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

TABLE 3. *Glyconeogenesis and glycogenesis 12 h after endotoxin (1 LD<sub>50</sub>) treatment in fasted mice*

| Precursor employed <sup>a</sup> | Liver glycogen (%)                            |                                  | Amt incorporated into liver glycogen |                                |                      |                                |
|---------------------------------|---|----------------------------------|--------------------------------------|--------------------------------|----------------------|--------------------------------|
|                                 | Control                                       | Endotoxin                        | Control <sup>b</sup>                 | Endotoxin <sup>b</sup>         | Control <sup>c</sup> | Endotoxin <sup>c</sup>         |
| None                            | 0.97 ± 0.11 <sup>d</sup><br>(10) <sup>f</sup> | 0.02 ± 0.01 <sup>e</sup><br>(10) | —                                    | —                              | —                    | —                              |
| Alanine- <sup>14</sup> C        | 0.75 ± 0.06<br>(10)                           | 0.02 ± 0.01 <sup>e</sup><br>(12) | 11.4 ± 0.2<br>(10)                   | 0.6 ± 0.1 <sup>e</sup><br>(12) | 10.6 ± 1.0<br>(10)   | 5.8 ± 0.4 <sup>e</sup><br>(12) |
| Pyruvate- <sup>2-14</sup> C     | 0.86 ± 0.14<br>(9)                            | 0.03 ± 0.01 <sup>e</sup><br>(10) | 7.7 ± 1.8<br>(9)                     | 0.7 ± 0.2 <sup>e</sup><br>(10) | 7.5 ± 2.3<br>(8)     | 2.7 ± 0.8 <sup>e</sup><br>(9)  |
| Glucose- <sup>14</sup> C        | 0.54 ± 0.08<br>(7)                            | 0.01 ± 0.01 <sup>e</sup><br>(11) | 4.3 ± 0.8<br>(10)                    | 0.7 ± 0.1 <sup>e</sup><br>(11) | 11.0 ± 2.5<br>(8)    | 5.6 ± 0.4 <sup>e</sup><br>(10) |

<sup>a</sup> One  $\mu$ Ci/20  $\mu$ mol of carrier injected i.p. at 30 min before sacrifice.

<sup>b</sup> Counts/min  $\times 10^{-3}$ /g of liver.

<sup>c</sup> Counts/min  $\times 10^{-3}$ /mg of glycogen.

<sup>d</sup> Mean  $\pm$  standard error.

<sup>e</sup> Significantly different from control values ( $P \leq 0.05$ ).

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

$^{14}\text{C}$ -label from alanine, pyruvate, or glucose into glycogen as well as the specific activity of the  $^{14}\text{C}$ -labeled glycogen isolated from poisoned mice was significantly reduced. Total incorporation of the  $^{14}\text{C}$ -label from alanine, pyruvate, and glucose was reduced to approximately 5%, 9%, and 16% of control values, respectively. Furthermore, the specific activity of the labeled glycogen was decreased to 55%, 36%, and 51% of controls when the precursors were, respectively, alanine, pyruvate, and glucose. Although some variation was observed, no significant increases in liver glycogen were seen in control animals given tracer amounts (1  $\mu\text{Ci}/20 \mu\text{mol}$ ) of  $^{14}\text{C}$ -labeled alanine, pyruvate, or glucose.

The effects of endotoxin on gluconeogenesis can be emphasized by comparing the product of the decrease in total incorporation (counts per gram of liver) and specific activity (counts per milligram of glycogen) with the total decrease in liver glycogen. In the case of alanine, this product was approximately 35 ( $11.4/0.6 \times 10.6/5.8 = 34.8$ ), whereas the reduction in liver glycogen was 37.5-fold ( $0.75/0.02 = 37.5$ ). With pyruvate the incorporation, similarly calculated, was decreased by a factor of approximately 30, whereas liver glycogen was reduced approximately 29-fold. The incorporation of  $^{14}\text{C}$ -glucose into liver glycogen in poisoned mice was reduced by a factor of 12, whereas liver glycogen levels were reduced from 25- to 50-fold. These data indicate that the direct conversion of glucose to glycogen was not as severely affected by endotoxin as the conversion of alanine and pyruvate to glycogen. Results with labeled alanine and pyru-

vate reflect both gluconeogenesis and glycogen synthesis, whereas use of labeled glucose requires glycogen synthesis only.

To characterize further gluconeogenesis and glycogenesis during endotoxin poisoning, shorter intervals after treatment were examined. The incorporation of alanine- $^{14}\text{C}$  into blood glucose and liver glycogen was examined at 0, 2, 4, and 6 h after the i.p. injection of an  $\text{LD}_{50}$  of endotoxin (Tables 4 and 5). The procedures were similar to those employed for the results in Tables 2 and 3, and the data are expressed in identical fashion. The 0, 2, 4, and 6 h after treatment correspond to 8:00 a.m., 10:00 a.m., 12:00 p.m., and 2:00 p.m., respectively.

Incorporation of the  $^{14}\text{C}$ -label from alanine into total glucose and specific activity of isolated blood glucose were both significantly reduced in poisoned mice at 2 and 4 h (Table 4). Significant decreases in blood sugar were not observed until 4 and 6 h after treatment. Although these changes were not as pronounced as those seen at 12 h (Table 2), they indicate early alterations in gluconeogenesis as a result of endotoxin poisoning. Total incorporation and specific activity were not significantly dissimilar in poisoned and control mice after 6 h, indicating a partial recovery of unexplained origin but possibly reflecting the animal's effort to maintain essential levels of blood sugar.

In contrast to the slight changes in alanine- $^{14}\text{C}$  incorporation into blood glucose at 0, 2, 4, and 6 h, the effects of endotoxin on the incorporation of the  $^{14}\text{C}$ -label into liver glycogen were most pronounced (Table 5). In endotoxin-treated mice

TABLE 4. Incorporation of the  $^{14}\text{C}$ -label from alanine- $^{14}\text{C}$  into blood glucose at 0, 2, 4, and 6 h after endotoxin (1  $\text{LD}_{50}$ ) treatment in fasted mice

| Time after treatment <sup>b</sup> (h) | Blood glucose (mg/100 ml)                        |                                    | Amt incorporated into blood glucose |                                      |                        |                                    |
|---------------------------------------|--|------------------------------------|-------------------------------------|--------------------------------------|------------------------|------------------------------------|
|                                       | Control  | Endotoxin                          | Control <sup>c</sup>                | Endotoxin <sup>c</sup>               | Control <sup>d</sup>   | Endotoxin <sup>d</sup>             |
| 0                                     | 62.0 $\pm$ 2.2 <sup>e</sup><br>(10) <sup>f</sup> | 63.8 $\pm$ 2.0<br>(9)              | 1.94 $\pm$ 0.37<br>(10)             | 1.86 $\pm$ 0.28<br>(7)               | 9.4 $\pm$ 0.5<br>(8)   | 10.3 $\pm$ 0.7<br>(8)              |
| 2                                     | 64.4 $\pm$ 4.1<br>(8)                            | 71.7 $\pm$ 4.2<br>(10)             | 2.66 $\pm$ 0.31<br>(8)              | 1.70 $\pm$ 0.24 <sup>g</sup><br>(9)  | 14.1 $\pm$ 1.5<br>(8)  | 8.5 $\pm$ 0.4 <sup>g</sup><br>(10) |
| 4                                     | 60.5 $\pm$ 3.1<br>(10)                           | 47.9 $\pm$ 4.3 <sup>g</sup><br>(9) | 2.26 $\pm$ 0.17<br>(10)             | 1.27 $\pm$ 0.30 <sup>g</sup><br>(10) | 13.8 $\pm$ 1.6<br>(10) | 7.7 $\pm$ 0.7 <sup>g</sup><br>(10) |
| 6                                     | 50.9 $\pm$ 2.4<br>(9)                            | 34.2 $\pm$ 1.7 <sup>g</sup><br>(9) | 2.17 $\pm$ 0.30<br>(10)             | 1.72 $\pm$ 0.34<br>(10)              | 14.6 $\pm$ 1.2<br>(9)  | 15.5 $\pm$ 1.3<br>(9)              |

<sup>a</sup> One  $\mu\text{Ci}/20 \mu\text{mol}$  of carrier injected i.p. at 30 min before sacrifice.

<sup>b</sup> All animals were fasted overnight before 0 h at 8:00 a.m.

<sup>c</sup> Counts/min  $\times 10^{-4}$ /ml of blood.

<sup>d</sup> Counts/min  $\times 10^{-4}$ /mg of glucose.

<sup>e</sup> Mean  $\pm$  standard error.

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

<sup>g</sup> Significantly different from control values ( $P \leq 0.05$ ).

TABLE 5. Incorporation of the  $^{14}\text{C}$ -label from alanine- $U\text{-}^{14}\text{C}$  into liver glycogen at 0, 2, 4, and 6 h after endotoxin ( $1\text{ LD}_{50}$ ) treatment in fasted mice

| Time after treatment <sup>b</sup> (h) | Liver glycogen (%)                            |                                 | Amt incorporated into liver glycogen |                                |                      |                                |
|---------------------------------------|---|---------------------------------|--------------------------------------|--------------------------------|----------------------|--------------------------------|
|                                       | Control                                       | Endotoxin                       | Control <sup>c</sup>                 | Endotoxin <sup>c</sup>         | Control <sup>d</sup> | Endotoxin <sup>d</sup>         |
| 0                                     | 0.73 ± 0.14 <sup>e</sup><br>(10) <sup>f</sup> | 0.69 ± 0.11<br>(10)             | 14.9 ± 1.2<br>(9)                    | 15.0 ± 1.4<br>(9)              | 20.4 ± 1.7<br>(9)    | 21.7 ± 3.3<br>(9)              |
| 2                                     | 0.84 ± 0.14<br>(7)                            | 1.68 ± 0.35 <sup>g</sup><br>(5) | 21.2 ± 2.4<br>(8)                    | 67.3 ± 3.8 <sup>g</sup><br>(8) | 25.2 ± 2.8<br>(8)    | 40.1 ± 5.3 <sup>g</sup><br>(8) |
| 4                                     | 0.66 ± 0.11<br>(10)                           | 0.05 ± 0.01 <sup>g</sup><br>(7) | 17.4 ± 2.0<br>(8)                    | 3.2 ± 0.4 <sup>g</sup><br>(8)  | 26.3 ± 2.1<br>(8)    | 6.4 ± 2.1 <sup>g</sup><br>(8)  |
| 6                                     | 0.44 ± 0.07<br>(9)                            | 0.03 ± 0.01 <sup>g</sup><br>(5) | 13.7 ± 1.7<br>(9)                    | 0.5 ± 0.1 <sup>g</sup><br>(9)  | 31.1 ± 7.9<br>(5)    | 1.7 ± 0.8 <sup>g</sup><br>(4)  |

<sup>a</sup> One  $\mu\text{Ci}/20\ \mu\text{mol}$  of carrier injected i.p. at 30 min before sacrifice.

<sup>b</sup> All animals were fasted overnight before 0 h at 8:00 a.m.

<sup>c</sup> Counts/min  $\times 10^{-3}$ /g of liver.

<sup>d</sup> Counts/min  $\times 10^{-3}$ /mg of glycogen.

<sup>e</sup> Mean  $\pm$  standard error.

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

<sup>g</sup> Significantly different from control values ( $P \leq 0.05$ ).

a significant increase in liver glycogen was observed at 2 h (twofold). Likewise, significant increases in both counts per minute per gram of liver and counts per minute per milligram of glycogen were seen in poisoned mice at the same time. These increases were possibly due to the transitory enhancement of adrenocortical activity early after the injection of endotoxin. At 4 h post-treatment, total incorporation of alanine into liver glycogen was reduced fivefold. The specific activity of  $^{14}\text{C}$ -labeled glycogen was similarly reduced. Further decreases in counts per minute per gram of liver and counts per minute per milligram of glycogen were observed in poisoned animals at 6 h, when liver glycogen levels were approximately 7% of the control values. These data indicate that glyconeogenesis is substantially impaired in mice treated with endotoxin as early as 4 h after the start of the experiment.

**Early changes in glycogen synthase.** Since an early impairment in the conversion of alanine into glycogen was observed in endotoxin-poisoned mice, one of the possible rate-limiting enzymes in the pathway, glycogen synthase, was assayed under conditions similar to those described above. The enzyme was measured in the presence of  $4\ \mu\text{mol}$  of glucose-6-phosphate so that the results correspond to total enzyme activity (I + D). The data plotted in Fig. 2 demonstrate that a progressive increase in enzyme activity occurred in fasted control mice during the 12-h period. This indicates induction of the enzyme in response to the stress of fasting. The data confirm the validity of the experimental animal employed in these studies,

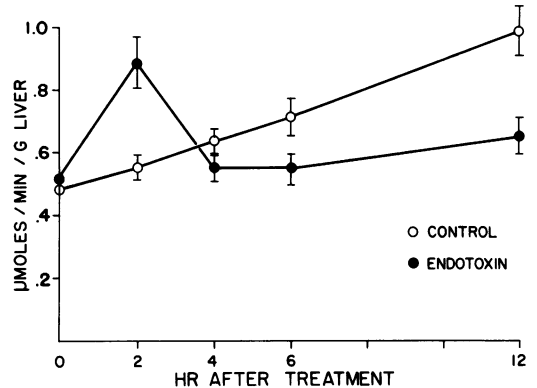


FIG. 2. Liver glycogen synthase activity in fasted control mice (○) and endotoxin-poisoned mice (●) at 0, 2, 4, 6, and 12 h after treatment. The points represent the mean values  $\pm$  standard errors obtained from 20 mice at each time interval. Enzyme activity = total (I + D), since glucose-6-phosphate ( $4\ \mu\text{mol}$ ) was added to the incubation mixture. *P* values: 0 h, not significant; 2 h,  $<0.01$ ; 4 h, not significant; 6 h,  $<0.05$ ; 12 h,  $<0.05$ .

since 12 h of fasting insure that carbohydrate synthesis will be predominantly toward glucose and glycogen. The results with endotoxin treatment differed considerably from those with controls. An initial increase in enzyme activity about twofold above the control value was observed in poisoned mice after 2 h. This early elevation in the enzyme parallels the increases in liver glycogen and in glyconeogenesis from alanine found in endotoxin-treated animals (Table 5). Although it was not significantly different from

controls, the enzyme appeared to be slightly reduced in poisoned mice at 4 h. However, beyond that time, significant reductions below controls were apparent even though the absolute activity was essentially constant. These data offer, in part, an explanation for why glycconeogenesis is impaired by endotoxin, as the results above indicate.

**Effect of pyruvate and glucose loads.** The work of Shands et al. (33) clearly demonstrated that a major defect in gluconeogenesis was present in endotoxin-poisoned mice and was sufficient to account for the observed hypoglycemia. This was confirmed in part by the failure of endotoxin-poisoned BCG-infected mice to raise the level of blood sugar after administration of a pyruvate load. In the present study, tracer amounts of pyruvate, alanine, and glucose (20  $\mu$ mol) were employed to detect alterations in gluconeogenesis and glycogen synthesis in endotoxin-poisoned mice; the results have been described. To confirm these alterations in the presence of larger amounts of precursor, 400  $\mu$ mol of pyruvate and 200  $\mu$ mol of glucose were administered to poisoned animals 30 min before sacrifice.

The injection of a pyruvate load increased significantly the level of blood glucose from 65.7 to 109.7 mg/100 ml in fasted control mice (Table 6). Likewise, the incorporation of the  $^{14}$ C-label from pyruvate-2- $^{14}$ C into blood glucose and the increase in specific activity of the isolated blood glucose increased, respectively, 23- and 12-fold in the controls. Although large increases in total incorporation and in specific activity were observed in poisoned mice, they were not as great as in controls and were insufficient to raise blood sugar to control levels. These data indicate that the pathway of gluconeogenesis in endotoxin-

treated mice is functioning at a rate lower than that observed in control animals.

Glyconeogenesis from pyruvate and glycconeogenesis from glucose were not normal when either a pyruvate or glucose load was given to mice 12 h after poisoning (Table 7). Liver glycogen in fasted control mice increased significantly after a large dose of either glucose or pyruvate (233 and 140% of controls, respectively). There were also significant amounts of the  $^{14}$ C-label of glucose incorporated into liver glycogen of control and poisoned mice as judged by counts per gram of liver or by specific activity. On the other hand, 400  $\mu$ mol of pyruvate did not augment liver glycogen levels, elevate the total incorporation into glycogen, nor increase the specific activity of labeled glycogen in endotoxemic mice. A glucose load of 200  $\mu$ mol increased glycogen levels and glycconeogenesis in poisoned animals, but these increases were not of sufficient magnitude to restore liver glycogen to control levels. Since administration of a glucose load caused some increase in glycogen synthesis in poisoned animals, this effect was further studied.

**In vivo activation of glycogen synthase by glucose.** De Wulf and Hers (6) noted a 15- to 20-fold increase in conversion of glucose to glycogen when mice were given a glucose load (50 mg) intravenously. Approximately a 10-fold increase in liver glycogen synthase was also observed. These two effects were explained by an increase in the conversion of the enzyme to a more active form, whereas recent evidence has indicated that this is true (7). Since a glucose load of 200  $\mu$ mol significantly increased liver glycogen in fasted control animals and to a much lesser extent in endotoxin-poisoned mice (Table 7), the ability of glucose to activate glycogen synthase under in vivo conditions was studied.

TABLE 6. Effect of a pyruvate load on gluconeogenesis 12 h after endotoxin (1  $LD_{50}$ ) treatment in fasted mice

| Treatment                     | Blood glucose (mg/100 ml)                        |                                     | Amt incorporated into blood glucose |                                     |                       |                                    |
|-------------------------------|--|-------------------------------------|-------------------------------------|-------------------------------------|-----------------------|------------------------------------|
|                               | Control  | Endotoxin                           | Control <sup>a</sup>                | Endotoxin <sup>a</sup>              | Control <sup>b</sup>  | Endotoxin <sup>b</sup>             |
| Pyruvate control <sup>c</sup> | 65.7 $\pm$ 5.9 <sup>d</sup><br>(10) <sup>f</sup> | 41.5 $\pm$ 4.5 <sup>e</sup><br>(10) | 1.2 $\pm$ 0.1<br>(10)               | 0.9 $\pm$ 0.1 <sup>e</sup><br>(8)   | 2.1 $\pm$ 0.2<br>(9)  | 2.8 $\pm$ 0.3<br>(9)               |
| Pyruvate load <sup>g</sup>    | 109.7 $\pm$ 6.6<br>(9)                           | 51.2 $\pm$ 7.6 <sup>e</sup><br>(10) | 27.6 $\pm$ 2.0<br>(9)               | 17.9 $\pm$ 1.6 <sup>e</sup><br>(10) | 25.1 $\pm$ 1.6<br>(9) | 35.1 $\pm$ 3.6 <sup>e</sup><br>(9) |

<sup>a</sup> Counts/min  $\times 10^{-4}$ /ml of blood.

<sup>b</sup> Counts/min  $\times 10^{-4}$ /mg of glucose.

<sup>c</sup> One  $\mu$ Ci/20  $\mu$ mol of carrier injected i.p. at 30 min before sacrifice.

<sup>d</sup> Mean  $\pm$  standard error.

<sup>e</sup> Significantly different from control values ( $P \leq 0.05$ ).

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

<sup>g</sup> One  $\mu$ Ci/400  $\mu$ mol of carrier injected i.p. at 30 min before sacrifice.



TABLE 7. Effect of a glucose or pyruvate load on glycogenesis and glyconeogenesis 12 h after endotoxin (1 LD<sub>50</sub>) treatment in fasted mice

| Treatment                     | Liver glycogen (%)                           |                                  | Amt incorporated into liver glycogen |                                 |                      |                                 |
|-------------------------------|--|----------------------------------|--------------------------------------|---------------------------------|----------------------|---------------------------------|
|                               | Control                                      | Endotoxin                        | Control <sup>a</sup>                 | Endotoxin <sup>a</sup>          | Control <sup>b</sup> | Endotoxin <sup>b</sup>          |
| Glucose control <sup>c</sup>  | 0.64 ± 0.08 <sup>d</sup><br>(9) <sup>f</sup> | 0.02 ± 0.01 <sup>e</sup><br>(11) | 4.5 ± 0.7<br>(10)                    | 0.6 ± 0.1 <sup>e</sup><br>(11)  | 11.0 ± 2.5<br>(8)    | 5.6 ± 0.4 <sup>e</sup><br>(10)  |
| Glucose load <sup>d</sup>     | 1.49 ± 0.18<br>(10)                          | 0.20 ± 0.06 <sup>e</sup><br>(10) | 36.7 ± 9.3<br>(10)                   | 13.1 ± 3.2 <sup>e</sup><br>(10) | 22.4 ± 0.5<br>(10)   | 11.4 ± 2.6 <sup>e</sup><br>(10) |
| Pyruvate control <sup>c</sup> | 0.86 ± 0.14<br>(9)                           | 0.03 ± 0.01 <sup>e</sup><br>(10) | 7.0 ± 1.1<br>(9)                     | 0.6 ± 0.2 <sup>e</sup><br>(10)  | 7.3 ± 2.1<br>(10)    | 2.4 ± 0.6 <sup>e</sup><br>(9)   |
| Pyruvate load <sup>d</sup>    | 1.20 ± 0.10<br>(9)                           | 0.03 ± 0.01 <sup>e</sup><br>(10) | 83.7 ± 10.5<br>(9)                   | 0.2 ± 0.1 <sup>e</sup><br>(10)  | 15.7 ± 1.7<br>(9)    | 0.8 ± 0.1 <sup>e</sup><br>(10)  |

<sup>a</sup> Counts/min × 10<sup>-2</sup>/g of liver.

<sup>b</sup> Counts/min × 10<sup>-3</sup>/g of glycogen.

<sup>c</sup> One μCi/20 μmol of carrier injected i.p. at 30 min before sacrifice.

<sup>d</sup> Mean ± standard error.

<sup>e</sup> Significantly different from control values ( $P \leq 0.05$ ).

<sup>f</sup> Numbers in parentheses refer to numbers of animals.

<sup>g</sup> One μCi/200 μmol of carrier injected i.p. at 30 min before sacrifice.

<sup>h</sup> One μCi/400 μmol of carrier injected i.p. at 30 min before sacrifice.

The results presented in Table 8 indicate a doubling in total enzyme activity (I + D) in control mice given a glucose load. No significant increase was observed, however, in poisoned mice (808 U without and 909 U with glucose loading). A slight but significant increase was noted in I activity (glucose-6-phosphate omitted from the assay mixture) in control animals given glucose to 154% of the values obtained from normal mice with no glucose load. A comparable increase in I activity was not seen in poisoned mice. Apparently, the increase in total enzyme activity due to glucose, measured in control mice, was due to increases in the D form (total - I). This contradicts the effect of glucose on glycogen synthetase conversion described by De Wulf and Hers (7) and possibly suggests that some activation of the enzyme occurred prior to assay. Gold and Segal (10) demonstrated that glycogen synthase activation in vitro is dependent on temperature of incubation. Although precautions were made in this study to prevent temperature activation of the enzyme, such a possibility could account for the increase in the D form due to glucose loading. Nevertheless, these data clearly indicate that glucose activation of liver glycogen synthase was impaired in mice 12 h after an LD<sub>50</sub> of endotoxin.

## DISCUSSION

Depletion of glycogen and alterations in the enzymes of the glycogen cycle have been reported in a wide variety of infections and pathological conditions. Liver glycogen is depleted in mice

TABLE 8. In vivo activation of liver glycogen synthase by glucose 12 h after treatment in endotoxin-poisoned (1 LD<sub>50</sub>) mice

| Treatment                                   | Glycogen synthase activity (nmol per min per g of liver) |                              |                               |
|---|--|------------------------------|-------------------------------|
|   | Total (+G6P) <sup>a</sup>                                | I-form (-G6P)                | D-form <sup>b</sup>           |
| Control                                     | 1163 ± 114 <sup>c</sup><br>(18) <sup>d</sup>             | 101 ± 5<br>(18)              | 1062 ± 124<br>(18)            |
| Endotoxin                                   | 808 ± 49 <sup>e</sup><br>(20)                            | 111 ± 8<br>(20)              | 697 ± 54 <sup>e</sup><br>(20) |
| Control 30 min after glucose <sup>f</sup>   | 2365 ± 261<br>(20)                                       | 155 ± 13<br>(20)             | 2210 ± 192<br>(20)            |
| Endotoxin 30 min after glucose <sup>f</sup> | 909 ± 104 <sup>e</sup><br>(20)                           | 117 ± 8 <sup>e</sup><br>(20) | 792 ± 70 <sup>e</sup><br>(20) |

<sup>a</sup> Glucose-6-phosphate (4 μmol) added to the incubation mixture.

<sup>b</sup> (I + D) activity - (I) activity.

<sup>c</sup> Mean ± standard error.

<sup>d</sup> Numbers in parentheses refer to numbers of animals.

<sup>e</sup> Significantly different from control values ( $P \leq 0.05$ ).

<sup>f</sup> Glucose (200 μmol) injected i.p. at 30 min before sacrifice.

infected with *S. typhimurium* (1) and *Staphylococcus aureus* (23). Marked depletion of hepatic glycogen during experimental tuberculosis in guinea pigs has been reported (34) and early

liver carbohydrate loss was observed in experimental *Listeria monocytogenes* infection in mice (25). Several investigators have attempted to correlate glycogen levels with altered activities of enzymes of the glycogen cycle. The metabolic abnormality responsible for depleted levels of glycogen in *Coxiella burnetti* infection includes both the suppressed activity of glycogen synthase and enhancement of glycogenolysis by increased levels of phosphorylase activity (30). The attempts of Wilder and Sword (38) to elucidate mechanisms responsible for glycogen depletion in listeriosis indicate decreased activity of both glycogen synthase and phosphorylase. Graybill et al. (11) showed that purified cholera enterotoxin produced hyperglycemia of at least 48-h duration in the dog. Further studies by Zieve et al. (42) showed that purified cholera enterotoxin markedly enhanced the rate of glycogenolysis in liver after intravenous injection in mice, a hormone-like effect most likely due to a cyclic 3',5'-adenosine monophosphate (AMP)-mediated mechanism. Bacterial endotoxins have long been known to deplete an animal of its carbohydrate reserves. Such a depletion could result from several causes. One of the most obvious would be an increased utilization of sugars because of an elevated metabolic rate. Woods et al. (40) demonstrated stimulation of cellular glycolysis in vitro by endotoxin, and more recently Snyder et al. (35) reported increased pyruvate kinase activities in mouse liver after treatment with endotoxin. Higher metabolism in animals that become febrile is to be expected, but in mice loss of carbohydrate is fully apparent even though they become hypothermic. Moreover, as Shands et al. (33) indicated, an elevated metabolic rate was not found in poisoned mice. The most likely remaining cause for carbohydrate loss in endotoxin poisoning is an impaired ability to carry out either gluconeogenesis or glycogen synthesis, or both. Impaired gluconeogenesis in endotoxin-poisoned animals has been demonstrated by Berry et al. (4), Shands et al. (33), and La Noue et al. (19). More recently, significant inhibition of the induction of hepatic phosphoenolpyruvate carboxykinase in endotoxin-treated mice has been observed (Rippe and Berry, *unpublished data*). Although it is beyond the scope of this study, several other possible causes for rapid liver carbohydrate depletion in mice after endotoxin administration deserve consideration. It seems unlikely that enhanced glycogenolysis is responsible. However, the idea that endotoxin may affect  $\beta$ -adrenergic receptors in the liver, thus leading to cyclic AMP-mediated activation of liver phosphorylase, is intriguing. Likewise, no evidence exists indicating whether early release

of lysosomal enzymes (e.g., amylases, glucosidases, etc.) may be responsible for hydrolysis of sugars in the liver. These possibilities are currently under investigation in this laboratory.

The results of this study clearly indicate that liver glycogen was rapidly depleted after injection of an LD<sub>50</sub> of endotoxin. The fasted animal was employed for two reasons: (i) to observe anabolic sugar production from noncarbohydrate precursors, and (ii) because mice treated with endotoxin fail to eat; therefore, fed animals would not be in a comparable metabolic state. The experiment in which liver glycogen was surveyed over a 24-h period established a defined experimental system which subsequently was used in all experiments. That is, when food was withdrawn from both control and treated mice prior to the usual evening eating period, carbohydrate stores were mobilized and utilized during the overnight period. As a result, the animals were forced to replenish carbohydrate stores from nonsugar sources, and, as Fig. 1 shows, they were actively synthesizing glucose and glycogen between 12 and 24 h after the withdrawal of food. For this reason, experiments were initiated at 8:00 a.m. the next morning, after an overnight fast. In endotoxin-treated mice, active liver glycogen synthesis was not observed, probably because of an impairment in glycogenesis and gluconeogenesis.

Glycogen synthase, also referred to as UDPG-glycogen transglucosylase, was discovered by Leloir and Cardini (21) and is believed to be the principle rate-limiting enzyme in the pathway leading from glucose to glycogen (36). The enzyme exists in two forms in various tissues and is controlled by a variety of complex regulatory factors. Through the work initiated by Hizukuri and Larner (13) and subsequently extended and developed by a number of investigators, it has become clear that in liver as well as muscle the enzyme exists in two forms interconverted by a phosphatase and a kinase. The original kinetic measurements of the D and I forms of rat liver glycogen synthase indicated that the D form was essentially inactive without glucose-6-phosphate and that the I form was almost fully active. In the presence of glucose-6-phosphate, the  $K_m$  of the D form for UDPG was  $9 \times 10^{-4}$  M, and  $6.7 \times 10^{-4}$  M for the I form. Interconversion was shown to be catalyzed by an enzyme in the smooth endoplasmic reticulum. In contrast to the small difference in  $K_m$  for UDPG which Hizukuri and Larner found with the D and I forms of the enzyme, Mersmann and Segal (27), Gold (9), and De Wulf et al. (8) studied the enzyme in concentrated homogenates with low concentrations of substrates presumably approaching more

physiological conditions. Activation was achieved by a conversion reaction subsequently shown to be identical with the conversion reaction of Hizukuri and Lerner (13). Under these conditions large differences in  $K_m$  values for UDPG for the two forms were noted. Lower  $K_m$  values were found for the active form of the enzyme (range, 0.007 to 0.2 mM) than for the inactive form (range, 0.34 to 5 mM). In the absence of glucose-6-phosphate, the active form had much higher  $K_m$  values (range, 0.48 to 1.8 mM) than in its presence. According to Hers et al. (12), liver glycogen synthase in normal mice is predominantly in the D form. It is converted into I within 3 to 5 min after the intravenous injection of glucose, or within 2 to 3 h after the administration of glucocorticoids. The I enzyme can be reconverted into D within 1 to 3 min after administration of glucagon, epinephrine, or cyclic AMP. The effect of these various effectors has also been demonstrated *in vitro*. The two main effectors, therefore, that act antagonistically on glycogen synthase and glycogen phosphorylase appear to be glucose and cyclic AMP. Due to their action, the synthesis of glycogen is inhibited whereas its degradation is stimulated, and vice versa.

In this study the enzyme was assayed in livers of fasted control and poisoned mice. It increased approximately twofold in mice fasted overnight or in animals given hydrocortisone. These increases were not observed in endotoxemic mice. Similar inhibition of the increase due to fasting or hormone injection in animals given actinomycin D suggests that both poisons may act by inhibiting induction (new enzyme synthesis).

To more fully characterize the observed defects in carbohydrate synthesis, detailed studies were initiated. Two approaches were employed: (i) radiolabeled carbohydrate precursors were given to mice, and the amount and rate of incorporation of label into blood glucose and liver glycogen were compared at intervals in control and endotoxin-treated mice; and (ii) total liver glycogen synthase activity was measured in both control and poisoned mice at similar times after treatment. The results in Tables 2 through 5 clearly indicate that the incorporation of the  $^{14}\text{C}$ -label of various precursors into sugar was significantly impaired in endotoxin-treated mice. This impairment becomes more dramatic when one considers the results in Tables 6 and 7, in which poisoned mice "loaded" with pyruvate or glucose failed to incorporate the substrates into blood glucose or liver glycogen at a normal rate. The results also indicate the relative rates of gluconeogenesis, and glycogen synthesis in control and treated animals. Gluconeogenesis was followed by the incorporation of the  $^{14}\text{C}$ -label from alanine- $U$ - $^{14}\text{C}$  or

pyruvate- $2$ - $^{14}\text{C}$  into blood glucose. The process of glyconeogenesis was evaluated by the incorporation of the same precursors into liver glycogen, whereas glycogenesis was measured as the direct incorporation of the  $^{14}\text{C}$ -label from glucose- $U$ - $^{14}\text{C}$  into liver glycogen. Tracer amounts of the precursors were employed initially and, to confirm an impaired flux in the pathway, pyruvate and glucose loads ( $1\ \mu\text{Ci}/400\ \mu\text{mol}$  of carrier and  $1\ \mu\text{Ci}/200\ \mu\text{mol}$  of carrier, respectively) were administered in both types of experiments 30 min before sacrifice.

The results establish that gluconeogenesis is more severely impaired by endotoxin than glycogen synthesis; however, this is difficult to evaluate since direct liver glucose to liver glycogen conversion was not measured. This is currently under investigation. In glyconeogenesis from alanine and pyruvate, a correlation between decrease in liver glycogen content and the changes in  $^{14}\text{C}$  incorporation into glycogen was observed in poisoned mice. Incorporation of the isotope is presented in two forms (counts per minute per gram of liver and counts per minute per milliliter of blood; and counts per minute per milligram of glycogen or counts per minute per milligram of glucose). The values listed in both columns of Tables 2 through 7 represent the total amount and rate of incorporation of the  $^{14}\text{C}$ -label into glucose or glycogen. The turnover rate of blood glucose or liver glycogen could be inferred from mathematical treatment of the data, but that is beyond the scope of this investigation. Even so, the results demonstrate altered gluconeogenesis, glyconeogenesis, and glycogen synthesis in endotoxin-treated mice.

Mention should be made of the early increases observed in liver glycogen and  $^{14}\text{C}$  incorporation 2 h after treatment in poisoned mice (Table 5). A similar increase was noted in liver glycogen synthase at 2 h (Fig. 2). It seems most likely that the "spike" at 2 h is due to enhanced adrenal activity after endotoxin treatment. Although adrenalectomized animals were not used to confirm this, previous evidence from this laboratory would indicate this to be the case. Studies are currently in progress to determine the role of adrenocorticoids and their early release in the mechanism of action of endotoxin. These results and others strongly argue for mediated mechanisms as responsible for some of the metabolic alterations produced by endotoxin treatment.

A crucial question which has received much attention in this laboratory concerns the response of inducible liver enzymes to endotoxin. Is the enzyme specifically inhibited by the poison, or is its synthesis directly impaired? Current evidence indicates the latter to be the most probable effect, at least in the case of tryptophan oxy-

genase and phosphoenolpyruvate carboxykinase (D. F. Rippe and L. J. Berry, unpublished data). Present results suggest that endotoxin impairs the synthesis of liver glycogen synthase as judged by the suppressed induction of the enzyme due to fasting (Fig. 2). This observation, however, deserves closer study. Evidence is presented here which may indicate that activation of liver glycogen synthase is altered by endotoxin. Glucose was shown by De Wulf and Hers (6) to enhance the conversion of liver glucose to liver glycogen by increasing the rate of activation of glycogen synthase. This effect of glucose may be due to stimulation of glycogen synthase phosphatase, as shown by Holmes and Mansour (14) in the case of muscle phosphorylase phosphatase. The results in Table 8 clearly indicate that *in vivo* activation of liver glycogen synthase by a glucose load is significantly inhibited in mice treated with endotoxin. As mentioned previously, the increase in the D form seen with glucose in fasted controls may be the result of nonspecific temperature activation prior to assay of the enzyme. This is currently under study. It could also be due to the route of glucose injection, intraperitoneally, versus intravenously, as employed by De Wulf and Hers (6). Nevertheless, activation (or increased activity) of the enzyme by glucose did not appear to take place *in vivo* in the poisoned animal. Whether the *in vitro* activation of glycogen synthase by caffeine or theophylline is affected by endotoxin remains to be studied.

The effects of endotoxin on carbohydrate synthesis have a rapid onset and appear relatively early in experimental endotoxemia in mice. A direct correlation between glycogen deposition and glycogen synthase activity in liver serves as a sensitive tool for understanding the molecular basis of endotoxin-induced metabolic damage. The significance of carbohydrate homeostasis and an animal's ability to overcome stress cannot be overemphasized. Since alterations in carbohydrate metabolism have been demonstrated in such a wide variety of infections, intoxications, and other conditions of harmful stress, disruption in the delicate balance of homeostasis may be one of the key events necessary for damage to develop.

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