The Impairment of Glucogenesis by Gram Negative Infection

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In vitro studies of gluconeogenesis in rat liver tissue have been carried out. The rates of glucogenesis of liver slices from rats suffering from a *Pseudomonas aeruginosa* infection and rats injected with a lethal dose of *Escherichia coli* endotoxin have been measured. The liver content of certain key glucogenic enzymes was also measured in these animals. The specific enzymes studied were glucose-6-phosphatase, fructose-1,6-diphosphatase and phosphoenol-pyruvate carboxykinase. The overall rate of glucogenesis in liver slices was shown to be impaired by infection and endotoxemia. The liver activity of glucose-6-phoshpatase was significantly lower in the infected and endotoxic animals than in controls. The other enzymes studied appeared unaffected. (Metabolism 17: No. 7, July, 606-611, 1968)

WE HAVE SHOWN in a previous report[1] that animals suffering from a fatal *Pseudomonas aeruginosa* infection die with depleted carbohydrate stores. This depletion is manifested by low blood sugar concentrations and depletion of liver glycogen. In vivo studies have indicated that the cause of this hypoglycemia is a failure of glucogenesis. A diminished capacity to synthesize glucose from pyruvic acid was found in these animals. Since the liver is the chief site of glucogenesis, in vitro studies of rat liver tissue were begun in order to delineate the specific metabolic lesion.

These studies included measurements of the capacity of surviving liver slices to synthesize glucose from sodium pyruvate and measurements of the activity of certain glucogenic enzymes in the liver.

Reversal of glycolysis requires the participation of several enzymes which are not operative in the forward sequence. These include glucose-6-phosphatase, fructose-1,6-diphosphatase, and phosphoenolpyruvate carboxykinase.[2] They are present in liver and not in muscle. In a situation characterized by diminished glucogenesis lowered activity of these enzymes is a likely cause.

MATERIALS AND METHODS

Sprague-Dawley male rats (200–300 Gm.) were given unlimited access to food and water until 24 hours before the experiment when food was withheld. All animals were killed by a sharp blow on the head followed by exsanguination.

The endotoxin used in these experiments was lipopolysaccharide W from *Escherichia*

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coli 0111:B4, commercially available from Difco Laboratories. We determined the LD_{50} of this preparation administered intravenously to be 1 mg./100 Gm. body weight for our rats. Severe hypoglycemia developed within three hours after injection in all rats given more than 0.5 mg./100 Gm. body weight. Death, when it occurred was at about five hours after injection, irrespective of dose.

The Walker technique[3] was used to produce infected rats. The animals were seeded with *Ps. aeruginosa*, SRU strain 12-4-4(59), beneath a 20 per cent split-thickness skin graft. The infected animals were studied on the second day postgraft. Control animals were sham-operated and also studied on the second day postgraft.

Measurement of glucogenesis in surviving liver slices. The livers were quickly removed from the rats after exsanguination and placed in a slightly modified[4] ice-cold Krebs Ringer bicarbonate solution. A block of tissue was cut from the liver and sliced with a Stadie-Riggs tissue slicer. The slices (0.4–0.5 mm. in thickness) were suspended in the Krebs solution and then two were placed in each Warburg vessel. The tissue used in each flask weighed between 100–250 mg. wet weight and 15–40 mg. dry weight.

The Warburg vessels each contained 2.5 ml. of Krebs-Ringer bicarbonate or Krebs-Ringer phosphate with added glucose (100 mg./100 ml.) and sodium pyruvate (10 mM). The side arm of the vessel held 0.5 ml. of the same solution plus 0.25 μc. of 2-C^{14} pyruvate The vessels were incubated for 10 minutes and gassed with 100 per cent O_2 or 95 per cent O_2–5 per cent CO_2 gas mixture. Then, the solution in the side arm with the 2-C^{14}-pyruvic acid was tipped into the main vessel and the reaction allowed to continue for two hours. Measurements of oxygen utilization were made with the Warburg manometer when the medium was Krebs-Ringer phosphate.

At the end of the experiment, the tissue slices were quickly removed and placed on copper planchets. These were dried overnight in a vacuum dessicator and weighed the next day.

A 2-ml. aliquot was taken from the reaction vessel immediately after removal of the slices, and deproteinized with $Ba(OH)_2$ and $ZnSO_4$. The protein free filtrate was analyzed for glucose by the *Nelson* technique.[5] Then a 2-ml. aliquot was removed and 4 mg. of carrier glucose added together with 48 mg. phenylhydrazine hydrochloride and 41 mg. sodium acetate. Phenylglucosazones were prepared by placing the tubes on a boiling water bath for 2–3 hours.[6] The crystals were collected by centrifugation, washed with water and ether, and then recrystallized five times with 30 per cent aqueous ethanol. The purified glucosazones were placed on tared copper planchets, weighed and counted in a thin window gas flow counter. The specific activity of the glucose in the medium at the end of the experiment was calculated from the formula.

$$\frac{q_G}{Q_G} = \frac{q_o}{Q_o} \times \frac{Mw(\text{glucosazone})}{Mw(\text{glucose})} \left[\frac{\text{mg. carrier glucose} + \text{mg. media glucose}}{\text{mg. media glucose}}\right]$$

where:

$\frac{q_G}{Q_G}$ = specific activity of medium glucose (cts/min.mg.).

$\frac{q_o}{Q_o}$ = specific activity of glucosazone (cts/min.mg.).

The specific activity of the medium glucose was multiplied by the total amount of glucose in the flask to obtain the total c.p.m. transferred from pyruvate to glucose.

A blank vessel from which the slices were omitted was processed with each experiment in order to check on the completeness of separation of the C^{14}-glucose from the 2-C^{14}-pyruvate. In each case the blank vessel produced glucosazone samples only a few (3–5) c.p.m. above background.

Assay procedures. Glucose-6-phosphatase was measured in whole liver homogenates. The method described by Swanson[7] was used to determine the enzymatic activity of the preparations. The reaction was stopped with 1 ml. 10 per cent TCA at 0, 5, 10, and 15 minutes. Inorganic phosphate was determined by the method of Chen.[8]

Fructose-1,6-diphosphatase was assayed in supernatant solutions of liver homogenates

Table 1.—Glucogenesis* in Liver Slices Suspended in Krebs-Carbonate Media

	Final Glucose conc. in media mg./100 ml.	Increase in amount glucose in media μmoles/2 hrs. mg.†	C^{14}-glucose synthesised μmoles/2 hrs. mg.†
Control rats (26)‡	126.9(121.2–132.6)	0.205(0.179–0.231)	0.127(0.116–0.138)
Infected rats (8)	112.0(106.6–117.4)	0.0632(0.047–0.079)	0.086(0.062–0.110)
Endotoxin injected rats (1 mg./100 Gm. body wt.) (8)	100.0(94.1–105.9)		0.089(0.065–0.113)
Endotoxin injected rats (2 mg./100 Gm. body wt.) (8)	95.2(90.0–100.4)		0.055(0.036–0.074)
Control liver + Endotoxin (4)	103.0(96.8–109.2)		0.102(0.080–0.124)

*Values are means (95 percent confidence limits of mean).
†Mg. dry weight of liver slices.
‡Numbers in parentheses refer to number of rats studied.

centrifuged at 30,000 Gm. for one hour. The method used was one modified from that of Taketa and Pogell.[9] The authors use a very low concentration (0.1 mM) of fructose diphosphate in the reaction mixture. The enzyme is maximally active at this concentration but at the same time the measured activity is very sensitive to changes in substrate concentration. In order to avoid this difficulty the procedure was modified by increasing the fructose diphosphate concentration to 1 mM and the incubation temperature from 30° C to 37° C. The reaction was stopped at 0, 5, 10, and 15 minutes by the addition of 0.2 ml. of 30% TCA. Inorganic phosphorus was determined by the method of Chen.[8]

Phosphoenolpyruvate carboxykinase was assayed by the procedure described by Nordlie and Lardy.[10]

Glucose was measured by the Nelson method.[5] Protein measurements were made by the Technicon Auto Analyzer utilizing the biuret technique.[11]

Results

The ability of surviving rat liver slices to metabolize 2-C^{14}-pyruvate to C^{14}-glucose was measured in Krebs-Ringer carbonate buffer.

Measurements were made using liver slices from control rats and from rats dying of a progressive surface infection of *Ps. aeruginosa*. Since it has been suggested that endotoxin may be a factor in the pathogenesis of gram negative infection, the glucogenic capacity was also measured in liver slices from rats injected with *E. coli* endotoxin. *E. coli* endotoxin was injected intravenously into two groups of fasted animals. One group received 1 mg./100 Gm. body weight and the other received 2 mg./100 Gm. body weight. The animals were sacrificed five hours after the injection and the liver slices studied immediately afterward.

One set of experiments using liver slices from control rats was carried out with endotoxin added to the medium (0.2 mg./3cc.) in the main chamber of the vessel. The results of these five sets of experiments are summarized in Table 1. Glucogenesis is significantly impaired in infected rat livers and in rats injected with endotoxin. There is no significant difference between the glucogenic capacity of rats injected with endotoxin (1 mg./100 Gm. body weight) and those infected with *Ps. aeruginosa* nor is there a statistically significant effect of endotoxin added to the media containing control liver slices. However, the higher dose of endotoxin does produce a more profound effect in vivo than the lower dose.

Table 2.—Glucogenesis and Oxygen Uptake* of Liver Slices Suspended in Krebs Phosphate Media

	Q_{O_2} µL./hr.mg.	Final Glucose conc. in media mg./100 ml.	Increase in amt. glucose µmoles/2 hrs. mg.†	C^{14}-glucose synthesized µmoles/2 hrs. mg.†
Control rats (7)‡	7.58(6.78–8.39)	125.0(118.3–131.8)	0.175(0.119–0.231)	0.116(0.098–0.134)
Infected rats (10)	7.41(6.72–8.10)	108.2(104.3–112.1)	0.053(0.039–0.067)	0.074(0.058–0.090)
Control rats + Endotoxin (4)	8.58(7.83–9.33)	135.2(107.7–162.7)	0.226(0.096–0.356)	0.131(0.118–0.144)

*Values are means (95 percent confidence limits of mean).
†Mg. dry weight of liver slices.
‡Numbers in parentheses refer to number of rats studied.

Table 3.—Blood Glucose* Concentrations of Rats Involved in Enzyme Study

	Blood glucose concentrations mg./100 ml.
Control rats (10)†	98.6 (94.2–102.3)
Infected rats (10)	54.0 (41.1–68.9)
Endotoxin rats (5) (2 mg./100 Gm. body wt.)	42.9 (13.1–72.7)

*Values are means (95 percent confidence limits of mean).
†Numbers in parentheses refer to numbers of rats studied.

The initial concentration of glucose in the media was 100 mg./100 ml. Net synthesis is represented by final media concentrations in excess of this amount Net synthesis of glucose occurred in the control and infected rat livers. Since this represents the sum of all glucose metabolism, the third column in the table, which measures incorporation of C^{14} from pyruvate, is considered a more accurate measure of glucogenesis.

If the livers of the infected and endotoxic rats were simply less viable than those from the control animals, these results might be expected. In order to test this possibility, the experiment was repeated in Krebs-Ringer phosphate, where oxygen utilization measurements were possible. Infected and control rat livers with and without added endotoxin (0.2 mg./3ml.) were studied and the results of the study are shown in Table 2. The impairment of glucogenesis is confirmed. Oxygen utilization by all the slices studied was normal. This suggests that the livers are equally viable and that reduced glucogenesis is not caused by lack of available energy from substrate oxidation. The results of this study appear to indicate that a major reason for the infected rats' diminished glucose production lies within the liver tissue itself.

Enzyme assays. The three glucogenic enzymes, glucose-6-phosphatase, fructose-1,6-diphosphatase and phosphoenolpyruvate carboxykinase, were assayed in control rat livers, in the livers of rats with *Ps. aeruginosa* infection, and in the rats injected intravenously with lethal doses of *E. coli* endotoxin (2 mg./100 Gm. body weight). The rats injected with endotoxin were killed five hours after the injection of the drug. Blood samples were taken from all animals immediately prior to sacrifice. These samples were assayed for glucose (Table 3).

Livers were removed from the experimental animals immediately after death and frozen within a few minutes. These frozen livers were stored in dry

Table 4.—Activities* of Glucogenic Rat Liver Enzymes

	Phosphoenolpyruvate carboxykinase†		Fructose-1, 6-diphosphatase‡		Glucose-6-phosphatase‡
	μmoles/min. mg. protein $\times 10^{-3}$	μmoles/min. Gm. liver	μmoles/min. mg. protein $\times 10^{-3}$	μmoles/min. Gm. liver	μmoles/min. Gm. liver
Control rats	6.38 (5.54–7.22) (8) §	7.38 (6.38–8.22) (8)	10.5 (9.58–11.42) (7)	12.0 (10.89–13.11) (7)	28.7 (27.0–30.4) (11)
Infected rats	5.32 (4.69–5.95) (6)	6.04 (5.31–6.77) (6)	9.34 (8.39–10.29) (4)	10.7 (9.59–11.11) (4)	12.4 (11.1–13.7) (11)
Endotoxic rats	6.00 (4.76–7.24) (3)	6.84 (5.10–8.58) (3)	10.5 (8.64–12.36) (5)	12.0 (9.89–14.11) (5)	22.5 (20.7–24.3) (6)

*Values are means (95 percent confidence limits of mean).
†Assay done at 30° C.
‡Assay done at 37° C.
§Numbers in parentheses are numbers of livers studied.

ice for a period not longer than four weeks. The results of the enzyme assays are shown in Table 4.

Phosphoenolpyruvate carboxykinase and fructose-1,6-diphosphatase are located in the soluble part of the cell. Therefore, the supernatant fractions of liver homogenate were assayed. Results of the assays are recorded in terms of units of activity per mg. of protein in this supernatant fraction. For comparison, the results are also recorded as units of activity per gram of liver. Since the enzyme glucose-6-phosphatase is attached to the microsomal fraction of the liver homogenate, the whole homogenate was assayed for activity of this enzyme and the results recorded only as units of activity per gram of liver.

Table 4 shows that the activity of glucose-6-phosphatase is significantly lower than normal in the infected and endotoxic rat livers.

There appears to be no impairment of the activity of the other two enzymes. Assay conditions were, of course, optimal for the measurement of carboxykinase and nearly optimal for fructose-1,6-diphosphatase. In vivo conditions in the infected rat might be such that one or both of these enzymes would not function optimally.

Discussion

Although the impairment in activity of glucose-6-phosphatase may be sufficient to account for the hypoglycemia of the infected animals, it does not seem to account for the equally severe hypoglycemia of the rats injected with endotoxin. Liver slices from endotoxic rats (2 mg./100 Gm. body weight) synthesize glucose at a lower rate than slices from infected rats, whereas the impairment in enzymatic activity is much less severe in the endotoxic animals. It would seem that some other factor must be responsible for the hypoglycemia caused by endotoxin.

It is possible that in both instances the key metabolic lesion in glucogenesis is at a lower level in the glycolytic pathway than glucose-6-phosphatase, perhaps involving a cofactor in the series of metabolic steps leading from pyruvate to phosphoenolpyruvate. The critical cofactors which have thus far been

identified in this series of reactions are inosine triphosphate,[5] acetyl CoA,[12] and perhaps cyclic AMP.[13] If the production of glucose were stopped at this level, it is conceivable that lowered substrate concentrations could impair the synthesis of glucose-6-phosphatase. The lowering of enzyme activity might be more severe in the infected animals since enzyme synthesis could have been slowed for a day or more, whereas the enzyme level in the animals injected with endotoxin would have had only five hours to diminish.

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