Effects of Endotoxin on Hepatic Glycogen Metabolism *in Vitro* (37009)

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(Introduced by J. R. Porter)
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Several investigators have demonstrated that the injection of endotoxin into animals results in an accelerated loss of liver glycogen (1–4). This depletion may result from (i) increased glycogenolysis, (ii) altered glyconeogenesis, or (iii) a combination of (i) and (ii). The mechanism of endotoxin action in causing glycogen depletion is unknown. Alterations in the glyconeogenic pathway in endotoxin-treated animals have been reported (5–9). Hamosh and Shapiro (4) reported elevated levels of the glycogenolytic enzyme phosphorylase (EC2.4.2.2) and Snyder, Deters and Ingle (10) demonstrated increased activity of the glycolytic enzyme, pyruvic kinase (EC2.7.1.40). Epinephrine stimulates the change of inactive phosphorylase to the active form (11) and has been implicated in endotoxic reactions (12–14). Other studies minimize the role of epinephrine in endotoxin-treated animals (3, 4, 15).

All the studies cited above were done on animals injected with endotoxin. Plaut and Goldman (16) used liver homogenates and reported a dose-related inhibition of oxidative metabolism, which they suggested caused an inhibition of the Krebs cycle or of the electron transport system.

The primary site for endotoxin action, if one exists, remains obscure. The purpose of this study was to determine the effects of endotoxin on glycogen metabolism, specifically the glycogenolytic pathway. This paper describes an *in vitro* system which offers a means of determining if the effects of endotoxin on glycogen metabolism are direct or indirect.

Materials and Methods. Endotoxins. Endotoxin extracted from *Salmonella typhimurium* by the phenol–water method of Westphal and Lüderitz was obtained commercially (Difco, Detroit, MI). Solutions were prepared the day of the experiment.

Endotoxin (LD_{50} = 500 μg/mouse) from *Serratia marcescens* and its endotoxoid (LD_{50} = 4000 μg/mouse) were kindly provided by Dr. A. Nowotny of Temple University.

The final concentration of endotoxin used in the homogenates differed with each lot and organism source. The preparations of commercial endotoxin varied from 300 to 600 μg/mg, whereas the Nowotny endotoxin was employed at 1600 μg/ml.

Animals. Female Swiss-Webster-Cox mice (Laboratory Supply, Indianapolis, IN) weighing 17–19 g were used in all studies. Tetracycline [1 g Terramycin Soluble Powder, Animal Formula, (Chas. Pfizer & Co., New York, NY)/liter H_2O] was added to the drinking water (10). After 2 days the tetracycline water was replaced with tap water. Mice were housed in metal cages on corn cob bedding at 22° and 50% relative humidity.

To minimize the effects of circadian rhythm and diurnal variation the mice were kept on a 12 hr day and 12 hr night cycle and all experiments were started at 8:30 AM unless noted otherwise.

Preparation of liver homogenates. Mice were killed by cervical dislocation; livers were removed and rinsed in the homogenizing solution (0°). Each liver was homogenized in a chilled Potter–Elvehjem homogenizer and diluted to 10 ml. The homogenizing fluid was 0.15 *M* KCl containing 0.002 *M* disodium ethylenediaminetetraacetate (EDTA). Liver homogenates were used immediately after preparation.

Development of an in vitro *model.* Livers from 10 normal mice were removed, homogenized, pooled, divided into 2 lots, and incu-

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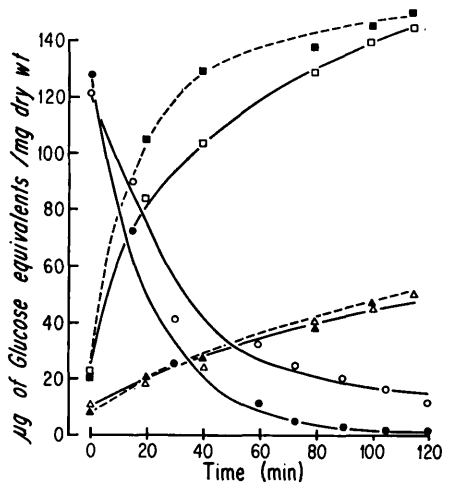

FIG. 1. *In vitro* effects of endotoxin on glycogen metabolism. Glycogen assay: (○—) control, (●—) endotoxin-treated; glucose assays: Sigma: (□—) control, (■- -) endotoxin-treated; Worthington: (△—) control, (▲- -) endotoxin-treated.

bated at 37° in a shaking water bath (model G76, New Brunswick Scientific, New Brunswick, N.J.). After 3 min incubation, the endotoxin from *S. typhimurium,* dissolved in homogenizing solution, was added to one homogenate and the other lot was treated with homogenizing solution. At various intervals, samples were removed and analyzed for glycogen and glucose.

The procedure for extraction of glycogen from liver homogenates was based on known methods (17–19).

The method of Johnson and Fusaro (20) was used for the enzymatic hydrolysis and assay of glycogen; however, double strength glucosidase reagent (PGO) (Sigma Chemical Co., St. Louis, MO) was substituted for the Worthington glucosidase reagent.

Protein was removed from tissue homogenates by the procedure described by Ashwell (21) prior to measurement of glucose and lactate.

Glucose was analyzed by two methods (i) PGO glucose kits of Sigma Chemical Co. (St. Louis, MO) and (ii) the Glucostat Special kits of Worthington Biochemical Co. (Freehold, N.J.). Lactic acid was measured by the method of Barker (22).

Total glucose phosphates were determined by measuring the phosphate released in glucose samples hydrolyzed with 0.5 ml 10.0 *N* H_2SO_4/ml test solution for 30 min at 100° (23, 24).

Thin layer chromatography. Tissue homogenates were deproteinized (21), concentrated by lyophilization, and reconstituted to one-seventh the original volume with water. Forty microliters of the test solutions and 20 μl of the standards were placed on thin layer chromatography plates of Absorbosil-1 (Applied Science Laboratories, State College, PA) impregnated with 0.2 *M* sodium acetate.

The solvent system was butanol:acetic acid:H_2O (5:4:1). The chromatograms were sprayed with diphenylamineaniline and developed by heating at 120° for 15 min.

Dry weight determination. Dry weights of liver homogenates were determined by adding a known volume of homogenate to a tared aluminum dish. After 24 hr at 110°, the dishes were cooled and weighed. Corrections were made for buffer, endotoxin, or other substances added to the liver homogenates.

Statistics. Data were analyzed by analysis of variance (25).

Results. Figure 1 shows that the endotoxin-treated liver homogenate lost glycogen faster than the control homogenate. Analysis of variance shows that there was a significant difference between treatments, time, and different homogenate pools. There was not a significant interaction between time and treatments. Data in Fig. 1 show that all of the glycogen lost was recovered as glucose when analyzed by the PGO method (Sigma) indicating an effect of endotoxin only on the glycogenolytic enzymes. Because of contamination of the Sigma product with maltase, the glucose levels in the homogenates were measured enzymatically with an assay which measured only free glucose (Worthington Glucostat Special). Results in Fig. 1 show no difference in glucose levels between the two homogenates and suggests that the effect of endotoxin was not limited to the glycogenolytic enzymes. The differences between the two methods may have been due to maltase degrading maltose moieties in glycogen or oligosaccharide present in the glucose sam-

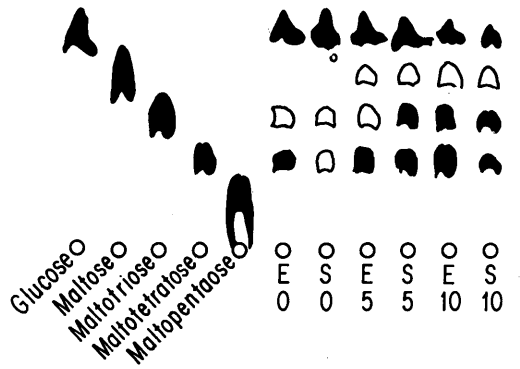

FIG. 2. Thin layer chromatogram of glucose samples at 0, 5, and 10 min. (E 0) endotoxin-treated, 0 time; (S 0) control, 0 time; (E 5) endotoxin-treated, 5 min; (S 5) control, 5 min; (E 10) endotoxin-treated, 10 min; and (S 10) control, 10 min.

ple. Addition of maltase to the Worthington product yielded results similar to that obtained with the Sigma product. There was no glycogen present in the glucose samples nor was there any glucose phosphates. The possibility of phosphatase acting on the glucose phosphates was eliminated because the glucose samples were deproteinized.

Thin layer chromatography (Fig. 2) showed the presence of oligosaccharides of the maltose series in the glucose samples. These results and the absence of a difference in lactate levels between the control and endotoxin-treated homogenates also suggested that the endotoxin effect was limited to the glycogenolytic enzymes.

To compare the *in vitro* effect of endotoxin on glycogen metabolism with endotoxin toxicity *in vivo* the effects of *S. typhimurium* and *S. marcescens* endotoxin and *S. marcescens* endotoxoid on glycogen metabolism were measured (Fig. 3). The *S. typhimurium* endotoxin caused a faster glycogen depletion than did the *S. marcescens* endotoxin and endotoxoid. Both the endotoxoid and endotoxin caused a more rapid depletion of glycogen than occurred in the control. No difference between the *S. marcescens* endotoxoid and endotoxin was observed.

Discussion. Zweifach, Nagler and Thomas (14) proposed an indirect effect of endotoxin mediated by released epinephrine, but Berry, Smythe and Young (3) did not find a relationship between epinephrine release and glycogen loss. Other studies (4) minimize the role of epinephrine in hepatic glycogen loss but they do not exclude the possibility that endotoxin may mimic the action of epinephrine.

The advantage of the model employed in this paper is that the level of endogenous substances, such as epinephrine, would be the same in both the control and endotoxin-treated homogenates. Thus, a method is provided to determine if the action of endotoxin is direct or indirect. The results reported here, also minimize the role of epinephrine as a major factor in the endotoxin-mediated loss of liver glycogen. Although it is not possible to rule out any effect of epinephrine in intact animals treated with endotoxin, these effects appear to be secondary responses to stress. Moreover, Hamosh and Shapiro (4) found that muscle glycogenolysis was markedly reduced in adrenalectomized rats treated with endotoxin.

In our study potassium methylate treatment of endotoxin (endotoxoid) did not abolish the accelerated glycogen loss produced by

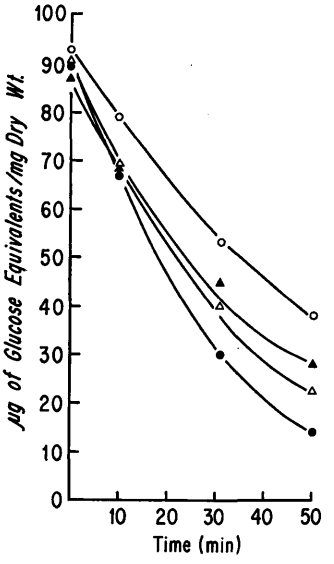

FIG. 3. Comparison of the effects of endotoxin from *S. typhimurium* with endotoxin and endotoxoid from *S. marcescens* on glycogen metabolism. (○—) Untreated control, (▲—) endotoxin from *S. marcescens*, (△—) endotoxoid from *S. marcescens*, (●—) endotoxin from *S. typhimurium*.

endotoxin in the *in vitro* system. The toxoid was as effective, or more so, than the parent toxin in accelerating glycogen loss. But potassium methylate "endotoxoid" unlike endotoxin, does not prevent cortisone-induced glyconeogenesis, and "endotoxoid" is inactive in altering enzyme activity *in vivo* (26). Skarnes (27) proposed that circulating plasma is the major site of endotoxin detoxification and it is possible that further detoxification of the "endotoxoid" may take place *in vivo*. It is equally possible that the loss of glycogen is not related to endotoxin toxicity.

Summary. An *in vitro* model employing homogenates of mouse liver was developed to study the effects of endotoxin from *Salmonella typhimurium* on glycogen metabolism. Studies with this model showed that endotoxin can act directly and that the accelerated loss of glycogen was not related to an increased release of epinephrine. These results indicated that endotoxin acts on the glycogenolytic enzymes.

Endotoxin from *Serratia marcescens* also caused a rapid loss of glycogen in the *in vitro* model but was not as effective as the typhimurium endotoxin. Potassium methylate treatment of the *S. marcescens* endotoxin did not eliminate the ability of the endotoxin to deplete glycogen.

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1. Cameron, G. R., Delafield, M. E., and Wilson, J., J. Pathol. Bacteriol. **51**, 223 (1940).

2. Kun, E., and Miller, E. P., Proc. Soc. Exp. Biol. Med. **67**, 221 (1948).

3. Berry, L. J., Smythe, D. S., and Young, L. G., J. Exp. Med. **110**, 389 (1959).

4. Hamosh, M., and Shapiro, M., Brit. J. Exp. Pathol. **41**, 372 (1960).

5. Kun, E., Proc. Soc. Exp. Biol. Med. **68**, 496 (1948).

6. Kun, E., and Abood, L. G., Proc. Soc. Exp. Biol. Med. **71**, 362 (1949).

7. Berry, L. J., and Smythe, D. S., J. Exp. Med. **110**, 407 (1959).

8. Shands, J. W., Miller, V., Martin, H., and Senterfitt, V., J. Bacteriol. **98**, 494 (1969).

9. Elliott, L., and Snyder, I. S., Proc. Soc. Exp. Biol. Med. **141**, 253 (1972).

10. Snyder, I. S., Deters, M., and Ingle, J., Infec. Immunity **4**, 138 (1971).

11. Krebs, E. G., and Fisher, E. H., Advan. Enzymol. Relat. Subj. Biochem. **24**, 236 (1962).

12. Dennis, E. W., Proc. Soc. Exp. Biol. Med. **42**, 553 (1939).

13. Thomas, L., J. Exp. Med. **104**, 865 (1956).

14. Zweifach, B. W., Nagler, A. L., and Thomas, L., J. Exp. Med. **104**, 881 (1956).

15. Meyer, M. W., and Ballin, H. M., Proc. Soc. Exp. Biol. Med. **100**, 288 (1959).

16. Plaut, M. E., and Goldman, J. K., Proc. Soc. Exp. Biol. Med. **133**, 433 (1970).

17. Good, C. A., Kramer, H., and Somogyi, M., J. Biol. Chem. **100**, 485 (1933).

18. Robbins, P. W., Traut, R. R., and Lipmann, F., Biochemistry **45**, 6 (1959).

19. Van Handel, E., Anal. Biochem. **11**, 256 (1965).

20. Johnson, J. A., and Fusaro, R. M., Anal. Biochem. **15**, 140 (1956).

21. Ashwell, G., *in* "Methods in Enzymology" (S. P. Colowick, and N. O. Kaplan, eds.), Vol. 3, p. 85. Academic Press, New York (1957).

22. Barker, S. B., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, p. 241. Academic Press, New York (1957).

23. Clark, J. M. "Experimental Biochemistry" p. 228. Freeman, San Francisco (1964).

24. Chen, P. S., Toribara, T. J., and Warner, H., Anal. Chem. **28**, 1759 (1956).

25. Steele, R. G. D., and Torre, J. H., "Principles and Procedures of Statistics," 481 pp. McGraw-Hill, New York (1960).

26. Berry, L. G., Smythe, D. S., and Colwell, L. L., J. Bacteriol. **96**, 1191 (1968).

27. Skarnes, R. C., J. Exp. Med. **132**, 300 (1970).

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