Effects of Actinomycin D on Rat Liver Alkaline Phosphatase (35295)

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(Introduced by Maurice S. Raben)

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Bile duct ligation in the rat causes a 5- to 10-fold increase in the activity of hepatic alkaline phosphatase and a lesser increase in the serum alkaline phosphatase (1, 2). The latter is due to leakage of the hepatic enzyme into the blood stream (3). The increase in hepatic alkaline phosphatase is dependent upon *de novo* protein synthesis and does not occur if protein synthesis is inhibited (2–4). This report examines the role of RNA synthesis in the changes in hepatic alkaline phosphatase induced by bile duct ligation. The data confirm a previous report (4) that the increase in this enzyme, both in liver and serum, is dependent upon intact RNA synthesis. In addition, actinomycin D has no paradoxical stimulatory effect on alkaline phosphatase induction (5, 6).

Methods. Animal experiments. Male Charles River CD^r rats, weighing approximately 100 g, were maintained on standard chow and tap water. On the day of an experiment they were anesthetized lightly with ether and the abdomen was opened through a midline incision. The common bile duct was isolated and then doubly ligated close to the liver to avoid damage to the pancreatic ducts (7). Control animals were sham operated. Groups of rats were sacrificed at varying times after operation, and the abdomen was reopened. Blood was withdrawn from the vena cava into a heparinized syringe and the livers were perfused with 20 ml of cold 0.25 *M* sucrose through a portal vein cannula. The livers were then excised, blotted dry, weighed, and minced with scissors. All animals had been fasted for 18 hr prior to sacrifice. In experiments involving inhibition of RNA synthesis, the above procedures were repeated, but actinomycin D (Lyovac-Cosmogen, Merck, Sharp and Dohme, West Point, Pa.), 50 μg/100 g of body weight, was given intramuscularly either 1 hr before surgery or at 1, 3, or 8 hr after surgery. Control animals received the normal saline carrier.

Enzyme preparations and assays. Livers were homogenized in a Waring blendor for 1 min at 2° in 5 vol of 0.25 *M* sucrose. For extraction of alkaline phosphatase, homogenates were vigorously stirred with *N*-butanol (20%, w/v) for 30 min at 2° (8). The mixture was centrifuged for 30 min at 20,000*g* and the aqueous phase was removed with a large syringe, and stored at —20° until assayed. For extraction of hepatic glutamic-pyruvic transaminase (GPT), the Waring blendor homogenates were centrifuged at 30,000*g* for 30 min at 2° and the supernatant was saved. Serum was separated from venous blood by slow speed centrifugation and similarly stored. Alkaline phosphatase activity was determined in a Gilford thermoregulated recording spectrophotometer at pH 10.2 and 30° with *p*-nitrophenyl phosphate as the substrate (9). The release of *p*-nitrophenol was followed by the rate of increase of absorption at 404 mμ. Serum glutamic-pyruvic transaminase (GPT) was determined by the method of Wroblewski and LaDue (10) and hepatic GPT as described by Kaplan and Righetti (3).

Measurement of RNA synthesis. Three hr prior to sacrifice, animals were injected intramuscularly with orotic acid-5-^{3}H (sp act 250 mCi/mmole; New England Nuclear Corp., Boston, Mass.), in doses of either 2.5 or 10 μCi/100 g of body weight. After sacrifice, the homogenized livers were divided into two equal portions for assay of alkaline phosphatase and for measurement of incorporation of radioactivity into RNA. RNA was extracted from liver homogenates using the

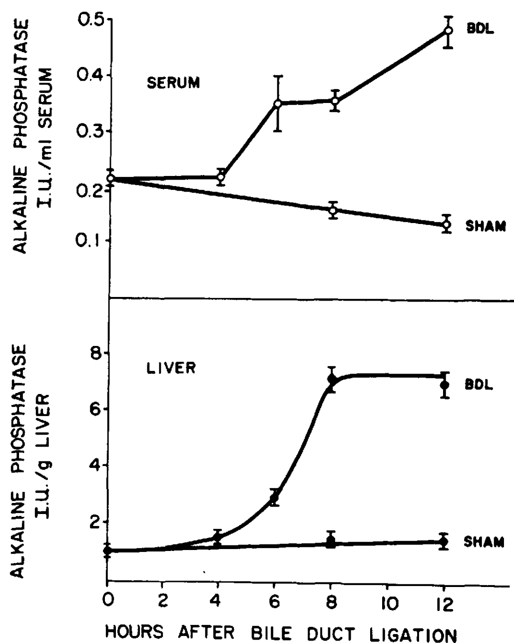

FIG. 1. Increase in serum and hepatic alkaline phosphatase after bile duct ligation: (vertical bars) mean ± 1 SEM.

method of Sleck and Munro (11). RNA concentration was estimated by measuring the absorption at 260 mμ. For determination of radioactivity, 0.5-ml aliquots of the RNA extracts were added to 15 ml of Bray's solution (12) and counted in a Nuclear Chicago Mark I liquid scintillation spectrometer. Counting efficiency, determined by the channels' ratio method, was 11.5%

Results. Bile duct ligation, as previously reported (1, 2), led to a rapid increase in hepatic alkaline phosphatase activity, which reached a plateau at 8 to 12 hr after ligation, that was 7 times greater than control values (Fig. 1). Actinomycin D, 50 μg/100 g of body weight, given 1 hr before ligation, inhibited this increase by 83% (Fig. 2). Actinomycin D had less effect when given 1 and 3 hr after ligation, causing 64 and 32% inhibition, respectively. It had no paradoxical stimulatory effect on alkaline phosphatase activity when it was given 8 hr after ligation, a time when this induced enzyme had reached plateau levels (Fig. 2). There is no statistical difference between the activities of hepatic alkaline phosphatase 12 hr after ligation in those animals receiving and not receiving actinomycin D ($p>0.2$ with Student's t test). The effects of actinomycin D on the serum alkaline phosphatase paralleled those in the liver (Fig. 3). Actinomycin D inhibited incorporation of orotic acid-5-^{3}H into hepatic RNA much less than it did induction of alkaline phosphatase, Tables I and II. Finally, this antibiotic had no effect on the

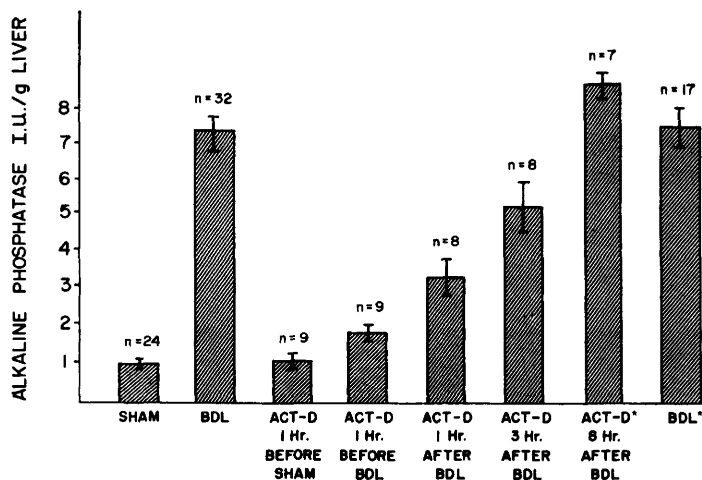

FIG. 2. Effect of Actinomycin D on hepatic alkaline phosphatase activity in bile duct ligated rats: The data are expressed as mean ± 1 SEM, with n equal to the number of animals in each group; Sham = sham operated; BDL = bile duct ligated animals. Actinomycin D (Act-D) was given either before or after operations as indicated. *These animals were sacrificed at 12 hr after operation rather than at 8 hr.

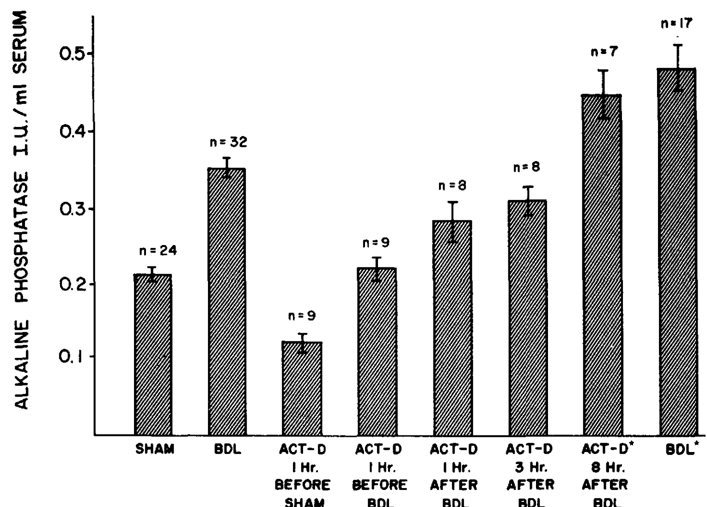

FIG. 3. Effect of Actinomycin D on serum alkaline phosphatase activity in bile duct ligated rats: The data are expressed as in Fig. 2.

bile duct ligation-produced changes in the serum and liver activities of another enzyme, glutamic-pyruvic transaminase (Fig. 4). The 5-fold increase in the serum GPT following bile duct ligation was not prevented by actinomycin D and there were no significant changes in hepatic GPT ($p>0.3$ with Student's t test).

Discussion. These data complement previously reported results with cycloheximide (3) and actinomycin D (4) and suggest that the increase in hepatic alkaline phosphatase activity following bile duct ligation is mediated by DNA-directed RNA synthesis. The increase in DNA-directed RNA synthesis must begin shortly after ligation since the inhibitory effect of actinomycin D is greatly lessened if this antibiotic is given after ligation rather than before. Its inhibitory effect when given 1 and 3 hr after ligation is only 75 and 42% of that when actinomycin D is given 1 hr after ligation.

TABLE I. Effect of Actinomycin D on the Incorporation of Orotic Acid-^{3}H into RNA.[a]

Surgical procedure	Actinomycin D	Orotic acid incorporation into RNA: dpm/mg RNA; mean ± SE	Orotic acid incorporation into RNA: % Inhibition
Sham (5)	0	6243 ± 436	—
Sham (4)	+	4294 ± 285	31
BDL (5)	0	6339 ± 306	—
BDL (4)	+	4923 ± 367	23

[a] The number of animals in each group is in parentheses. Actinomycin D, 50 μg/100 g of body weight, was given 1 hr before operation and rats were sacrificed 8 hr after surgery. Orotic acid-5-^{3}H, 10 μCi/100 g of body weight, was given 3 hr prior to sacrifice.

There was no paradoxical stimulatory effect of actinomycin D on hepatic alkaline phosphatase induction as has been described for other rat liver enzymes. Garren *et al.* (5) noted an anomalous stimulation of actinomycin D on hepatic tryptophan pyrrolase synthesis if this antibiotic was given when the induced enzyme had reached a plateau level. Thompson *et al.* (6) noted a similar effect of actinomycin D on hepatoma cell tyrosine transaminase. We looked for paradoxical stimulation of hepatic alkaline phosphatase induction but did not find it. Actinomycin D had no effect on hepatic alkaline phosphatase activity when it was given 8 hr after ligation, a time when alkaline phosphatase activity had plateaued. It inhibited the increase when it was given 3 hr after ligation, a time when hepatic alkaline activity was rapidly increasing.

The apparent discrepancy between the large degree of inhibition of alkaline phosphatase induction, 83%, and the lesser de-

TABLE II. Relation Between Time of Administration of Actinomycin D and Incorporation of Orotic Acid-5-^{3}H into RNA.[a]

Surgical procedure	Actinomycin D: Dose (μg/100 g of body wt)	Actinomycin D: Time given (hr after operation)	Orotic acid incorporation into RNA: (dpm/mg RNA; mean ± SE)	Orotic acid incorporation into RNA: % Inhibition
Sham (4)	0	—	1517 ± 103	—
Sham (4)	50	1	810 ± 51	47
BDL (5)	50	1	718 ± 112	53
Sham (3)	50	3	1075 ± 180	29
BDL (3)	50	3	731 ± 85	52

[a] The number of animals in each group is in parentheses. Orotic acid-5-^{3}H, 2.5 μCi/100 g of body weight, was given 3 hr before sacrifice and animals were sacrificed 8 hr after ligation.

gree of inhibition of RNA synthesis, 22%, in rats receiving actinomycin D 1 hr before ligation, may be due to the fact that total hepatic RNA synthesis was measured rather than cytoplasmic RNA. Paul and Struthers (13) and Revel and Hiatt (14) have shown that nuclei contain an RNA whose synthesis is resistant to actinomycin D. The persistence of RNA synthesis in this actinomycin D-resistant nuclear RNA may have masked the greater inhibitory effect of this antibiotic on DNA-directed messenger RNA. Conceivably, the messenger RNA specific for alkaline phosphatase is particularly sensitive to actinomycin D. Previous data suggests that the dosage used here, 50 μg/100 g, is sufficient to inhibit the induction of other enzymes in the liver. The same degree of inhibition of induction of rat liver tryptophan pyrrolase and tyrosine transaminase has been reported by Greengard and Acs using a slightly larger dose of actinomycin D, 70 μg/100 g of rat (15). They did not measure RNA synthesis. It was not possible to give larger amounts of actinomycin D to our rats. The liver plays a prominent role in the metabolism of actinomycin D, and bile duct ligation interferes with its detoxification (16). The dose used in

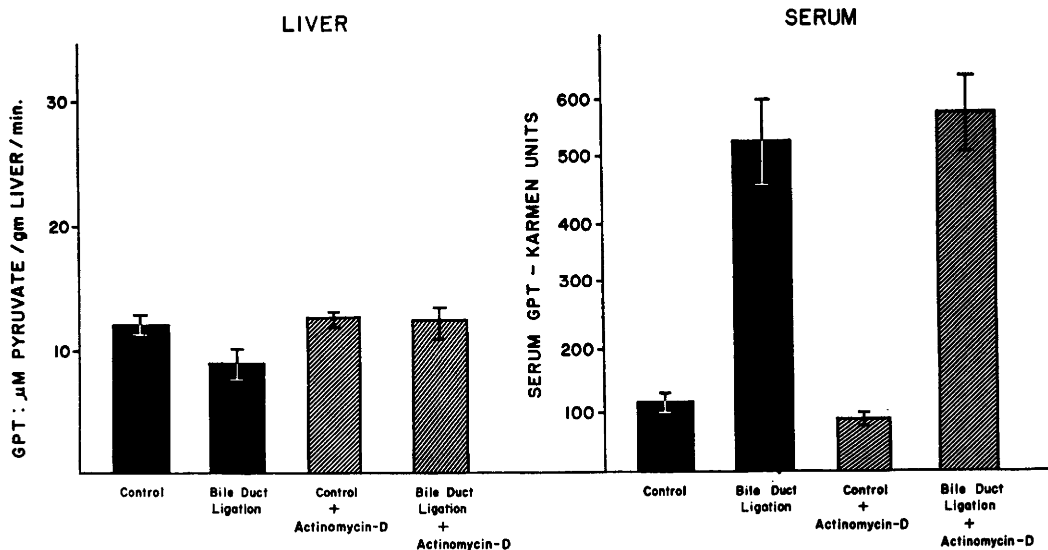

FIG. 4. Effect of actinomycin D on serum and hepatic glutamic-pyruvic transaminase: actinomycin D was given 1 hr before surgery and the animals were sacrificed 8 hr after surgery. The data are expressed as mean ± 1 SEM.

our experiments was close to the LD_{50} reported for bile duct ligated rats (16). With larger doses, the animals became extremely ill and many died several hours after injection.

Many previous studies have indicated that actinomycin D has numerous actions in addition to its effect on DNA-directed RNA synthesis (17, 18). Due to the extreme toxicity and serious side effects of this drug, interference with induction by this compound is only suggestive evidence that RNA synthesis is specifically required (19). We attempted to rule out some of the nonspecific effects of actinomycin D by examining its effect on the serum concentrations of another enzyme whose increase is thought not to be due to enzyme induction. We chose glutamic-pyruvic transaminase (GPT), whose serum increase is caused by leakage from damaged liver cells (3). Eight hr after bile duct ligation, there was a 5-fold increase in serum GPT values and a decline in liver values (Fig. 4). Actinomycin D had no effect on the response of this enzyme, either in serum or liver, to bile duct ligation. Thus, the inhibition of the alkaline phosphatase increase in serum by actinomycin D was due to more than a nonspecific poisoning of liver cells by this antibiotic.

While these data sugggest that bile duct ligation may stimulate the synthesis of a messenger RNA specific for hepatic alkaline phosphatase, they also raise other questions. First, what is the mechanism through which bile duct ligation effects this induction? Is it possible that ligation causes the accumulation of a substance normally excreted in bile and that this substance is the actual chemical inducer of alkaline phosphatase? If such a substance exists and can be identified, it might provide a clue to the function of hepatic alkaline phosphatase, an enzyme with no known physiological role. Secondarily, while enzyme induction is a reasonable explanation for the above data, other interpretations, such as enzyme activation, can be invoked. Indeed, Griffin and Cox have demonstrated that enzyme activation is responsible for the apparent induction of another alkaline phosphatase, that in HeLa cells (20). A similar explanation has been suggested for the progressive increase in duodenal alkaline phosphatase activity during neonatal development in the chick and mouse (21). Answers to these questions may be forthcoming when hepatic alkaline phosphatase is prepared in a homogeneous state.

1. Kaplan, M. M., and Righetti, A., Biochim. Biophys. Acta **184**, 667 (1969).

2. Börnig, H., Stepan, J., Horn, A., Giertler, R., Thiele, G., and Vecerek, B., Hoppe-Seyler's Z. Physiol. Chem. **348**, 1311 (1967).

3. Kaplan, M. M., and Righetti, A., J. Clin. Invest. **49**, 508 (1970).

4. Börnig, H., Horn, A., and Müller, W., Acta Biol. Med. Ger. **22**, 537 (1969).

5. Garren, L. D., Howell, R. R., Tomkins, G. M., and Crocco, R. M., Proc. Nat. Acad. Sci. U.S.A. **52**, 1121 (1964).

6. Thompson, E. B., Tomkins, G. M., and Curran, J. F., Proc. Nat. Acad. Sci. U.S.A. **56**, 296 (1966).

7. Lambert, R., "Surgery of the Digestive System in the Rat" p. 110, 127. Thomas, Springfield, Ill. (1965).

8. Morton, R. K., Biochem. J. **57**, 595 (1954).

9. Bessey, O. A., Lowry, O. H., and Brock, M., J. Biol. Chem. **164**, 321 (1946).

10. Wroblewski, F., and LaDue, J. S., Proc. Soc. Exp. Biol. Med. **91**, 569 (1956).

11. Sleck, A., and Munro, H. N., Biochim. Biophys. Acta **55**, 571 (1962).

12. Bray, G. A., Ann. Biochem. **1**, 279 (1960).

13. Paul, J., and Struthers, M. G., Biochem. Biophys. Res. Commun. **11**, 135 (1963).

14. Revel, M., and Hiatt, H. H., Proc. Nat. Acad. Sci. U.S.A. **51**, 810 (1964).

15. Greengard, O., and Acs, G., Biochim. Biophys. Acta **61**, 652 (1962).

16. Lahiri, S. R., Bolton, N., Brown, B. L., and Rosenoer, V. M., Gastroenterology **58**, 1021 (1970) Abstr.

17. Yamado, C., Clark, A. J., and Swendseid, M. E., Science **158**, 129 (1967).

18. Pastan, I., and Friedman, R. M., Science **160**, 316 (1968).

19. Harris, H., "Nucleus and Cytoplasm." Oxford Univ. Press (Clarendon), London (1968).

20. Griffin, M. J., and Cox, R. P., Proc. Nat. Acad. Sci. U.S.A. **56**, 946 (1966).

21. Moog, F., and Grey, R. D., Biol. Neonatorum **9**, 10 (1966).

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