Induction of Rat Liver Alkaline Phosphatase by Bile Duct Ligation

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Bile duct ligation causes a five- to sevenfold increase in the activity of rat liver alkaline phosphatase within 12 hours after ligation and a similar rise in the activity of alkaline phosphatase in serum. The increased serum activity is due entirely to the appearance of a new isoenzyme that has the properties of rat liver alkaline phosphatase. The increase in both serum and liver alkaline phosphatase is prevented by the prior administration of cycloheximide in a dose that inhibits protein synthesis by 70%. Rat liver alkaline phosphatase was then purified to homogeneity. Antibody was raised to purified rat liver alkaline phosphatase in rabbits. The antibody was coupled to sepharose 4B and affinity columns made. 3-H-leucine was then injected into the portal veins of sham operated rats and rats with bile duct ligation four hours after ligation. One hour after injection and five hours after ligation, animals were sacrificed. Liver alkaline phosphatase was purified by means of affinity chromatography and double immunoprecipitation with rabbit antibody to rat liver alkaline phosphatase and goat anti-rabbit gamma globulin. Bile duct ligation increased the incorporation of 3-H-leucine into liver alkaline phosphatase more than threefold compared with sham operated rats, 164 CPM/mg protein vs. 49 CPM/mg protein (p < .001). The data indicate that the increased activity of rat liver alkaline phosphatase after bile duct ligation is due to enzyme induction rather than to activation of a pre-existing, relatively inactive enzyme.

Doctor Klatskin, colleagues, and guests, I am pleased to be here, to be able to share in these festivities, and, most important, to repay, in part, the debt I owe Gerry for the training, guidance, and inspiration he provided. Above all, I want to thank him for serving as a role model, a function that he undoubtedly provided to all the Fellows who had the good fortune to train with him.

I would like to review today some of the work I have done on alkaline phosphatase (AP) regulation in liver disease. I first became interested in alkaline phosphatase when I was a trainee with Gerry in the mid 60's. At that time there was still some question about the cause of the elevated serum alkaline phosphatase in liver disease. Most data indicated that alkaline phosphatase elevation in obstructive jaundice was due to leakage of liver alkaline phosphatase into serum [1,2]. However, many still held to an earlier belief, that the liver cleared alkaline phosphatase from serum much as it did bilirubin and that the obstructed or sick liver was unable to do this [3]. To

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resolve this question, we used as a model rats with bile duct ligation. The rat proved to be a fortuitous model because there is no liver alkaline phosphatase in the serum of normal rats [4]. Rather, all of the serum alkaline phosphatase comes from bone and intestine [4].

In the experiments that will be described below, rat livers were homogenized in a Waring blender for one minute in 0.25 M sucrose, 1 part liver to 5 parts sucrose (w/v), and then extracted for 30 minutes in 1-butanol, 20% v/v. This extract was then spun at 20,000 × g for 20 minutes in a Sorvall refrigerated centrifuge. The aqueous phase was removed and dialyzed overnight against several changes of 0.01 M Tris-HCl, pH 7.5.

Figure 1 shows the change in alkaline phosphatase isoenzymes in serum after bile duct ligation. Alkaline phosphatase isoenzymes were separated by means of polyacrylamide gel electrophoresis and stained specifically for alkaline phosphatase activity as previously described [5]. The control animals demonstrated only one band of alkaline phosphatase activity. This actually consists of two isoenzymes, one from intestine and one from bone [4]. After 4 hours of bile duct ligation, a new isoenzyme is visible which migrates with liver alkaline phosphatase and is distinct from intestinal alkaline phosphatase. This isoenzyme then increases dramatically with time, peaking between 12 to 24 hours. We have purified this isoenzyme and shown that it is identical to liver alkaline phosphatase [6]. Figure 2 shows the results of a densitometric scan of the gel. It demonstrates that the liver isoenzyme increases dramatically and that the activity of the other isoenzymes decreases. The loss of alkaline phosphatase reflects the decline in intestinal alkaline phosphatase following bile duct ligation [7].

We next quantitated the activity of alkaline phosphatase both in serum and in liver. This is demonstrated in Fig. 3. The activity of rat liver alkaline phosphatase

![FIG. 1. Electrophoresis of serum, liver and intestinal alkaline phosphatase on polyacrylamide gel slabs. Sera were pooled from rats which had undergone bile duct ligation and 10 microliter aliquots applied to the gel. The times shown refer to the duration of bile duct ligation. Alkaline phosphatase bands were developed by sequential incubation in alpha naphthyl phosphate and a diazonium dye at pH 10.](image-url)
increased from 1 to 7 units per gram liver after bile duct ligation. There was a similar increase in the liver isoenzyme of alkaline phosphatase in serum. The serum assays were done after liver alkaline phosphatase had been separated from the other isoenzymes by polyacrylamide gel electrophoresis.

We next asked the question: Is this increase dependent upon intact protein synthesis? The answer is yes. Figure 3 shows that the increase in both liver and serum alkaline phosphatase is prevented if protein synthesis is inhibited. The bottom curves were derived from animals with bile duct ligation that had received cycloheximide in a dose that inhibited protein synthesis by 70 percent [6]. Cycloheximide had no effect on either liver or serum alkaline phosphatase activity in control animals [4].

While it is always dangerous to extrapolate from the rat to man, Table 1 indicates that the same increase in AP activity occurs in people with obstructive jaundice. In this case we assayed alkaline phosphatase in the livers of 12 individuals who had no obvious liver disease and who were explored primarily for Hodgkin's disease staging. In these 12 patients, alkaline phosphatase activity was 2.9 units per gram of liver. This was significantly lower than the alkaline phosphatase activity in the livers of 10 individuals who had bile duct obstruction, 11 units per gram, $p < .001$.

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**FIG. 2.** Relative change in alkaline phosphatase isoenzymes in serum after bile duct ligation. Total serum alkaline phosphatase activity was quantitated by densitometric scanning of the gels. Activity was expressed as a percentage of the total activity at each time point. * Percent alkaline phosphatase activity in slow (control) band shown in Fig. 1. o Percent alkaline phosphatase activity in more anodal (liver) band shown in Fig. 1.

**FIG. 3.** Change in alkaline phosphatase activity in serum and liver after bile duct ligation and the administration of cycloheximide, 1 mg/kg body weight. * Bile duct ligation alone; o bile duct ligation plus cycloheximide given one hour before operation.
We next sought to find the mechanism of this increased alkaline phosphatase activity; that is, whether it was due to increased de novo synthesis of alkaline phosphatase, activation of a proenzyme or due to retention within the liver of alkaline phosphatase that is normally secreted into bile. The latter explanation was easily disproved. We cannulated bile ducts of 6 rats and measured alkaline phosphatase excretion in bile, Table 2. We found that the accumulation of alkaline phosphatase in the liver was 246-fold greater than that secreted into bile during the first 12 hours of ligation, a time when liver alkaline phosphatase activity had peaked [6]. In recovery experiments, we showed that bile did not inactivate liver, intestinal, or bone alkaline phosphatase activity.

To decide between increased de novo synthesis and activation (a mechanism believed by others to be responsible for the increased activity in rat liver [8] and in HeLa cell alkaline phosphatase stimulated by prednisolone [9]), we next purified rat liver alkaline phosphatase to homogeneity. Rat liver alkaline phosphatase was purified 45,000-fold with a 25 percent recovery [10]. Figure 4 is one of several demonstrations that the enzyme was pure. As much as 100 micrograms of purified enzyme migrated as one band on analytical polyacrylamide gel electrophoresis. The purified enzyme also stained for alkaline phosphatase activity [10]. Figure 5 shows the results of a meniscus depletion sedimentation equilibrium experiment with the purified enzyme. It was performed on a Beckman-Spinco model E ultracentrifuge at 5°C using an external loading, 6-channel centerpiece and a critically aligned Rayleigh optical bench. Molecular weights were calculated from plots of the natural logarithm of the vertical fringe displacement against the square of the distance from the center of rotation. The fact that the points fall on a straight line is an indication of the purity of the enzyme. The data indicated a protein of 154,000 Daltons that was at least 98 percent homogeneous.

We next raised antibodies to this purified enzyme in rabbits and purified the antiserum using standard immunologic techniques. Following that we made specific

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**TABLE 1**
Alkaline Phosphatase Activity in Human Liver

| Patients          | n | AP IU/g liver | AP IU/mg protein |
|-------------------|---|---------------|------------------|
| "Normal"          | 12| 2.9 ± 0.4     | 0.044 ± 0.005    |
| Bile duct obstruction | 10| 11.1 ± 1.2    | 0.137 ± 0.034    |

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**TABLE 2**
Alkaline Phosphatase (AP) Output in Bile Compared to Accumulation in Liver Following Bile Duct Ligation

| Units/g liver                      |
|------------------------------------|
| 1. Base Level Hepatic AP Activity  | 0.800 |
| 2. Hepatic AP Activity after 12 Hours of Bile Duct Ligation | 7.000 |
| 3. Increase in Hepatic AP Activity Following 12 Hours of Ligation | 6.200 |
| 4. AP Output in Bile per 12 Hours  | 0.025 |
| 5. Increase in Hepatic AP/Bile AP Output | 6.200/0.025 = 246 |
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FIG. 4. Analytical polyacrylamide gel electrophoresis of purified rat liver alkaline phosphatase. The top gel was stained for alkaline phosphatase activity and the lower gel stained for protein with Coomassie Blue. Fifty micrograms of purified alkaline phosphatase were applied to the gel.

alkaline phosphatase affinity columns by coupling the purified antiserum to sepharose 4B. When this was accomplished, we were set for our critical experiment, to see if there was increased synthesis of alkaline phosphatase. Since alkaline phosphatase comprises only a tiny fraction of liver protein, approximately 1/500,000, we had to inject preposterous amounts of radioactivity into the portal veins of rats, 5 millicuries per rat. We did so 4 hours after ligation or sham operation. One hour later we sacrificed the rats and extracted alkaline phosphatase from the livers.

Figure 6 demonstrates the results of one phosphatase affinity column. In this experiment 3.5 units of alkaline phosphatase and between 10 to 20 million counts

FIG. 5. Meniscus depletion sedimentation equilibrium analysis of purified rat liver alkaline phosphatase. *Top panel*, variation in the weight average molecular weight as a function of position. **Lower panel**, natural log (fringe displacement) as a function of position for rat liver alkaline phosphatase in 0.01 M Tris-HCl, pH 7.4. The protein concentration was 0.3 mg per ml. The ultracentrifuge was operated at 20,000 rpm for 28 hours at 4.8°.
were applied to each of 10 identical columns. The columns were washed with a dilute tris buffer which eluted 99.8 percent of the radioactivity and less than 30 percent of the alkaline phosphatase. After counts in the eluant had reached background, the columns were washed with a 1 molar phosphate buffer, pH 11. This removed about one-tenth of 1 percent of radioactivity and 30 percent of alkaline phosphatase. In subsequent experiments, using larger columns and sodium triacetate instead of phosphate, closer to 90 percent of the alkaline phosphatase has been recovered.

We next purified further this alkaline phosphatase by sequential immunoprecipitation using our rabbit antiserum and goat anti-rabbit antibody. The double antibody technique precipitated approximately 95 percent of alkaline phosphatase activity, Table 3. In the BDL animals, .0089 of .01 units were precipitated and slightly more in the sham rats. Only 37 percent of the counts were precipitated from the bile duct ligated animals, versus 26 from the sham operated. This suggests that at least 65 percent of the material we had recovered from our affinity column was not alkaline phosphatase. We were able to assay the alkaline phosphatase in the immunoprecipitate and to express the \( ^3 \)H-leucine in the immunoprecipitate as CPM/mg protein, CPM/gm wet weight liver and CPM/unit alkaline phosphatase. The data in Table 4 indicate that bile duct ligation does cause an increased synthesis of alkaline phosphatase; that is, an increased incorporation of tritiated leucine into alkaline phosphatase.

**TABLE 3**

| Experimental Condition | n | AP Added (units) | AP Precipitated (units) | CPM Added | CPM Precipitated | % CPM Precipitated |
|------------------------|---|------------------|-------------------------|-----------|------------------|-------------------|
| BDL                    | 5 | 0.01             | 0.0089 ± 0.0002         | 310 ± 54  | 116 ± 17         | 37 ± 4 |
| Sham                   | 5 | 0.01             | 0.0098 ± 0.0003         | 341 ± 36  | 88 ± 9           | 26 ± 1 |
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TABLE 4
Incorporation of $^3$H-leucine into Alkaline Phosphatase

| Experimental Condition | n  | CPM/mg prot  | CPM/g liver | CPM/unit AP |
|------------------------|----|--------------|-------------|-------------|
| BDL                    | 5  | 164 ± 17     | 32,128 ± 3,358 | 12,891 ± 1,668 |
| Sham                   | 5  | 49 ± 8       | 9,070 ± 1,446  | 8,939 ± 898   |

$p < 0.001$  $p < 0.001$  $p < 0.1$

When expressed as counts per minute per milligram protein, there were 164 counts in the ligated animals versus 49 in the sham ($p < .001$). When expressed as counts per gram liver, the difference was equally striking, approximately 32,000 versus 9,000 counts ($p < .001$). Expressed as counts per minute per unit alkaline phosphatase, there was no significant difference between ligated and sham rats. This is what one would expect in pulse label experiments if enzyme induction were responsible for an increase in enzyme activity. With enzyme activation or decreased enzyme catabolism, there would be a significantly greater amount of radioactivity in the sham animals.

As a final proof of increased synthesis, we had to demonstrate that the radioactivity we were measuring was truly in alkaline phosphatase. We did this by purifying further on preparative polyacrylamide gel electrophoresis the alkaline phosphatase which we had eluted from our affinity columns. We were then able to show by means of sodium dodecylsulfate polyacrylamide gel electrophoresis that the $^3$H-leucine and alkaline phosphatase activity migrated as one peak. We calculated the radioactivity in alkaline phosphatase isolated by this technique. The data were almost identical to those in the earlier double antibody experiments; that is, three times as much tritiated leucine in ligated rat alkaline phosphatase as in shams.

I believe these data show clearly that alkaline phosphatase is one of the few enzymes whose synthesis is increased in the liver in response to bile duct ligation. While there is little data in humans as yet, our preliminary data indicate that a similar mechanism pertains.

REFERENCES

1. Polin SG, Spellberg MA, Teitelman L, et al: The origin of elevation of serum alkaline phosphatase in hepatic disease: an experimental study. Gastroenterology 42:431–438, 1962
2. Sebesta DG, Bradshaw FJ, Prockop DJ: Source of the elevated serum alkaline phosphatase in biliary obstruction: studies using isolated liver perfusion. Gastroenterology 47:166–170, 1964
3. Gutman AB: Serum alkaline phosphatase activity in diseases of the skeletal and hepatobiliary systems. A consideration of the current status. Am J Med 27:875–901, 1959
4. Righetti ABB, Kaplan MM: The origin of the serum alkaline phosphatase in normal rats. Biochim Biophys Acta 230:504–509, 1971
5. Kaplan MM, Rogers L: Separation of the serum alkaline phosphatases by electrophoresis on polyacrylamide gel slabs. Lancet 2:1029–1031, 1969
6. Kaplan MM, Righetti A: Induction of rat liver alkaline phosphatase: the mechanism of serum elevation in bile duct obstruction. J Clin Invest 49:508–516, 1970
7. Fishman WH, Green S, Inglis N: Decline in rat-serum alkaline phosphatase following bile duct ligation. Biochim Biophys Acta 6:429–431, 1962
8. Ferwerda W, Stepan J: Study on the mechanism of induction of alkaline phosphatase activity in rat liver after bile flow obstruction. Hoppe-Seyler's Z Physiol Chem 354:1462–1472, 1973
9. Griffin MJ, Cox RP: Studies on the mechanism of hormone induction of alkaline phosphatase in human cell cultures. II. Rate of enzyme synthesis and properties of base level and induced enzymes. Proc Natl Acad Sci USA 56:946–953, 1966
10. Ohshuku A, Langerman N, Kaplan MM: Rat liver alkaline phosphatase—purification and properties. J Biol Chem 249:7174–7180, 1974