Induction of Rat Liver Alkaline Phosphatase: the Mechanism of the Serum Elevation in Bile Duct Obstruction

MARSHALL M. KAPLAN and ADRIANA RIGHETTI

From the Gastroenterology Unit, Department of Medicine, Tufts-New England Medical Center, Boston, Massachusetts 02111

ABSTRACT Bile duct ligation in the rat leads to a rapid increase in hepatic and serum alkaline phosphatase activity. Within 12 hr after bile duct ligation, hepatic alkaline phosphatase has increased 7-fold and serum alkaline phosphatase activity 2½-fold. The elevation in the serum activity is completely due to an increase in an isozyme that appears to originate in the liver. This serum isozyme and liver phosphatase, both partially purified by DEAE-cellulose column chromatography, have identical Michaelis constants, pH optima, and rates of heat denaturation. These isozymes migrate identically when subjected to electrophoresis on polyacrylamide gel, and their migration rates are equally slowed after neuraminidase digestion. The data suggest that the rise in hepatic alkaline phosphatase activity is dependent on *de novo* protein synthesis. Cycloheximide, in a dose that inhibited incorporation of leucine-^{14}C into protein by 68%, inhibited the rise in liver phosphatase by 98% and that in serum by 80%. The rise in liver phosphatase activity could not be accounted for by simple retention of alkaline phosphatase that would normally appear in bile. The rise in liver activity after bile duct ligation was 240 times greater than the amount of phosphatase that normally appears in bile over a similar period of time. Cycloheximide had no effect on the bile duct ligation–induced changes in the serum and liver glutamic pyruvic transaminase.

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INTRODUCTION

Elevation of alkaline phosphatase activity in the serum has been associated with obstructive liver disease for almost 40 yr (1). Despite numerous investigations of this problem, the mechanism of this elevation is still unclear. Two opposing theories have been proposed. The first, the "retention" theory, states that the elevated serum phosphatase results from inability of the damaged liver to excrete phosphatases made in other tissues such as bone and intestine (2). The second, the "regurgitation" theory, assumes that the elevated serum phosphatase originates in the liver (3). Most recent studies have supported the latter theory (4–7). In a preliminary communication we have suggested that the rise in the serum alkaline phosphatase after bile duct ligation is dependent on a simultaneous increase in the activity of hepatic phosphatase (8).

This current study demonstrates that the elevated serum phosphatase in bile duct obstruction originates in the liver and that its rise in serum is due to induction of this enzyme within the liver.

METHODS

Animal experiments. Male Charles River CD rats, weighing approximately 100 g, were anesthetized with ether. Through a midline incision, the common bile duct was isolated and doubly ligated as close to the liver as possible to minimize damage to the pancreatic ducts (9). Control animals were sham operated. Groups of 10 rats were sacrificed at 0, 2, 4, 6, 8, 12, 18, and 24 hr after operation and then groups of five rats daily up to 5 days. Immediately after sacrifice, the abdomens were opened and blood withdrawn from the inferior vena cava. After the blood had clotted, it was centrifuged and the serum removed and stored at −15°C. The livers were excised, weighed, minced with scissors, and washed for 30 min in ice cold 0.25 M sucrose. In some experiments, kidneys, ribs, and small intestines were also removed. All animals had been maintained on standard

chow and tap water before surgery and were fasted for 18 hr before sacrifice.

In other experiments, rats were similarly anesthetized, but the bile duct was cannulated close to the liver with polyethylene tubing, Type PE 10 (Clay-Adams, Inc., New York). The cannula was secured with silk ties and led out through the lower end of the midline incision. Bile was collected at room temperature and stored at −15°C. These animals were restrained in Bollman cages (10) and fed regular chow and tap water.

Enzyme preparations. Livers, small intestines, kidneys, and ribs were excised and the intestinal mucosa was scraped free from the muscular coat with glass slides. Tissues were minced with scissors, washed in cold 0.25 M sucrose, homogenized in a Waring Blendor for 1 min at 2°C in 5 volumes of 0.25 M sucrose, and then extracted with *N*-butanol, 25%, w/v for 30 min at 2°C (11). The butanol mixture was centrifuged for 30 min at 20,000 *g* and the aqueous phase removed with a large syringe and stored at −20°C.

For extraction of liver GPT (glutamic pyruvic transaminase) Waring Blendor homogenates, prepared as above, were centrifuged at 30,000 *g* for 30 min at 2°C and the supernatant saved. To determine the intracellular localization of alkaline phosphatase, intracellular fractions of liver were isolated from Potter-Elvehjem homogenates by sedimentation in sucrose as previously described (12).

Assays. Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate (13) at 30°C and pH 10.2 in a Gilford thermostated spectrophotometer. Serum GPT was determined by the method of Wroblewski and LaDue (14). For determination of GPT activity in liver extracts this method was modified as follows: lactic dehydrogenase and NADH (reduced diphosphopyridine nucleotide) were omitted from the incubation mixture; after 20 min at 30°C, the reaction was stopped by the addition of 1.8 M trichloroacetic acid, 0.1 ml/1.2 ml of incubation mixture; after the denatured protein was spun down, aliquots of the supernatant were assayed for pyruvate with NADH and lactic dehydrogenase.

Protein was assayed by the method of Lowry, Rosebrough, Farr, and Randall (15).

Measurements of protein synthesis. To determine the relationship between *de novo* protein synthesis and increases in liver alkaline phosphatase activity, rats were injected intramuscularly with cycloheximide (Sigma Chemical Co., St. Louis, Mo.), 100 μg/100 g body weight, 1 hr before bile duct ligation and sacrificed 8 hr after ligation. Control animals were injected with normal saline. 3 hr before sacrifice, each animal was injected intramuscularly with 2 μg of DL-leucine-1-^{14}C (SA 25.8 mCl/mmole, (New England Nuclear Corp., Boston, Mass.). After sacrifice, protein was extracted from livers and its radioactivity measured as described by Posner and Fanburg (16). Counting efficiency, determined by the channel's ratio method, was 65%.

Identification of alkaline phosphatase isozymes. Electrophoreses were carried out as previously described on 3-mm thick, 7½% polyacrylamide-gel slabs in a water-cooled vertical gel electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.) (17). Only nonspecific alkaline phosphatase stains and the phosphatase isozymes appear as brown bands. Isozyme activity was quantitated by densitometry. Photographs of the gels were taken on Polaroid Type 46-L projection film (Polaroid Corporation, Cambridge, Mass.) and the transparencies scanned on a microzone densitometer (Beckman Instruments, Inc., Fullerton, Calif.).

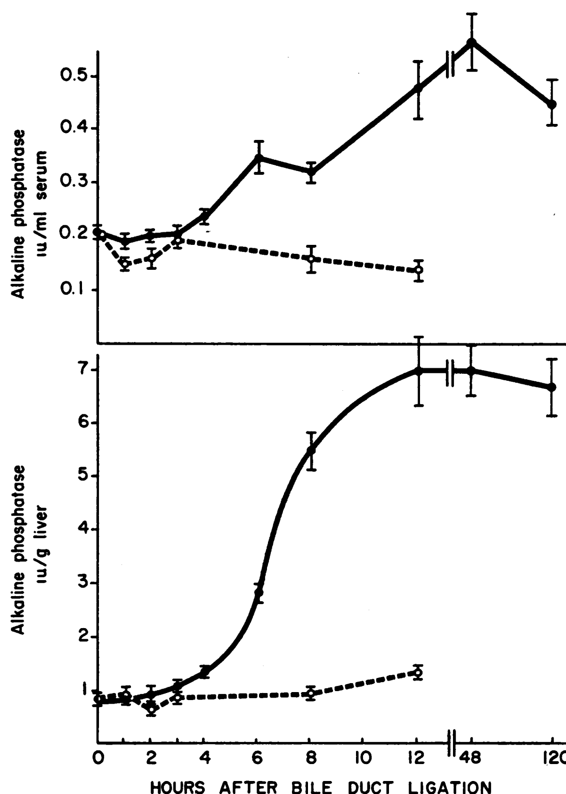


FIGURE 1 Changes in alkaline phosphatase activity in serum and liver after bile duct ligation. ●——●, bile duct ligated animals; ○- - -○, sham-operated animals.

Alkaline phosphatase isozymes in serum and liver were partially purified by chromatography on DEAE-cellulose (Whatman, DE 52, H. Reeve Angel & Co., Inc., Clifton, N. J.). Columns, 44 × 2.3 cm, were packed by gravity and equilibrated with 0.01 M Tris-HCl, pH 7.5. Serum and liver extracts were dialyzed overnight against this same buffer and then applied to the column. Alkaline phosphatase was eluted with a linear gradient of sodium chloride, 0–0.4 moles/liter, in 0.01 M Tris-HCl, pH 7.5. Fractions containing alkaline phosphatase were pooled and concentrated by ultrafiltration through ¼ inch dialyzer tubing (Arthur H. Thomas Co., Philadelphia, Pa.).

Heat inactivation studies (18), neuraminidase digestions (19), and determination of Michaelis constants (20) were done as previously described. Neuraminidase was Sigma type V (Sigma Chemical Co.).

RESULTS

Effects of bile duct ligation on liver and serum alkaline phosphatase. Hepatic alkaline phosphatase activity began to rise within 2 hr after bile duct ligation and continued to do so for approximately 12 hr, at which time alkaline phosphatase activity was more than seven times that of control values (Fig. 1). This new level was maintained for at least 5 days. The change in the

Table I
Intracellular Distribution of Rat Liver Alkaline Phosphatase

Cell, fraction	Alkaline phosphatase			
	Bile duct ligated		Sham operated	
	IU/g liver	*% total*	*IU/g liver*	*% total*
Nuclei and cell membranes	2.84 ±0.25	54.0	1.00 ±0.13	65.0
Mitochondria	0.28 ±0.05	6.3	0.04 ±0.03	2.6
Microsomes	1.65 ±0.37	35.8	0.44 ±0.06	28.5
Supernatant	0.18 ±0.01	3.9	0.06 ±0	3.9

serum alkaline phosphatase activity was similar to that in the liver (Fig. 1). However, it rose more slowly and to a lesser degree, about 2½ times that of control animals.

Approximately 90% of the alkaline phosphatase activity within the liver was found in the microsomal and cell membrane fractions. There was little change in this distribution after bile duct ligation (Table I). Larger rats, weighing 375 g, were used in this latter experiment, and the increase in liver alkaline phosphatase 12 hr after bile duct ligation was only three times that of control animals. Although the cell membranes were not separated from the nuclei in these experiments, it has already been shown that alkaline phosphatase activity in this so-called nuclear fraction is actually confined to the cell membranes (21).

Identification and isolation of the elevated alkaline phosphatase in serum. The increase in alkaline phosphatase activity in the serum correlated with the appearance of a new isozyme which had the same electrophoretic mobility on polyacrylamide gel as hepatic alkaline phosphatase and different mobility than bile, intestine, bone, and kidney alkaline phosphatase (Fig. 2). Densitometric analysis indicated that the rise in the serum phosphatase was due completely to this new isozyme (Fig. 3). The activity of the other serum isozyme identified with gel electrophoresis declined during the same period. This isozyme is presumed to be of intestinal origin (22). Its decline is thought to reflect the lack of entry of this isozyme from intestinal lymph into serum because of fasting (23).

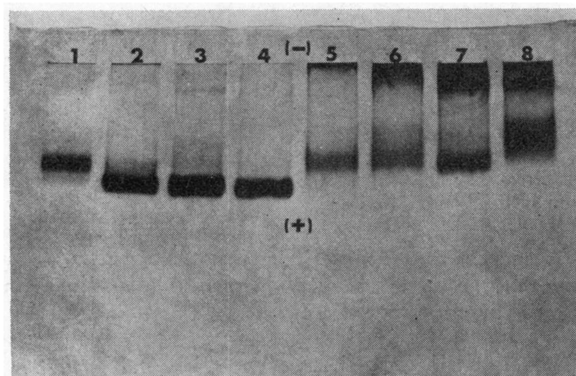
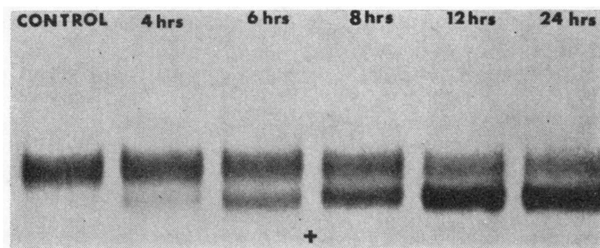


Figure 2 Electropherogram of rat serum and tissue alkaline phosphatases. Alkaline phosphatase was extracted from tissues as described in Methods. Preparations were adjusted to a concentration of approximately 0.15 IU/ml with 20% sucrose and 10-μl aliquots were applied to the gel. Sample slot: 1, serum from sham-operated rat; 2, serum after 24 hr of bile duct ligation; 3, liver from sham-operated animal; 4, liver after 24 hr of bile duct ligation; 5, bile; 6, intestinal mucosa; 7, bone; 8 kidney.



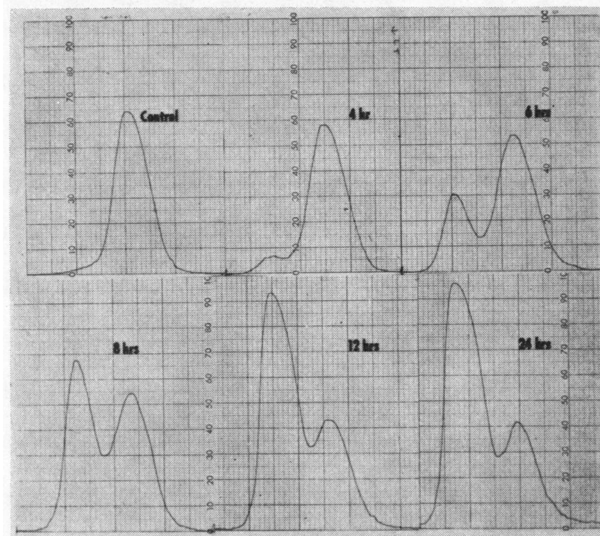


Figure 3 Densitometry of serum alkaline phosphatase isozymes after bile duct ligation. Sera from animals used in Fig. 1 were pooled and 10-μl samples of each were subjected to electrophoresis. After the isozyme bands were developed, the gel was photographed and scaned as described in Methods. The anodal band is on the left of each scan and first appears after 4 hr of bile duct ligation.

To determine whether this new isozyme produced by bile duct ligation was of hepatic origin, it was purified by chromatography on a DEAE-cellulose column (Fig. 4). The serum phosphatase isolated in this manner was free of other phosphatase isozymes when subjected to electrophoresis on polyacrylamide gel. Its specific activity increased from 0.0076 to 0.0434 IU/ml. Alkaline phosphatase extracted from liver was similarly purified. Its specific activity increased from 0.36 to 1.37 IU/mg.

Both of these electrophoretically homogeneous enzymes were used in subsequent experiments.

Properties of serum and liver phosphatases purified on DEAE-cellulose. The serum and liver phosphatases have similar pH optima (Fig. 5), identical Michaelis constants (Fig. 6), and both were inactivated at equal rates by heating at 56°C (Fig. 7). The electrophoretic mobilities of both were equally slowed after incubation overnight in neuraminidase, although incubation at pH 5.6 without neuraminidase revealed slight differences in stability between the serum and liver isozymes (Fig. 8). By these criteria, the new serum alkaline phosphatase appearing after bile duct ligation was virtually identical with the phosphatase isolated from liver.

Mechanism of the serum phosphatase elevation. When it was established that the elevated serum alkaline phosphatase after bile duct ligation originated in the liver, experiments were undertaken to determine the biochemical mchanism responsible for this serum elevation. Two possibilities were considered: (*a*) that the rise resulted from simple regurgitation into serum of alkaline phosphatase normally excreted into the bile; and (*b*) that the increase in the serum phosphatase was dependent on an increase in the activity of this enzyme in the liver which, in turn, was dependent on *de novo* protein synthesis.

To test the first alternative, simple regurgitation of alkaline phosphatase from bile, a comparison was made between the amount of alkaline phosphatase normally excreted in bile and the increase in liver phosphatase resulting from bile duct ligation. Bile was collected from six rats through bile fistulas and alkaline phosphatase activity was determined. 200-g rats were used in this experiment to facilitate the bile duct cannulation. The results were normalized by expressing excretion as enzyme units per gram of liver. These rats excreted approximately 0.025 IU of alkaline phosphatase per gram of liver per 12 hr (Table II). During the same period of time, the increase in liver alkaline phosphatase activity resulting from bile duct ligation was 6.0 IU/g liver (Fig. 1) and that in serum was 0.28 IU/g liver. This latter figure can be derived directly from Fig. 1 since the serum volume in ml of the 100 g rat is approximately equal to the liver weight in grams (24, 25). Since the

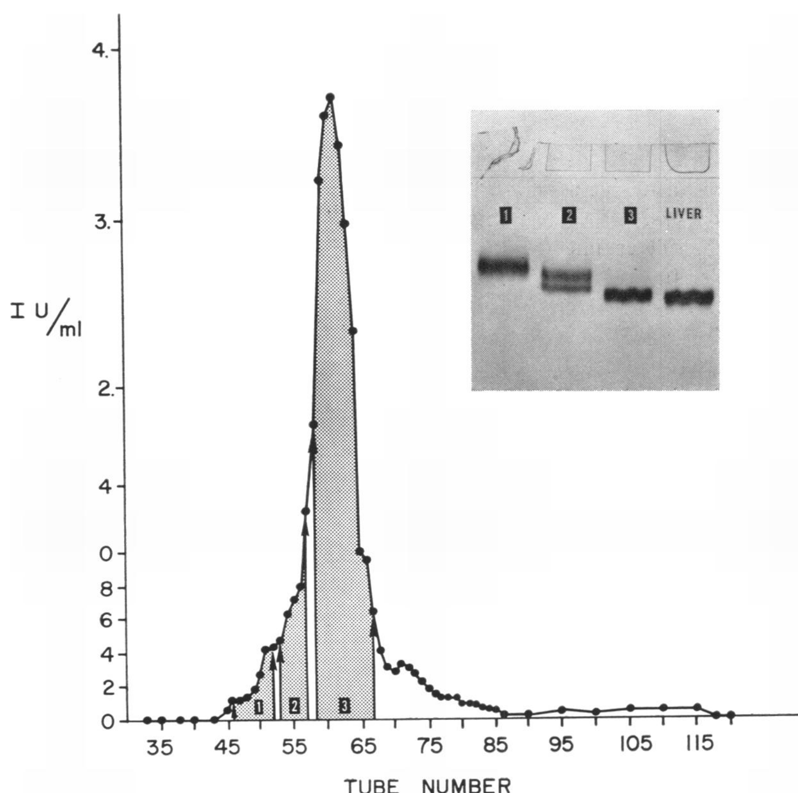


FIGURE 4 DEAE-cellulose chromatography of serum alkaline phosphatase. Tubes 46-52 (No. 1), 53-57 (No. 2), and 58-67 (No. 3) were pooled, concentrated by pressure dialysis, and subjected to polyacrylamide-gel electrophoresis with liver alkaline phosphatase similarly purified. The electropherogram is shown in the inset. Fraction 3 was used in subsequent experiments.

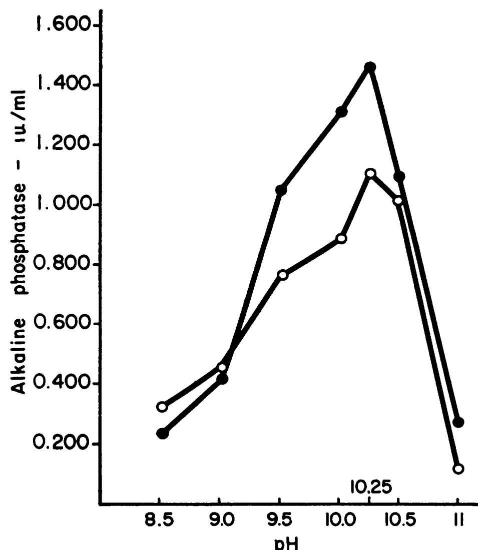


FIGURE 5 pH optima of liver and serum alkaline phosphatases purified by DEAE-cellulose chromatography. ●——●, liver; ○——○, serum.

increase in liver phosphatase was more than 240 times greater than that excreted in bile over a similar period of time and that in serum 11 times greater, simple regurgitation of bile alkaline phosphatase could not account for the serum elevation.

To eliminate the possibility that the low output of alkaline phosphatase observed in bile was an artifact produced by either inactivation or inhibition of this enzyme, experiments were done to determine the stability of alkaline phosphatase in bile. In the first experiment, alkaline phosphatases extracted from liver, bone, and intestine were added to rat bile in small enough amounts so that the bile was diluted less than 20%. This mixture was kept at 20°C, and alkaline phosphatase activity was

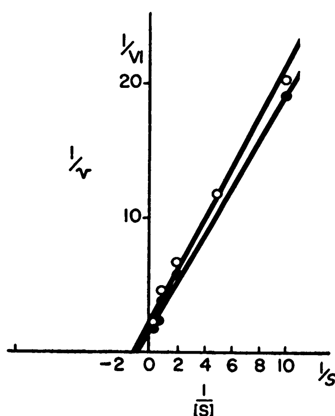


FIGURE 6 Michaelis constants of liver and serum alkaline phosphatase. Conditions are given in Methods. ●——●, liver; ○——○, serum.

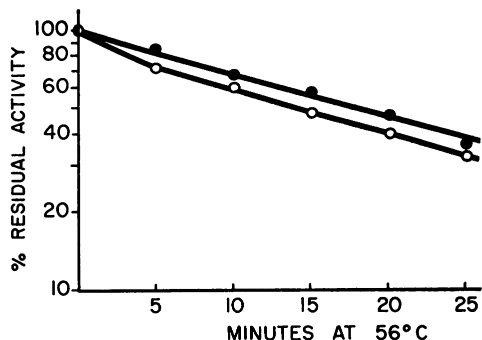


FIGURE 7 Heat lability of liver and serum alkaline phosphatases. Samples were kept at 56°C and aliquots removed and assayed for residual enzyme activity every 5 min. ●——●, liver; ○——○, serum.

measured at 2, 4, 8, and 24 hr. There was no significant change in enzyme activity under these conditions (Table III). To test for the presence of alkaline phosphatase inhibitors in bile, bile was assayed for alkaline phosphatase before and after overnight dialysis and passage through Sephadex G-50 columns. Again, there was no significant change in enzyme activity.

Alternative 2, dependency of the serum phosphatase rise on induction of liver phosphatase, was investigated by examining the effect of an inhibitor of protein synthesis on the changes in liver and serum alkaline phosphatase. Cycloheximide, in a dose that inhibited protein synthesis by 68%, inhibited the rise in liver phosphatase by 98% and that in serum by 80% (Fig. 9 and Table IV). Thus, the rise in liver activity and serum activity was dependent on *de novo* protein synthesis.

To investigate the possibility that cycloheximide was a general hepatotoxin that nonspecifically interfered with

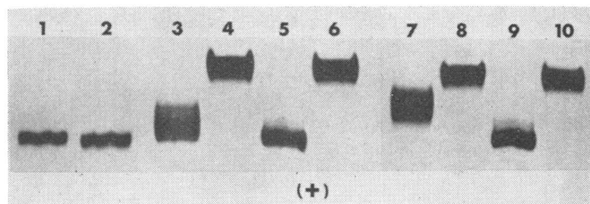


FIGURE 8 Polyacrylamide-gel electrophoresis of serum and liver alkaline phosphatase before and after digestion with neuraminidase. Liver and serum alkaline phosphatase isozymes were prepared as described in Methods and Fig. 4. Sample slot: 1, liver; 2, serum. Sample slots 3, 4, 5, and 6 were kept for 16 hr at pH 5.6 and 20°C while sample slots 7–10 were kept for 40 hr at pH 5.6 and 20°C. Sample slots 3 and 7, liver phosphatase; sample slots 4 and 8, liver phosphatase plus neuraminidase; sample slots 5 and 9, serum phosphatase; sample slots 6 and 10, serum phosphatase plus neuraminidase. Note that the liver phosphatase's electrophoretic mobility is progressively slowed at pH 5.6 while the serum phosphatase is not affected. After neuraminidase digestion the electrophoretic mobilities are identical.

TABLE II

Alkaline Phosphatase in Rat Bile

Rat	Liver weight	Bile flow	Bile alkaline phosphatase	
	g	*ml/12 hr*	*IU/ml*	*IU/12 hr per g liver*
1	9.2	9.0	0.041	0.042
2	6.2	5.5	0.016	0.014
3	9.4	5.8	0.063	0.039
4	6.8	4.5	0.017	0.011
5	7.0	5.0	0.012	0.008
6	7.0	9.2	0.027	0.037
Mean	7.6 ±1.2	6.6 ±1.9	0.029 ±0.018	0.025 ±0.014

enzyme changes after bile duct ligation, the behavior of another enzyme, GPT, was examined. GPT activity rose in the serum after bile duct ligation and fell slightly in the liver (Fig. 10). Its serum elevation is thought to reflect leakage from damaged cells and to be independent of increased *de novo* synthesis of protein (26). Cycloheximide did not alter the response of serum and liver GPT to bile duct ligation (Fig. 10). This experiment would rule against the possibility that cycloheximide was merely acting as a nonspecific enzyme inhibitor.

DISCUSSION

These results demonstrate that the elevated serum alkaline phosphatase in obstructive jaundice originates in the liver and thus support the "regurgitation" theory. However, simple regurgitation of liver phosphatase is only a partial explanation since concomitant increase in liver phosphatase activity was essential for the serum elevation to occur, and this increase was several orders of magnitude greater than the amount of phosphatase that would normally be excreted in bile and hence might accumulate in the liver because of bile duct obstruction.

The entire elevation in activity was due to the steady increase of a new isozyme that had the same properties as liver alkaline phosphatase. Both the new serum isozyme and liver phosphatase migrated identically on polyacrylamide-gel electrophoresis and their electrophoretic mobilities were equally slowed after neuraminidase digestion; both enzymes had identical pH optima, identical Michaelis constants, and both were inactivated at similar rates by heating at 56°C. Although these data indicate that this new serum isozyme is similar to liver phosphatase and almost certainly originates in the liver, these two isozymes are not completely identical. At least one difference was detected. The stability of the serum isozyme was not affected by passage over DEAE-cellulose. In contrast, similar treatment of liver phosphatase produced an alteration in its molecular configuration such that subsequent incubation at pH 5.6 slowed its electrophoretic migration rate. Hydrolysis of exposed sialic acid residues at pH 5.6 is the most likely explanation for this slowing, since liver phosphatase is known to contain such residues that can be completely removed by neuraminidase (19). Other properties of the liver enzyme remained unchanged. It is not surprising that such minor differences might exist between these two enzymes. The vigorous extraction with *N*-butanol required to solubilize liver alkaline phosphatase in the test tube is undoubtedly very different from the manner in which this enzyme is released into serum in the intact animal. Such differences could lead to changes in molecular structure.

The data indicate that the rise in the serum alkaline phosphatase in obstructive jaundice was intimately re-

TABLE III

Stability of Alkaline Phosphatase in Bile

Source	Alkaline phosphatase: Tissue, Activity	Tissue, Vol. added	Bile, Activity	Bile, Vol. added	Estimated alkaline phosphatase in mixture	Recovery*: 0 hr		24 hr	
	IU/ml		*IU/ml*		*IU/ml*	*IU/ml*	%	*IU/ml*	%
Liver	0.45	0.2	0.02	0.8	0.11	0.12	109	0.10	91
Bone	0.38	0.2	0.02	0.8	0.10	0.11	110	0.11	110
Intestine	2.78	0.1	0.02	0.9	0.30	0.22	73	0.22	73

* The values at 2, 4, and 8 hr were the same as those at 0 and 24 hr and are not shown.

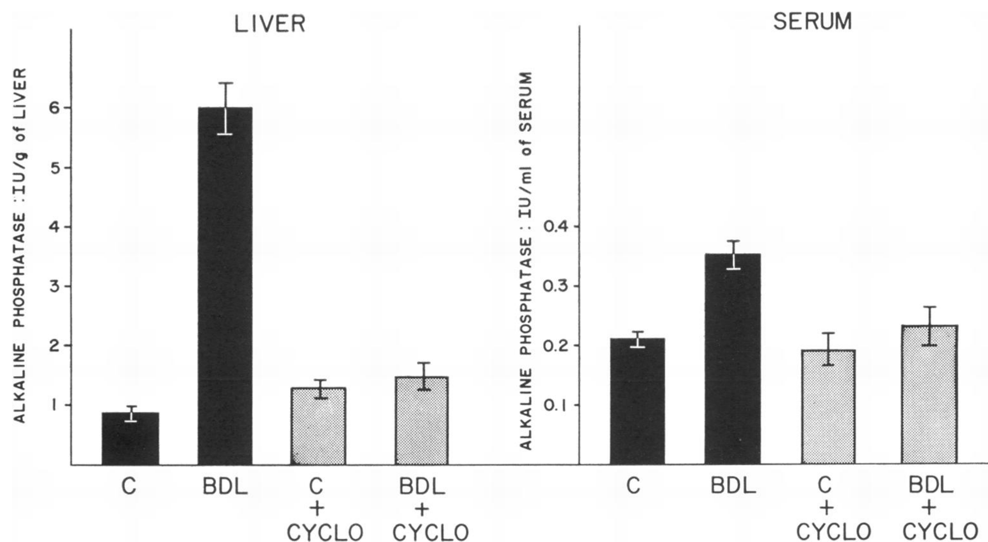


FIGURE 9 Effect of cycloheximide on the bile duct ligation–induced changes in the serum and liver alkaline phosphatase. Each bar represents the mean of five rats ±1 SE. C, control, sham-operated rats; BDL, bile duct–ligated rats; cyclo, cycloheximide.

lated to *de novo* synthesis of this enzyme by liver. Cycloheximide, in amounts that inhibited the enzyme increase in liver, prevented the expected rise in the serum. This agent had no effect on the response of GPT to bile duct ligation, an observation which supports the belief that the elevation of this latter enzyme in serum is due to simple leakage of preformed enzyme from damaged cells (26, 27). Conversely, the data indicate that alkaline phosphatase elevation in serum is dependent on simultaneous induction of this enzyme in liver. If this is so, it is the first instance that we are aware of in which enzyme induction has been shown to be a mechanism in the elevation for a serum enzyme, although the data of Polin, Spellburg, Teitelman, and Okumura in dogs with partial biliary obstruction (4) and of Sebesta, Bradshaw, and Prockop in isolated perfused cat livers (5) suggested that this might be so. In neither instance was increased production actually documented, nor was the role of *de novo* enzyme synthesis examined.

Although enzyme induction is a logical explanation for the increase in alkaline phosphatase activity observed in the obstructed liver, other mechanisms fit the data. The prevention of increase in enzyme activity by agents that inhibit protein and RNA synthesis can no longer be taken as unequivocal proof that such increases are synonymous with true enzyme induction. Thus, Griffin and Cox found that the apparent induction of alkaline phosphatase in HeLa cells by adrenocorticoids was actually due to activation of a preexisting enzyme rather than to increased synthesis of this enzyme (28). As in the above studies, an inhibitor of protein synthesis, puromycin, prevented the steroid-mediated increase in enzyme activity. However, the rise in phosphatase activity was thought to be due to a change in the conformation of a preexisting enzyme since there was no increase in the quantity of enzyme protein as measured by immunoprecipitation. A similar mechanism has been postulated by Moog and Grey (29, 30) to explain the progressive increase in duodenal alkaline phosphatase activity in the chick and mouse during their early development.

Another explanation for the alkaline phosphatase elevation observed in the obstructed liver is decreased degradation of alkaline phosphatase by a proteolytic enzyme with a very rapid turnover. Such a mechanism has been demonstrated in rat liver to produce the apparent induction of tryptophan pyrrolase by tryptophan (31) and has been postulated to account for the paradoxical induction of tyrosine transaminase by actinomycin D (32). Our data do not permit evaluation of this possi-

TABLE IV
Incorporation of Leucine-^{14}C into Rat Liver Protein

Experimental condition	No. of rats	Liver protein	% Inhibition of leucine incorporation
		dpm/mg	%
Sham operated	5	1478 ±236	—
Bil duct ligated	5	1065 ±226	—
Sham operated + cycloheximide	5	762 ±31	48.5
Bile duct ligated + cycloheximide	5	487 ±70	67.0

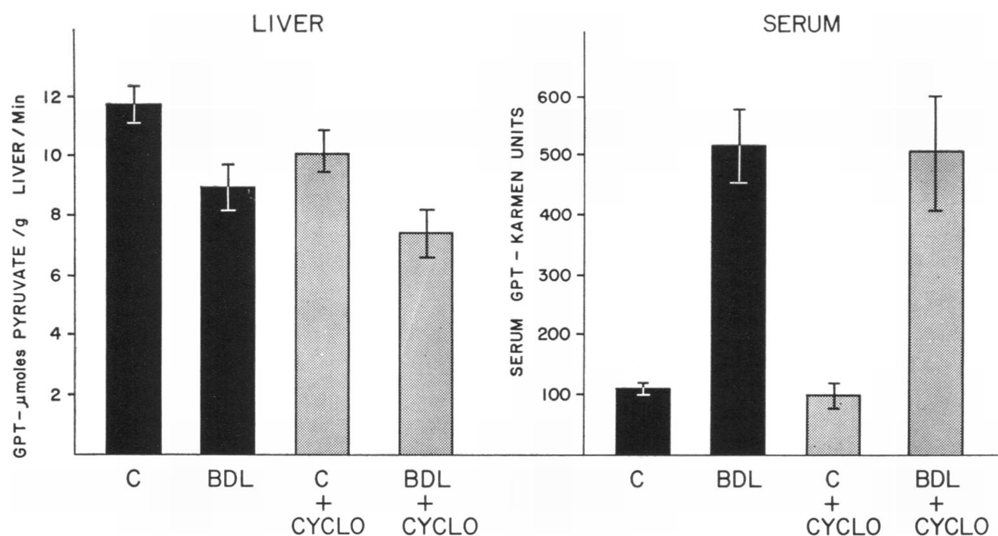


FIGURE 10 Effect of cycloheximide on the bile duct ligation-induced changes in the serum and liver GPT. The same animals as in Fig. 9 were used, and bars represent the mean of five rats ±1 SE.

bility, and unequivocal assignment of a mechanism for the increase in alkaline phosphatase activity awaits complete purification of this enzyme.

A final comment should be made on the relationship of these studies to the situation in humans. There is considerable evidence that the elevated serum alkaline phosphatase in human with liver disease is due to a similar mechanism as that in the rat. Many studies have shown that the alkaline phosphatase circulating in patients with hepatic disorders is similar to liver phosphatase and different from that found in the serum of patients with skeletal disorders. These studies have identified isozymes by electrophoretic separation (7, 33, 34) and by differential rates of enzyme inactivation (18, 35). In another approach, liver biopsies obtained from patients with obstructive jaundice have been found to contain greatly increased amounts of alkaline phosphatase when compared with livers obtained from normal subjects.[1] Finally, there is little evidence that the human liver excretes serum alkaline phosphatase into the bile. The alkaline phosphatase found in bile is different from the serum enzyme (36), and obstruction of the common duct does not impede the clearance from serum of infused human placental alkaline phosphatase (6). Most arguments that the liver normally clears alkaline phosphatase from the serum and excretes it in the bile are based on the unproven assumption that the liver excretes large protein molecules as it does smaller molecules such as bilirubin and bromsulphthalein (2). When this hypothesis has been directly tested, it has been shown not to be true for the human liver (6), the dog liver (4), and the isolated, perfused rat liver (37).

[1] Unpublished observations.

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