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ENZYME CHANGES IN EXPERIMENTAL BILIARY OBSTRUCTION

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SUMMARY

Changes in the activities of alkaline phosphatase, 5'nucleotidase and γ -glutamyl transpeptidase in serum and liver of bile-duct ligated rats were studied over a period of 8 days after operation. All three enzymes showed increased activity in the liver, but for 5'nucleotidase and γ -glutamyl transpeptidase the increases were not detectable before 48 h, whereas for alkaline phosphatase the maximum rise was at 12 h. All three activities in the serum were significantly elevated at 12 h after operation. Maximum levels were reached at 24 h for alkaline phosphatase and 5'nucleotidase but not before 48 h for γ -glutamyl transpeptidase. These results show that bile duct ligation causes an early increase in synthesis of hepatic alkaline phosphatase whereas the increase in 5'nucleotidase and γ -glutamyl transpeptidase is delayed until the phase of bile duct proliferation. The early changes in serum activities of 5'nucleotidase and γ -glutamyl transpeptidase do not appear to be due to a rapid increase in hepatic synthesis of these enzymes.

INTRODUCTION

Clinical observations have shown that changes in serum γ -glutamyl transpeptidase activity in hepatobiliary diseases are closely correlated with changes in serum alkaline phosphatase and 5' nucleotidase¹⁻³. Experimentally-produced bile-duct obstruction in animals also results in a rapid elevation of all three enzymes in the serum. Recent studies suggest that the increase in serum alkaline phosphatase in these animals results from a *de novo* synthesis of hepatic alkaline phosphatase, with an overflow of the enzyme in the blood⁴⁻⁶. The increased production of liver enzyme is prevented by cycloheximide, an inhibitor of protein synthesis at the translation stage, or by actinomycin D, which inhibits transcription of DNA to RNA⁶. When the synthesis of hepatic alkaline phosphatase is prevented in this way, no rise in serum alkaline phosphatase is observed.

These studies were later extended by Righetti and Kaplan to include 5'nucleotidase, with rather different results⁷. In the case of this enzyme, bile duct ligation in rats caused a prompt increase in the serum activity, but no significant rise in hepatic

activity during the 24-h period of observation. Inhibitors of protein synthesis did not abolish the increase in serum 5'-nucleotidase which was consequently ascribed to leakage of pre-existing enzyme from damaged liver cells. However, the persistence of elevated levels of 5'-nucleotidase and γ -glutamyl transpeptidase for periods of weeks or months in the serum of patients with chronic liver disease suggests that some long-term alteration in the production or release of liver enzymes must be sought to account for these changes. We have therefore studied changes in serum and liver alkaline phosphatase, 5'-nucleotidase and γ -glutamyl transpeptidase during the 8-day period following bile-duct ligation in rats. Significant increases in both 5'-nucleotidase and γ -glutamyl transpeptidase activities in the liver occurred between 24 h and 8 days after operation, preceded in each case by increases in serum activity of both enzymes which were detectable within 12 h.

EXPERIMENTAL

The experiments were performed on an inbred strain of male Wistar rats weighing 150–250 g, fed on a standard chow diet and fasted overnight before operation. The animals were anaesthetised lightly with ether and the common bile duct was isolated and ligated close to the junction of the hepatic ducts. A second ligature was tied immediately distal to the first ensuring complete occlusion of the duct without damaging the pancreatic ducts. Groups of 5–10 animals were killed at 12, 24, 48, 96 and 192 h after operation. Other groups of animals received cycloheximide (1 μ g/g body weight) or actinomycin D (0.5 μ g/g body weight) one hour before bile-duct ligation and were killed at 24 h. Control groups of animals were not operated on but two groups received cycloheximide or actinomycin D 25 h before being killed.

At the end of the experiment, animals were individually anaesthetised with ether and exsanguinated by withdrawing blood from the aorta. Serum was subsequently separated from the withdrawn blood and stored at -20° until analysis. The liver of each rat was perfused via the portal vein with 10 ml of cold 0.25 M sucrose solution immediately after the animal had been exsanguinated. It then was excised, rinsed in sucrose, blotted dry, weighed, minced with scissors and homogenised in a knife-blade blender for 3 min in 5 volumes of cold 0.25 M sucrose solution. The homogenate was divided into two portions, one for extraction of alkaline phosphatase and the other for extraction of γ -glutamyl transpeptidase and 5'-nucleotidase.

To extract alkaline phosphatase, two parts of the homogenate were mixed with one part by volume of *n*-butanol (ref. 8), mixed on a roller mixer for 1 h, and centrifuged. The aqueous layer was used for estimation of alkaline phosphatase with phenyl phosphate as substrate⁹. For extraction of the other two enzymes, 3 ml of the second portion of the homogenate was mixed with 5 ml of 1% sodium deoxycholate in 1% sodium bicarbonate solution and homogenised for 3 min in a Potter-Elvehjem homogeniser. Experiments in which the duration of homogenisation and the quantity of added deoxycholate were varied showed that this procedure gave maximal and reproducible extraction of the enzyme activities. γ -Glutamyl transpeptidase activity was measured in this homogenate by the method of Szasz¹⁰ and 5'-nucleotidase by the method described by Campbell¹¹. Protein concentrations of suspensions of homogenised livers were estimated by the method of Lowry *et al.*¹². Enzyme activities were measured in the serum by the methods used for the estimations on liver extracts.

RESULTS

The results obtained for liver and serum alkaline phosphatase, 5'nucleotidase and γ -glutamyl transpeptidase are shown in Figs. 1-3. The numbers of animals in each group differ because of the variable postoperative mortality. The ratio of liver: serum enzyme activities has been calculated for each animal and the changes in this ratio with time after bile-duct ligation are shown in Fig. 4.

The activities of all three enzymes in liver tissue showed a marked increase after bile duct ligation. However, the response of both 5'nucleotidase and γ -glutamyl transpeptidase was delayed, the earliest significant change being detectable after 48 h. At 8 days after operation enzyme levels were approx. twice and three times their basal levels, respectively, and the activity of γ -glutamyl transpeptidase may not have reached a maximum by this time. In contrast, alkaline phosphatase showed a maximum rise in 12 h after operation as reported previously⁸. Enzyme activities in the liver are represented as units per g protein in Figs. 1-3: however, when these data were expressed in terms of units per g wet weight of liver, the changes in enzyme activity with time were not significantly different from those seen with g protein as the base.

When enzyme activities in serum are considered the behaviour of alkaline phosphatase and 5'nucleotidase was similar, maximum values for both being reached at 24 h. γ -Glutamyl transpeptidase in serum also rose rapidly, but with this enzyme the

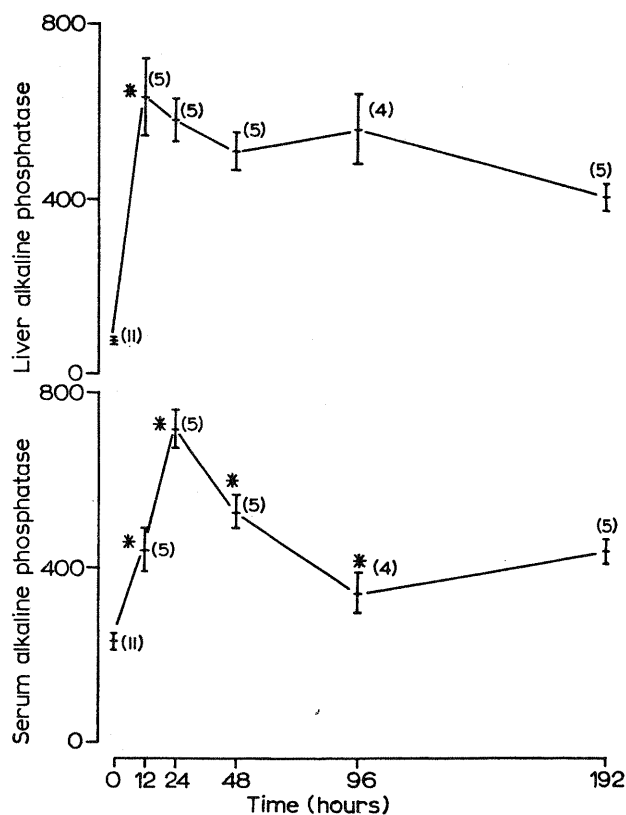


Fig. 1. Alkaline phosphatase activity (mean \pm 1 S.E.M.) in the serum and liver of rats following bile-duct ligation. Serum activity is in I.U./l: liver activity in I.U./g of protein. Means indicated by an asterisk are significantly different ($P < 0.05$) from the preceding mean value. In this and other Figs., numbers in parentheses are the numbers of animals in each group.

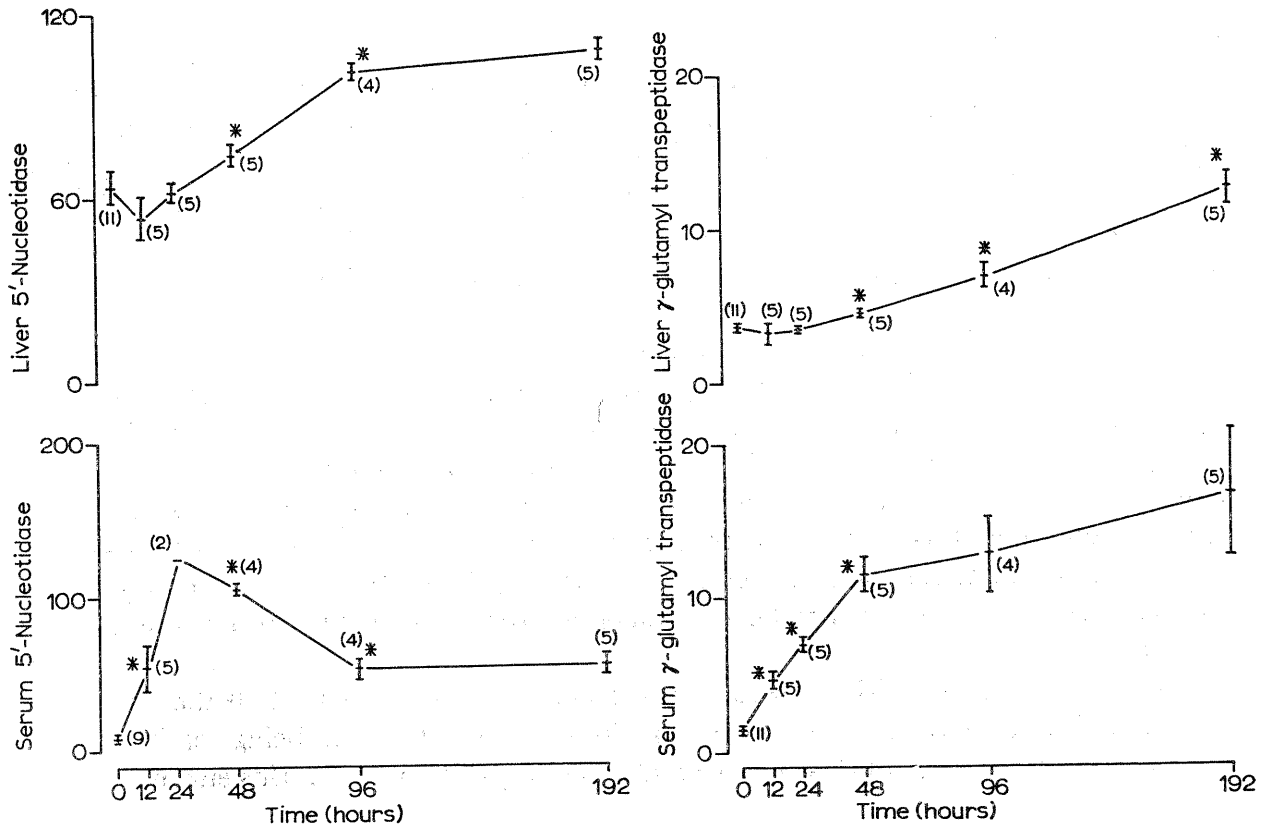


Fig. 2. 5'-Nucleotidase activity (mean \pm 1 S.E.M.) in the serum and liver of rats following bile-duct ligation. Serum activity is in I.U./l: liver activity in I.U./g of protein. Means indicated by an asterisk are significantly different ($P < 0.05$) from the preceding mean value.

Fig. 3. γ -Glutamyl transpeptidase activity (mean \pm 1 S.E.M.) in the serum and liver of rats following bile-duct ligation. Serum activity is in I.U./l: liver activity in I.U./g of protein. Means indicated by an asterisk are significantly different ($P < 0.05$) from the preceding mean value.

peak level was not reached before 48 h. and possibly even later. The differences in response between alkaline phosphatase on the one hand and 5' nucleotidase and γ -glutamyl transpeptidase on the other are strikingly emphasised by the liver:serum activity ratios. In these ratios the steep rise observed for alkaline phosphatase contrasts markedly with the initial sharp fall seen with the other two enzymes (Fig. 4).

The results of administration of cycloheximide and actinomycin D are summarised in Figs. 5, 6 and 7. As expected, both cycloheximide and actinomycin D partially inhibit the increases in alkaline phosphatase activity in the liver and serum after bile-duct ligation. Both these agents also caused a significant reduction in serum alkaline phosphatase activity in control rats, which may be due to interference with the production of intestinal phosphatase since this isoenzyme comprises a significant part of the normal serum activity in this species¹³. There was a small, but significant, fall in liver phosphatase activity in control rats which received actinomycin D.

Calculation of enzyme activities in liver in terms of units per g wet weight of tissue, instead of as units per g protein, confirmed the slight fall in alkaline phosphatase in the livers of control rats given actinomycin D, as well as the inhibition by both this agent and cycloheximide of the rise in liver alkaline phosphatase induced by bile-duct ligation. Calculation on the basis of wet weight of tissue did not significantly alter the results of 5'-nucleotidase and γ -glutamyl transpeptidase estimations on the livers of rats with bile-duct ligations.

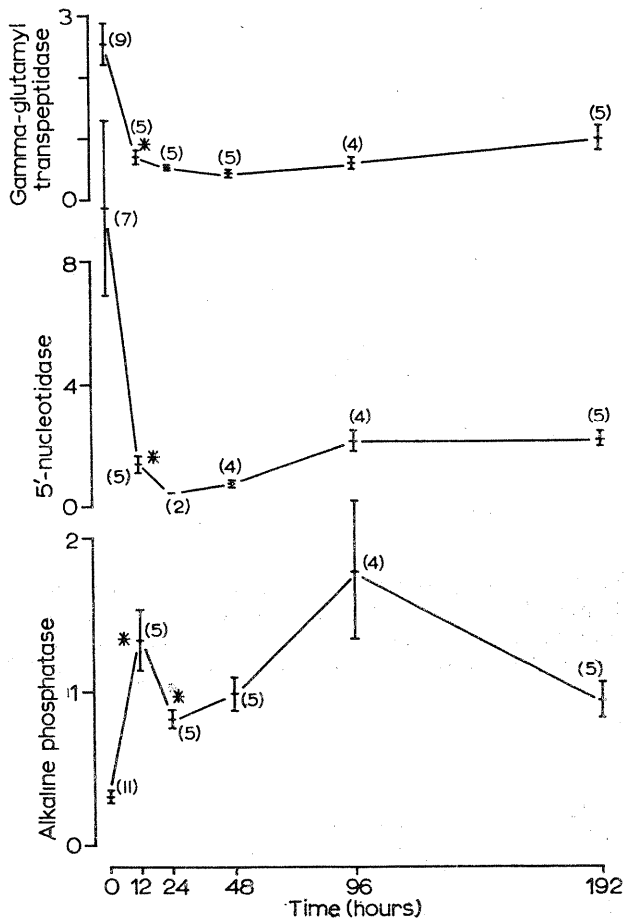


Fig. 4. Changes in the ratio liver activity:serum activity with time after bile-duct ligation for alkaline phosphatase, 5'-nucleotidase and γ -glutamyl transpeptidase (mean \pm 1 S.E.M.). Means indicated by an asterisk are significantly different ($P < 0.05$) from the preceding mean value.

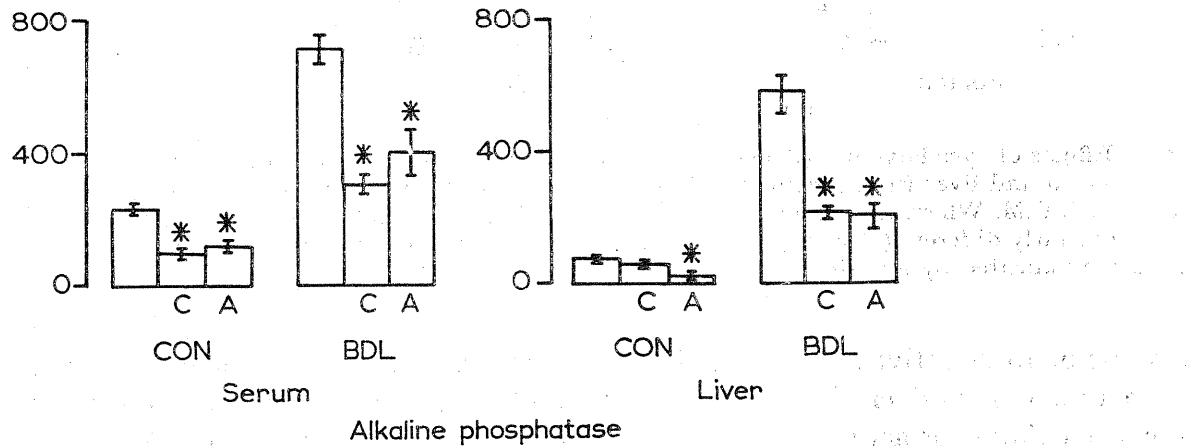


Fig. 5. Effects of cycloheximide (C) and actinomycin D (A) on alkaline phosphatase activity in the serum and liver from control (CON) and 24-h bile-duct ligated (BDL) rats. All values as mean \pm 1 S.E.M. Where the means for groups of animals given cycloheximide or actinomycin D are significantly different ($P < 0.05$) from the groups not given these inhibitors of protein synthesis, this is indicated by an asterisk.

The effects of the inhibitors of protein synthesis on serum and liver 5'-nucleotidase and γ -glutamyl transpeptidase activities were rather complex. Neither cycloheximide nor actinomycin D had any effect on the activities of these two enzymes in livers of bile-duct ligated animals at 24 h, but this was not unexpected because of the

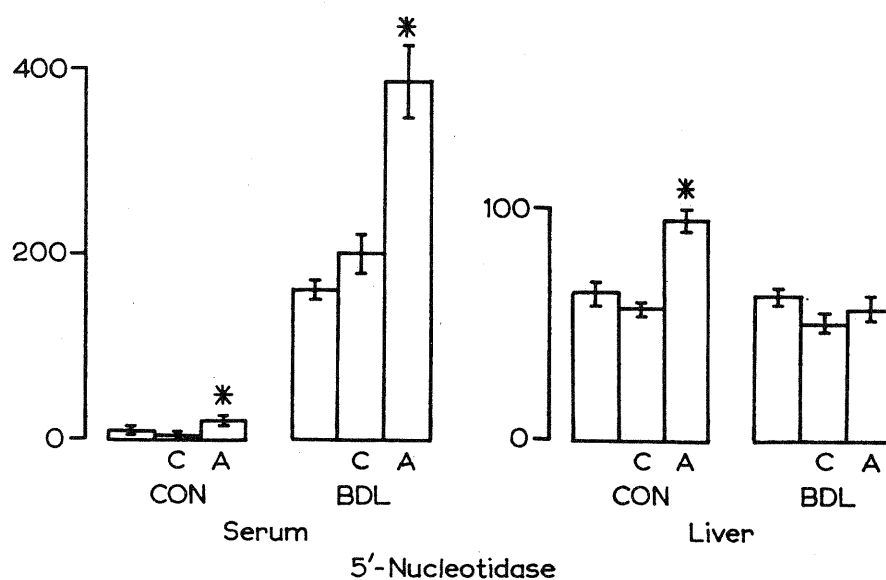


Fig. 6. Effects of cycloheximide (C) and actinomycin D (A) on 5'-nucleotidase activity in the serum and liver from control (CON) and 24-h bile-duct ligated (BDL) rats. All values as mean \pm 1 S.E.M. Where the means for groups of animals given cycloheximide or actinomycin D are significantly different ($P < 0.05$) from the groups not given these inhibitors of protein synthesis, this is indicated by an asterisk.

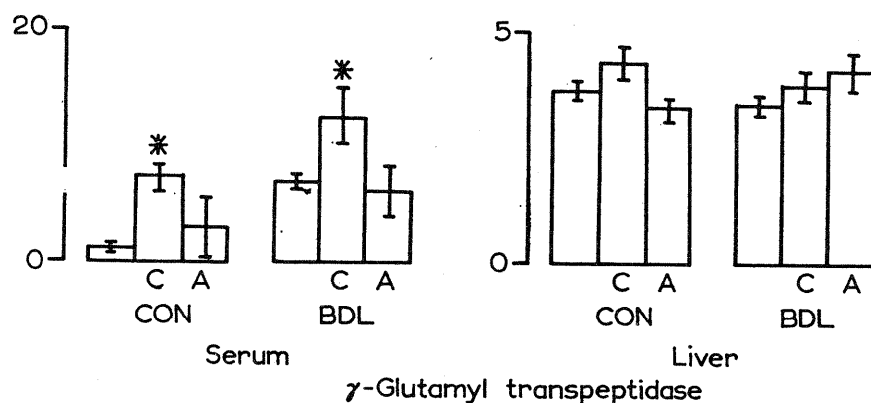


Fig. 7. Effects of cycloheximide (C) and actinomycin D (A) on γ -glutamyl transpeptidase activity in the serum and liver from control (CON) and 24-h bile-duct ligated (BDL) rats. All values as mean \pm 1 S.E.M. Where the means for groups of animals given cycloheximide or actinomycin D are significantly different ($P < 0.05$) from the groups not given these inhibitors of protein synthesis, this is indicated by an asterisk.

slow rise of these activities. It would have been of interest to determine whether the delayed increase of liver 5'-nucleotidase and γ -glutamyl transpeptidase was inhibited by cycloheximide or actinomycin D, but the animals could not be maintained for a sufficient length of time on these toxic compounds. 5'-Nucleotidase activity in the serum of both control and bile-duct ligated rats was found to be increased approximately two-fold, 24 h after administration of actinomycin D, and a smaller rise also occurred in the livers of control animals. Significant increases, of the order of 2-3-fold, in serum activity of γ -glutamyl transpeptidase were observed 24 h after administration of cycloheximide to control and bile-duct ligated animals.

DISCUSSION

The results of these investigations show that although circulating and hepatic alkaline phosphatase, 5'nucleotidase and γ -glutamyl transpeptidase all increase in response to bile-duct ligation, the changes do not parallel one another. Moreover, if drugs known to inhibit protein synthesis are given to the rat immediately before ligating the common bile-duct, the enzyme responses are also disparate. These experimental results support clinical observations that statistical correlations of activities of these enzymes in the sera of large groups of patients with hepatic disease mask real differences in their behaviour¹⁴.

The differences in response of the three enzymes cannot be correlated readily with their distribution within the liver as determined histochemically. In the rat, alkaline phosphatase and 5'nucleotidase are apparently bound to lipid membranes within the hepatocytes whereas γ -glutamyl transpeptidase appears to be associated with the biliary epithelium^{15,16}. It has been suggested previously that the proliferation of biliary epithelium which begins two days after bile-duct obstruction may explain the rise in serum 5'nucleotidase^{17,18}, a hypothesis which may be extended to include γ -glutamyl transpeptidase, but at present there is insufficient evidence to sustain this view. However, even if the relatively slow proliferation of ductular epithelium is capable of providing an explanation for the delayed increase of 5'nucleotidase and γ -glutamyl transpeptidase in the liver it cannot account for the rapid changes in serum activities of these two enzymes and the sharp fall in liver:serum activity ratio. Several explanations may be considered to account for this phenomenon.

The elevated serum enzyme activities may represent an accumulation of enzyme either released from non-hepatic sources, by some secondary effect of bile stasis on these tissues, or retained in the circulation because of a failure of normal excretion in the bile. Although there are several rich extrahepatic sources of γ -glutamyl transpeptidase (*e.g.* the kidney), no marked immediate effect of bile stasis on these tissues has been demonstrated, apart from an effect on lysosomal stability in the kidneys of bile-duct ligated rats¹⁹. Rich, non-hepatic sources of 5'nucleotidase are less plentiful. Furthermore, earlier views which emphasized biliary excretion as a factor in the clearance of plasma enzymes (*e.g.*, alkaline phosphatase) have not been supported by later work. Accumulation of non-hepatic γ -glutamyl transpeptidase and 5'nucleotidase therefore seems unlikely.

A second possibility is that obstruction of the flow of bile rapidly damages the enzyme-containing cells, with consequent leakage of the two enzymes into the plasma. The loss of enzyme would not necessarily reduce significantly the total enzyme activity of the liver, particularly as the total hepatic activity is 100-fold greater than that in the circulation. A slight fall in liver 5'nucleotidase was seen in our experiments, but this was not statistically significant. However, if damage to cells is invoked to account for the increase in serum γ -glutamyl transpeptidase and 5'nucleotidase activities, this must be of a different nature, or must affect different cells, from the damage which leads to release of enzymes such as aspartate transaminase or isocitrate dehydrogenase into the plasma, since many clinical studies on patients with liver diseases have demonstrated a poor correlation between changes in serum transaminase and 5'nucleotidase or γ -glutamyl transpeptidase activities³. The latter enzymes are apparently firmly bound to cellular elements (*e.g.* membranes) and require vigorous

treatment, such as ultrasonication or treatment with detergents or organic solvents, to free them into solution in the laboratory. Righetti and Kaplan⁷ suggest that accumulation of bile salts may effect this solubilization *in vivo* in the bile-duct ligated animal, and this seems a probable explanation.

The early increase in the serum activities of 5'nucleotidase and γ -glutamyl transpeptidase might be due to a rapid synthesis of the two enzymes which exceeds the capacity of the enzyme-binding sites in the cells. However, our observations that inhibitors of protein synthesis did not prevent, and perhaps slightly enhanced, the rises in serum enzyme activity, do not support this view. It is also possible that accumulation of some substance which is normally excreted in the bile may activate enzyme molecules in the serum, or release them from an inactive complex but experiments with a mixture of sera from control and bile-duct ligated animals provided no evidence for this.

The data presented here may provide an experimental basis for the clinical observation that alkaline phosphatase, 5'nucleotidase and γ -glutamyl transpeptidase in serum behave in a similar, though not identical way in patients with diseases affecting the biliary tract, and that in chronic diseases of this nature, 5'nucleotidase and γ -glutamyl transpeptidase measurements become more sensitive tests than alkaline phosphatase^{3,14}. However, extrapolation from short-duration experiments involving acute biliary obstruction in rats to chronic diseases in man which probably result from several causative factors, must be made with caution.

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