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Evaluation of Key Gluconeogenic Enzymes in Experimental Biliary Obstruction

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In order to evaluate the usefulness of key gluconeogenic enzymes, in relation to the markers commonly used (alkaline phosphatase and gamma-glutamyl transpeptidase) for the diagnose of cholestasis the serum activity of phosphoenolpyruvate carboxykinase, fructose 1,6 bisphosphatase and glucose-6-phosphatase has been measured in rats with bile-duct ligation. Among the gluconeogenic enzymes studied only phosphoenolpyruvate carboxykinase activity increased significantly in the first 48 hours after cholestasis, decreasing thereafter to normal values. Both alkaline phosphatase and gamma-glutamyl transpeptidase activities showed a very significant increase which persisted throughout the experiment. These results seem to indicate that in spite of the high organ specificity of these enzymes they do not appear to be useful for the diagnosis of cholestasis.

Key words: Gluconeogenic enzymes, Bile duct ligation, Serum.

In the last few years a number of enzymes have been measured in serum in search of a valid test for the diagnosis of hepatic and biliary system diseases (18). Of the enzymes studied, alkaline phosphatase (AP) and gamma-glutamyltranspeptidase (GGT) have proven to be the best markers for cholestasis while aminotransferases (aspartate aminotransferase, AST and alanine aminotransferase, ALT) are markers of hepatocellular injury. Nevertheless several studies have demonstrated that the overlap is such that the determination of the activity of these enzymes in serum is at present of little value in the differential diagnosis of jaundice (2).

Due to the relatively specific localization of some enzymes in cellular compartments, a number of studies seem to indicate that for several enzymes the determination of their serum activity could

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indicate damage to specific cellular structures, i.e. these measurements could be considered as «biochemical biopsies» (1, 11). Although several theories have been put forward to explain this, the precise mechanism by which some enzymes of the intermediate metabolism under a number of aggressions are released from their subcellular compartments, escape through the citoplasmic membrane and gain the extracellular space remains unknown (3).

To date the serum activities of key enzymes of the gluconeogenic pathway such as phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6 bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) and their relationship to hepatobiliary pathology have received little attention. The preferential hepatic localization of these enzymes and their typical topografic distribution in this organ (mostly in periportal cells) (4, 7, 8) led us to study their potential diagnostic value in cholestasis in comparison with that of two other enzymes (i.e. alkaline phosphatase and gamma-glutamyltranspeptidase).

Materials and Methods

Animal experiments.— Wistar female rats weighing 150-250 g were fed with standard laboratory diet and tap water *ad libitum*. Food was withheld for 24 h before surgery which was performed under anesthesia induced with 0.35 ml/100 g body wt of 8 % chloral hydrate. The bile duct was doubly ligated near the liver and excised through a midline abdominal incision. Sham-operated animals served as control. After the operation the rats were again given food and water *ad libitum*. Test and control animals were weighed before sacrifice and killed by exsanguination on days 1, 2, 3, 4, 5, 6, 7, 12 or 15 after the operation. Only 1 of 3 survived between 5 and 15 days, the remainder succumbing to rupture of the bile duct and bile peritonitis.

Preparation of serum.— Blood samples were collected from the abdominal aorta after anaesthetizing the animals. The serum was separated after centrifugation at 1,500 g for 15 min at 4 °C and immediately used for enzyme activity determinations. Hemolized sera were discarded. A small piece of the liver was fixed in 10 % formalin for histopathological evaluation.

Enzyme assay.— The serum gluconeogenic enzymes tested were assayed at 37 °C according to previously published procedures: PEPCK (EC 4.1.1.32) (16), FBPase (EC 3.1.3.11) (12) and G6Pase (EC 3.1.3.9) (5).

Measurement of bilirubin and activity of classical hepatic enzymes.— The serum levels of bilirubin and the activity of the enzymes AP (EC 3.1.3.1.), GGT (EC 2.3.2.2), AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were measured by optimized methods of the German Society for Clinical Chemistry using an Hitachi-737 Analyzer (Boehringer Mannhein, GmbH).

Statistics.— Statistical evaluation was performed by Student's t-test.

Results

The rats whose bile ducts were ligated became promptly jaundiced, reduced their food intake and lost weight in comparison to sham-operated animals. When the ligated rats were killed, markedly distended common bile ducts were observed but both ligatures were found intact and no reconstitution of bile flows was found in any animal.

Histological changes.— The hematoxylin-eosin stained sections showed the following alterations. Samples obtained 24, 48 and 72 h after the ligation of the bile duct showed discrete portal inflamation, with distention and proliferation of bile

ducts as well as signs of degeneration of hepatocytes. No significant differences were observed on the samples obtained between day 4 and day 6 after ligation. In this period portal inflammation and proliferation of bile ducts were seen. Necrosis and degeneration of the hepatocytes was more evident on day 6. On day 12 and mostly on day 15 the degeneration and necrosis of hepatocytes were less evident. No histological changes were observed in the sham-operated rats.

Changes in gluconeogenic enzymes (fig. 1).— PEPCK activity in serum increased above normal limits at 24 and 48 h after ligation, the higher values being obtained 62 and 54 U/l respectively. These values decreased steadily thereafter to near control values.

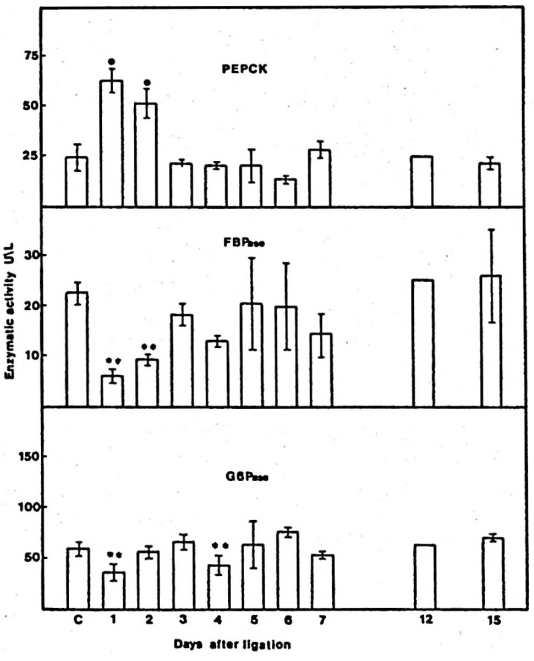

Fig. 1. *Gluconeogenic enzyme activities in the serum of rats following bile duct ligation.*
Bars show the average of 2 to 7 animals and vertical lines represented the SEM; only 1 rat survived 12 days. C: Control group. * = $p < 0.001$, ** = $p < 0.05$.

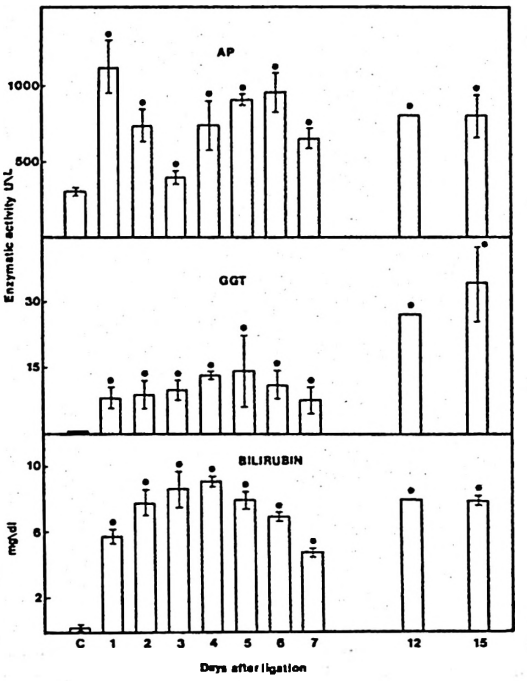

Fig. 2. *Alkaline phosphatase and gamma-glutamil transpeptidase activities and bilirubin levels in the serum of rats following bile duct ligation.*
See fig. 1 for explanations. C: Control group. * = $p < 0.001$.

Serum FBPase activity decreased significantly below control values 24 and 48 h after ligation (7 and 9 U/l respectively) control values being reached for the rest of the experiment.

G6Pase activity in serum only showed significant changes on the first and fourth day after ligation.

Changes in bilirubin, AP and GGT (fig. 2).— Bilirubin rose steadily to reach a plateau on the 4th day after ligation. AP and GGT increased very significantly from the first day and stayed elevated throughout the experiment. GGT showed a progressive increase from day 1 onward.

Changes in AST and ALT (fig. 3).— Serum aminotransferases showed a signif-

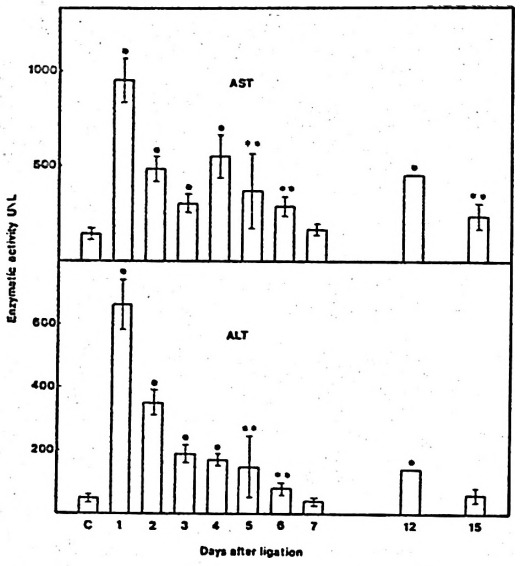

Fig. 3. *Aspartate and alanine aminotransferase activity in the serum of rats following bile duct ligation.*
See fig. 1 for explanations. C: Control group. * = $p < 0.001$, ** = $p < 0.05$.

icant increase on the first few days after ligation decreasing thereafter to near control values.

Discussion

The clinical usefulness of AP and GGT for the diagnosis of cholestasis even in the absence of jaundice is well documented (6, 9). Nevertheless the low specificity of these two enzymes when used separately has motivated the search of more specific enzymatic markers. Since gluconeogenesis is a biochemical pathway operating almost exclusively in hepatic periportal cells (13) (it also occurs in renal cortex during severe liver failure) the key enzymes of this pathway could be useful markers for hepatobiliar disease.

To date, few reports exist relating the serum activity of these enzymes (particularly on FBPase and PEPCK), and their clinical applications (14, 15). Our results show that although the gluconeogenic enzymes are at very low concentrations in the hepatic cells they can be detected in serum. In the rat FBPase and PEPCK are cytoplasmic enzymes while G6Pase is bound to the endoplasmic reticulum.

In our experimental conditions the bile duct ligation leads to a significant decrease in FBPase on the first and second day after the operation while PEPCK increases significantly in the same period of time. As it has been reported (11) and as the present results show, animals with induced cholestasis have only a discrete degree of hepatocellular necrosis. For this reason rupture of cells following necrosis does not explain by itself the increase in PEPCK and an alternative mechanism to explain this phenomenon should be found. The fact that PEPCK increases in serum very significantly 24 and 48 hours after ligation seems to indicate that the rise of biliar pressure in some way increases the permeability of the hepatocyte to this large protein allowing its access to the plasma. Similar mechanism of escape has been proposed by MORITZ *et al.* (11) after measuring the serum levels of a different set of enzymes in animals with surgically-induced cholestasis. These authors reported a significant increase in the cytoplasmic enzyme sorbitol dehydrogenase and in the mitochondrial enzyme ornithine carbamyl transferase as early as 1 h after ligation declining only 48 h later. Another possible explanation for the increase of PEPCK observed is that the tissue damage could be the consequence of the detergent effect exerted by the accumulated bile salts. The effect of the increase of bile salts upon the lipid bilayer probably modifies the permeability of cell membranes allowing the access of PEPCK to the plasma. The gradual reduction in specific activity of PEPCK after bile duct ligation is probably due to dilution by proliferating bile

duct epithelium which does not contain this enzyme (11).

In cholestasis, bile acids and bile salts accumulation in millimolar concentrations has also been shown *in vitro* to decrease reduced nicotinamide adenine dinucleotide-linked respiration to uncouple oxidative phosphorylation and to increase adenosine triphosphatase activity (10). An increase in adenosine triphosphatase activity would induce a rise of adenosine diphosphate (ADP) and adenosine-5'-monophosphate (AMP) in the cell. Of these two metabolites AMP is a specific inhibitor for FBPase as shown with FBPase isolated from a variety of sources (19). This increase of AMP, caused by the bile acids and bile salts accumulation, could probably explain the FBPase inhibition found in our experiments and perhaps the wide range of variations observed in the activity of this enzyme at different times after ligation. In this respect TSUGE *et al.* (20) found a statistically significant decrease of ATP levels in the liver of bile duct-ligated rats, as well as a non significant increase of AMP and ADP in relation to control animals.

The lack of changes in the activity of G6Pase seen during cholestasis contrasts with the results obtained for PEPCK and FBPase. Nevertheless, the electron microscopy after bile duct ligation shows only early dilation and later hypertrophy of the smooth endoplasmic reticulum and a mild decrease in rough endoplasmic reticulum (17). This mild damage is less apparent in the endoplasmic reticulum than in mitochondria and this could explain why an enzyme such as G6Pase localized mainly in endoplasmic reticulum does not show significant alterations after bile duct ligation. Similar results have been reported by MORITZ *et al.* (11). These authors obtained a negative serum response from the microsomal liver esterase.

From these results we can conclude that the serum measurement of the gluconeogenic enzymes studied are of poor value for the diagnosis of cholestasis.

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Resumen

Con el fin de evaluar la utilidad de los enzimas gluconeogénicos clave para el diagnóstico de la colestasis, en relación a otros marcadores usados comúnmente (fosfatasa alcalina y gamma-glutamil transpeptidasa), se ha medido la actividad sérica de fosfoenolpiruvato carboxikinasa, fructosa 1,6-bifosfatasa y glucosa-6-fosfatasa en un grupo de ratas tras ligadura del coledoco. De los enzimas gluconeogénicos estudiados, sólo aumentó significativamente la actividad de la fosfoenolpiruvato carboxikinasa en las primeras 48 horas de colestasis, descendiendo posteriormente a valores normales. Tanto la fosfatasa alcalina como la gamma-glutamil transpeptidasa mostraron un aumento significativo que se mantuvo a lo largo del experimento. Los resultados obtenidos indican que estos enzimas, a pesar de su alta especificidad de órgano, no parecen ser de utilidad en el diagnóstico de la colestasis.

Palabras clave: Enzimas gluconeogénicas, Suero, Ligadura del colédoco.

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