Glycogen and fructose 2,6-bisphosphate levels in rat liver decreased quickly after partial hepatectomy. After 7 days the glycogen level was normalized and fructose 2,6-bisphosphate concentration still remained low. The 'active' (non-phosphorylated) form of 6-phosphofructo-2-kinase varied in parallel with fructose 2,6-bisphosphate levels, whereas the 'total' activity of the enzyme decreased only after 24 h, similarly to glucokinase. The response of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from hepatectomized rats (96 h) to m-glycerol 3-phosphate and to cyclic AMP-dependent protein kinase was different from that of the enzyme from control animals and similar to that of the foetal isoenzyme.

INTRODUCTION
In the liver tissue which remains after partial hepatectomy, marked changes in the composition and the energy metabolism develop even before the occurrence of increased mitotic activity. The hepatic metabolism is shifted from a predominant utilization of carbohydrates to an increased utilization of lipids (Simek et al., 1967). An increase in gluconeogenesis and a decrease in glycolysis take place in order to maintain the glucose homoeostasis during the phase of rapid cell proliferation (Leffert et al., 1979; Katz, 1979). The changes in the main carbohydrate metabolic pathways occur in parallel with an increase in glucagon (Morley et al., 1975; Bucher & Weir, 1976; Leffert et al., 1979), corticosterone (Leffert et al., 1979) and catecholamines (Cruise et al., 1987), and with a slight decrease in insulin in perportal blood (Morley et al., 1975; Bucher & Weir, 1976; Leffert et al., 1979). These hormonal variations suggest that the rapid change in the glycolytic/gluconeogenic flux is probably due to an increase in cyclic AMP (Koide et al., 1978), which induces phosphorylation of the enzymes susceptible to modification by the cyclic AMP-dependent protein kinase. However, Fru-2,6-P₂ could also play an important role in this system. Fru-2,6-P₂, which is the most potent allosteric activator of 6-phosphofructo-1-kinase and inhibitor of fructose-1,6-bisphosphatase, has a significant function in the regulation of the glycolytic/gluconeogenic pathway in the liver. The synthesis and the breakdown of Fru-2,6-P₂ are produced by the bifunctional enzyme PFK-2/FBPase-2. The regulation of this enzyme is a complex function of the influence of substrates and effectors, as well as its phosphorylation state via cyclic AMP-dependent protein kinase, in addition to the control of the enzyme levels (Hue & Bartrons, 1985; Van Schaftingen, 1987; Hue & Rider, 1987; Pilks & El-Maghrabi, 1988).

During hepatic regeneration, some transitions from adult liver-type isoenzymes to other isoenzyme patterns corresponding to a less differentiated state have been described. This is the case for glucokinase (Sato et al., 1969) and L-type pyruvate kinase (Bonney et al., 1973; Garnett et al., 1974), which undergo transitions to isoenzymic forms similar to the foetal enzymes. In this experimental model, we have analysed the variations in glycogen and Fru-2,6-P₂ levels and in PFK-2 activity during the first week of liver regeneration. We have also determined the changes in the main regulatory properties of PFK-2/FBPase-2, in order to detect a possible isoenzymic transition similar to that described in hepatoma cells (Loiseau et al., 1988).

EXPERIMENTAL

Chemicals
Enzymes and biochemical reagents were from either Boehringer Mannheim or Sigma. All other chemicals were of analytical grade.

Animals
Fed male Sprague–Dawley rats (180–220 g) were subjected to a 12 h-light/12 h-dark cycle (light periods starting at 08:00 h). Partial hepatectomy (comprising laparotomy and removal of two-thirds of the liver) or sham operation (laparotomy) was performed between 08:00 and 10:00 h, under diethyl ether anaesthesia and by the procedure described by Higgins & Anderson (1931). Control rats were not subjected to either anaesthesia or surgery. The animals were killed by decapitation, and the livers were removed and quickly freeze-clamped in liquid N₂.

Metabolite assays
Fru-2,6-P₂ was extracted and measured as described by Van Schaftingen et al. (1982). Liver glycogen was isolated as described by Carrol et al. (1956), and the amount of glucose produced by acid hydrolysis was determined as described in Kunst et al. (1984). Glycerol 3-phosphate was extracted and measured as described by Lang (1984). Concentrations of glucose 6-phosphate and fructose 6-phosphate were measured fluorimetrically in 0.5 M-HCl extracts by the method of Lang & Michal (1974). Cyclic AMP was measured in these extracts with the cyclic AMP assay kit from Amersham. Ribonucleotide concentrations were determined with a h.p.l.c. system. The liver was homogenized in 10 vol. of 5\% (w/v) trifluoroacetic acid and, after

Abbreviations used: Fru-2,6-P₂, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46).

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were measured as described by Bartrons et al. (1983). FBPase-2 activity was measured by the production of \([^{32}P]P\) from \([2-^{32}P]Fru-2,6-P_2\), which was synthesized as described by El-Maghribi et al. (1982). The reaction was carried out at 30 °C in 50 mM-Hepes buffer (pH 7.5) containing 50 mM-KCl, 5 mM-KH₂PO₄, 2 mM-EDTA, 1 mM-dithiothreitol, 2 mM-MgCl₂, 0.1 mM-NADP⁺, 5 μM-[2-³²P]Fru-2,6-P₂ (200 000 c.p.m./assay), 9 units of phosphoglucoisomerase/ml, 4 units of glucose-6-phosphate dehydrogenase/ml, and in the presence or absence of 5 mM-phenylmethanesulphonyl fluoride. Blanks typically did not exceed 0.1% of the applied radioactivity. Hexokinase activity was calculated as the glucose-phosphorylation capacity at 0.5 mM-glucose, and glucokinase activity as the difference between the glucose-phosphorylation capacity at 100 mM- and at 0.5 mM-glucose, by using the continuous assay described by Davidson & Arion (1987).

As shown in Fig. 1, after partial hepatectomy both the level of Fru-2,6-P₂ and glycogen stores decreased very quickly to very low values. By 7 days after hepatectomy, liver weight and glycogen content were nearly normalized (91% and 76%, respectively), whereas Fru-2,6-P₂ levels still remained low (40%). In the sham-operated group of animals, the metabolites diminished to a much lesser extent. Fru-2,6-P₂ content was restored after 6 h, whereas glycogen stores needed 24 h to regain control values, coinciding with the refeeding period of the animals (dark cycle).

The decrease observed in Fru-2,6-P₂ concentration after partial hepatectomy, we have determined the activity responsible for the synthesis of the metabolite, using kinetic measurements of the ‘total’ and the ‘active’ (non phosphorylated) form of the enzyme. As shown in Fig. 2, after partial hepatectomy the ‘total’ PFK-2 activity decreased only after 24 h, attained significantly lower values at day 4, and did not recover to normal levels during the period studied. In sham-operated animals the ‘total’ PFK-2 activity was not modified with respect to control rats. The ‘active’ PFK-2 activity rapidly decreased in hepatectomized animals, reached 23% of sham-operated activity after 6 h, and recovered moderately (56%) after 7 days of liver resection. In sham-operated animals the ‘active’ PFK-2 varied significantly only after 24 h.

In hepatectomized animals the ‘active’/‘total’ PFK-2 activity ratio paralleled the changes in the ‘active’ form of the enzyme, reaching minimum values 6 h after the surgical resection, and was normalized after 7 days.

The levels of cyclic AMP rapidly increased in the remnant liver after resection (Fig. 3). This suggests that the decrease in the ‘active’ PFK-2 activity after partial hepatectomy is mainly a consequence of the phosphorylation of the enzyme by the cyclic AMP-dependent protein kinase.

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**RESULTS**

**Fru-2,6-P₂ and glycogen contents during liver regeneration**

As shown in Fig. 1, after partial hepatectomy both the level of Fru-2,6-P₂ and glycogen stores decreased very quickly to very low values. By 7 days after hepatectomy, liver weight and glycogen content were nearly normalized (91% and 76%, respectively), whereas Fru-2,6-P₂ levels still remained low (40%). In the sham-operated group of animals, the metabolites diminished to a much lesser extent. Fru-2,6-P₂ content was restored after 6 h, whereas glycogen stores needed 24 h to regain control values, coinciding with the refeeding period of the animals (dark cycle).

**Time course of PFK-2 activity**

In order to explain the decrease observed in Fru-2,6-P₂ concentration after partial hepatectomy, we have determined the
Fructose 2,6-bisphosphate during liver regeneration

Fig. 2. Time course of PFK-2 activity

Hepatectomized (●), sham (○) and control (□) animals were assayed for (a) the 'total' PFK-2 activity (pH 8.5), (b) the 'active' form of the enzyme (pH 6.6) and (c) the 'active'/total' activity ratio. Each point shows the mean ± S.E.M. for 5–8 rats. Statistically significant differences between the hepatectomized and sham operated animals are indicated by * P < 0.001, † P < 0.01 and ‡ P < 0.05.

Fig. 3. Cyclic AMP levels after partial hepatectomy

The concentrations of cyclic AMP were measured in hepatectomized (●), sham-operated (○) and control (□) animals. Each point shows the mean ± S.E.M. for 4–10 rats. Statistically significant differences between the hepatectomized and sham operated animals are indicated by † P < 0.01 and ‡ P < 0.05.

Fig. 4. Effect of sn-glycerol 3-phosphate on PFK-2/FBPase-2 activities

Partially purified PFK-2/FBPase-2 from 96 h-hepatectomized (●) and sham-operated (○, □) animals was used. PFK-2 was assayed in the presence of 0.1 mM-fructose 6-phosphate and 0.5 mM-ATP-Mg<sup>2+</sup>, and with the indicated concentrations of sn-glycerol 3-phosphate, at pH 7.1. FBPase-2 was determined as reported in the Experimental section. The 100% activities (Δ) for hepatectomized and sham-operated animals were 3.9 ± 1.2 and 15.4 ± 2.9 μ-units/mg of protein for PFK-2, and 9.3 ± 1.2 and 9.2 ± 0.5 μ-units/mg of protein for FBPase-2. The values are means ± S.E.M. for 4–5 rats. Statistically significant differences between hepatectomized and sham-operated animals are indicated by * P < 0.001, † P < 0.01 and ‡ P < 0.05.

Changes in the effects of sn-glycerol 3-phosphate and the catalytic subunit of cyclic AMP-dependent protein kinase on PFK-2 activity

In order to establish if PFK-2 in regenerating liver underwent an isoenzymic transition similar to that found in rat hepatoma cells (Loiseau et al., 1988), we have compared the effects of sn-glycerol 3-phosphate and the catalytic subunit of cyclic AMP-dependent protein kinase on the hepatic PFK-2 activity from sham-operated and hepatectomized animals. It has been reported that PFK-2 isozyme from adult liver is more sensitive than the isozyme forms from heart (Rider et al., 1985), hepatoma cells (Loiseau et al., 1988) and foetal liver (Martín-Sanz et al., 1987) to sn-glycerol 3-phosphate inhibition. In contrast with the hepatic isozyme, those isoenzymes are not inactivated by cyclic AMP-dependent protein kinase (Hue & Rider, 1987; Loiseau et al., 1988; Martín-Sanz et al., 1987).

The hepatectomized or sham-operated animals were tested 96 h after surgical treatment. This time corresponded to the highest levels of hexokinase activity observed during the hepatic regeneration (Fig. 5) and to the maximal isoenzymic transition described for pyruvate kinase (Bonney et al., 1973). As shown in Fig. 4, PFK-2 activity from regenerating rat liver was markedly less inhibited by sn-glycerol 3-phosphate than was the activity from sham-operated animals. In contrast, the FBPase-2 activity was more sensitive and more activated. The PFK-2/FBPase-2 activity ratio (at pH 8.5 for the kinase activity, and at pH 7.5 with 5 mM-glycerol 3-phosphate for the phosphatase activity) increased from 5.3 ± 0.9 in hepatectomized to 8.0 ± 1.0 in sham-operated animals (P < 0.05).

Incubation with the catalytic subunit of cyclic AMP-dependent
Table 1. Effect of treatment of PFK-2 with the catalytic subunit of protein kinase

<table>
<thead>
<tr>
<th>Animals</th>
<th>Addition</th>
<th>pH 6.6</th>
<th>pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomized</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+catalytic subunit</td>
<td>43 ± 8</td>
<td>120 ± 31</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+catalytic subunit</td>
<td>101 ± 5</td>
<td>144 ± 5</td>
</tr>
</tbody>
</table>

PFK-2 activity (µ-units/mg of protein) was determined in the presence of 5 mM-dithiothreitol. The mean ± S.E.M. for 3–5 rats is shown. Statistically significant differences between the hepatotmized and sham-operated animals are indicated by * P < 0.01.

As shown in Fig. 5, glucokinase activity decreased 12 h after hepatectomy, attained a minimum value (25% of sham-operated activity) at 48 h and recovered partially after 96 h. In contrast, the hexokinase activity showed higher activities after 12 h of liver resection and attained a maximal value at 96 h, corroborating the isoenzymic transition. The total (glucokinase + hexokinase) activity did not change in sham-operated animals and followed a parallel pattern to that of glucokinase in hepatotmized rats.

DISCUSSION

It is known that after partial hepatectomy the decrease in liver tissue is accompanied by complex hormonal changes, qualitatively resembling those observed in response to starvation, which produce in the liver remnant an increase in glycogenolysis and gluconeogenesis, and a decrease in glycolysis, in order to maintain the blood glucose concentration even during food restriction (Katz, 1979; Petenucsi et al., 1983; Holness et al., 1989). After partial hepatectomy, lactate formation from glucose was decreased to less than 20% of the control, whereas lactate formation from fructose remained unaltered (Katz, 1979). In addition, it has been demonstrated that regenerating liver has an abnormal response of hepatic lipid synthesis to glucose in the fed state (Holness et al., 1989). The altered glycolytic rate with glucose as substrate was ascribed to a decrease in hepatic glucokinase (Katz, 1979), whereas the insensitivity of hepatic lipid synthesis to changes in the carbohydrate supply has been attributed to inactivation of the hepatic pyruvate dehydrogenase (Holness et al., 1989).

The results reported herein indicate that, in addition to the glucokinase and pyruvate dehydrogenase restriction, the fructose 6-phosphate/fructose 1,6-bisphosphate cycle can be an important regulatory step, modulating the glycolytic/gluconeogenic flux during liver regeneration. We have observed that in the first 24 h, before initiation of DNA synthesis, the contents of glycogen and Fru-2,6-P_2 decreased quickly to very low values (Figs. 1b and 1c), probably as a consequence of the rise in cyclic AMP levels (Fig. 3) secondary to the changes in the glucagon/insulin ratio (Leffert et al., 1979) and in catecholamine levels (Cruise et al., 1987). Under these conditions, stimulation of glycogen phosphorylase kinase and concomitant inactivation of PFK-2 and activation of FBPase-2, resulting from phosphorylation of these enzymes by cyclic AMP-dependent protein kinase (Bartrons et al., 1983), would cause an increased degradation of glycogen and a decrease in Fru-2,6-P_2 levels. The fast decrease in the ‘active’ form of PFK-2 observed after hepatectomy (Fig. 2b) would agree with this hypothesis.

Other factors involved in PFK-2 inactivation could be the variations in the concentration of different metabolites that modulate the bifunctional PFK-2/FBPase-2. We have found that hexose 6-phosphates decreased transiently after 6 h hepatectomy (from 578 ± 37 nmol/g in sham-operated animals to 100 ± 29 nmol/g; P < 0.001). ATP levels did not change significantly (results not shown) and glycerol 3-phosphate content was not significantly altered after 24 h hepatectomy (from 1.2 ± 0.5 µmol/g in sham-operated animals to 0.90 ± 0.07 µmol/g). It has been reported that phosphoenolpyruvate (Schofield et al., 1986) and citrate (Schofield et al., 1987) levels were increased after 24 h of partial liver resection. These changes could be especially important, since both phosphoenolpyruvate and citrate are known to exert a dual inhibitory action on PFK-2: in addition to inhibiting PFK-2 activity directly, they promote the cyclic AMP-dependent phosphorylation of the bifunctional PFK-2/FBPase-2 (Van Schaftingen et al., 1984).

The fall in Fru-2,6-P_2 concentration produced by the mechanisms discussed above could lead to an inhibition of 6-phospho-
Fructose-2,6-bisphosphate during liver regeneration

fructose-1-kinase and to an activation of fructose-1,6-bisphosphatase. In this situation, gluconeogenesis occurs and glycolysis is stopped, so that futile cycling of metabolites is avoided. The significance of the restriction of the glycolytic flux at the level of fructose 6-phosphate/fructose 1,6-bisphosphate cycle must be essential during the first day of liver resection, when glyco-
genesis is activated mainly to provide glucose in order to maintain glycaemia, and when glucokinase is not yet decreased. The role of glucokinase to lower the glycolytic flux should be more important after 12 h of liver regeneration, since it is in this phase when the enzyme begins to decrease.

After 24 h of liver resection, the stimulation of gluconeogenesis seems to be maintained through changes in the concentration of regulatory enzymes, in addition to the enzyme cyclic AMP-dependent phosphorylations. As reported herein ‘total’ PFK-2 (Fig. 2a) and glucokinase (Fig. 5) activities remained decreased, even 7 days after hepatectomy. Pyruvate kinase and pyruvate dehydrogenase activities were also decreased in regenerating liver (Schofield et al., 1986), whereas phosphoenolpyruvate carboxy-
kinase and glucose-6-phosphatase activities were increased (Katz, 1979). All these results can contribute to explain the higher glucose production seen in the regenerating liver (Petensuc et al., 1983).

The results reported herein, showing that in hepatectomized rats glycogen stores are replenished faster than Fru-2,6-P_{2} levels, suggest that glycogenesis could be sustained through the indirect pathway, the bulk of liver glycogen being of gluconeogenic origin. Fru-2,6-P_{2} levels remain low during glycogen repletion, as has been found during refeeding (Katz & McGariry, 1984; Kurland & Pilks, 1989). In contrast, in the sham-operated group of animals Fru-2,6-P_{2} is recovered faster than glycogen (Fig. 1). This suggests that after the first hours of surgical stress, when the active/total PFK-2 activity ratio is not significantly modified (Fig. 2), glycogen breakdown could produce the observed recovery of Fru-2,6-P_{2} levels by providing substrate for its synthesis. Glycogen seems to be deposited essentially (Fig. 2a) and glucokinase (Fig. 5) activities remained decreased, even 7 days after hepatectomy. Pyruvate kinase and pyruvate dehydrogenase activities were also decreased in regenerating liver (Schofield et al., 1986), whereas phosphoenolpyruvate carboxy-
kinase and glucose-6-phosphatase activities were increased (Katz, 1979). All these results can contribute to explain the higher glucose production seen in the regenerating liver (Petensuc et al., 1983).

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