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Fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase during liver regeneration

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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 *sn*-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 periportal 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-P_2 could also play an important role in this system. Fru-2,6-P_2, 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-P_2 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; Pilkis & 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-P_2 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 N_2.

Metabolite assays

Fru-2,6-P_2 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-P_2, 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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centrifugation for 5 min at 12000 *g*, the supernatant was neutralized with 0.5 M-acetic acid/acetate buffer, pH 4.0. Then 20 μl of the neutralized sample was used for the determination (Itakura *et al.*, 1986).

Enzyme assays

The 'total' PFK-2 activity and the 'active' PFK-2, corresponding to the activity of the non-phosphorylated enzyme, were measured as described by Bartrons *et al.* (1983). FBPase-2 activity was measured by the production of $[^{32}P]P_i$ from [2-^{32}P]Fru-2,6-P_2, which was synthesized as described by El-Maghrabi *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-KH_2PO_4, 2 mM-EDTA, 1 mM-dithiothreitol, 2 mM-$MgCl_2$, 0.1 mM-$NADP^+$, 5 μM-[2-^{32}P]Fru-2,6-P_2 (200000 c.p.m./assay), 9 units of phosphoglucoisomerase/ml, 4 units of glucose-6-phosphate dehydrogenase/ml, and in the presence or absence of *sn*-glycerol 3-phosphate. 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).

One unit of enzyme activity represents the activity that catalyses the formation of 1 μmol of product/min under the assay conditions, except for the catalytic subunit of cyclic AMP-dependent protein kinase, which is defined (Sigma P-2645) as the activity that transfers 1 pmol of phosphate from [γ-^{32}P]ATP to hydrolysed and partially dephosphorylated casein/min at pH 6.5 and 30 °C.

Partial PFK-2 purification

Liver was homogenized in 10 vol. of 20 mM-KH_2PO_4 buffer, pH 7.1, containing 10 mM-EDTA, 100 mM-KF and 1 mM-dithiothreitol. After centrifugation at 27000 *g* for 30 min, the supernatant was fractionated with poly(ethylene glycol) (6–21 %). The pellet was resuspended in 1 ml of homogenizing medium and used to measure PFK-2 activity. To measure FBPase-2 activity, the enzyme was purified by the same procedure, but in 50 mM-Hepes/50 mM-KCl/5 mM-KH_2PO_4/0.1 mM-EDTA/1 mM-dithiothreitol/0.5 mM-phenylmethanesulphonyl fluoride, at pH 7.5, as homogenizing medium.

Other methods

Proteins were measured as described by Bradford (1976), with bovine serum albumin as standard. Statistical significance of differences was assessed by Student's unpaired *t* test.

RESULTS

Fru-2,6-P_2 and glycogen contents during liver regeneration

As shown in Fig. 1, after partial hepatectomy both the level of Fru-2,6-P_2 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-P_2 levels still remained low (40 %). In the sham-operated group of animals, the metabolites diminished to a much lesser extent. Fru-2,6-P_2 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-P_2 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.

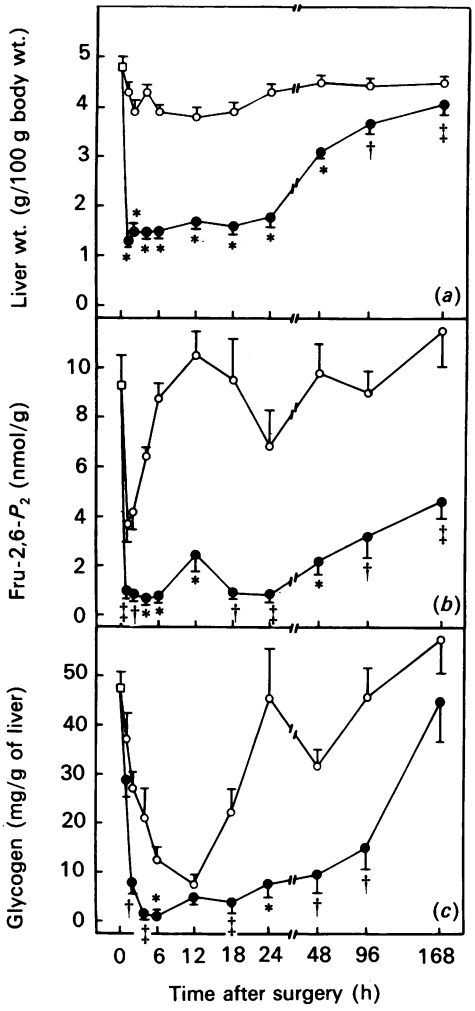

Fig. 1. Fru-2,6-P_2 and glycogen contents during liver regeneration

Liver weight (*a*), Fru-2,6-P_2 (*b*) and glycogen (*c*) concentrations from hepatectomized (●) and sham-operated (○) animals and controls (□) are shown. Each point shows the mean ± S.E.M. for 4–9 rats. Statistically significant differences between the hepatectomized and sham-operated animals are indicated by * $P < 0.001$, † $P < 0.01$ and ‡ $P < 0.05$.

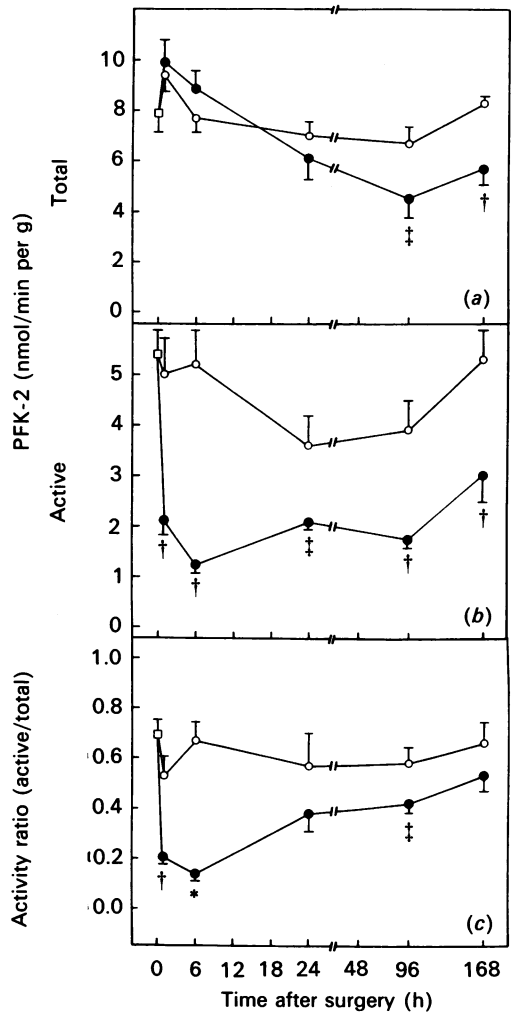

Fig. 2. Time course of PFK-2 activity

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 ‡ $P < 0.05$.

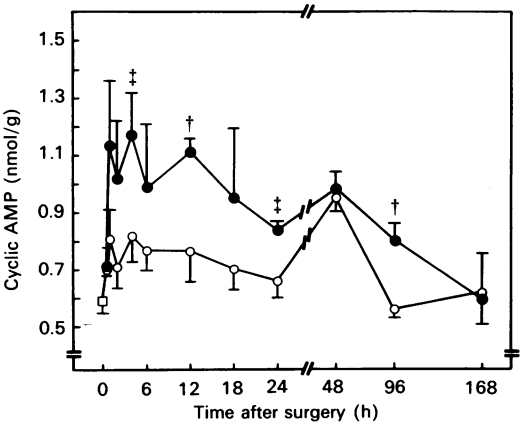

Fig. 3. Cyclic-AMP levels after partial hepatectomy

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 ‡ $P < 0.05$.

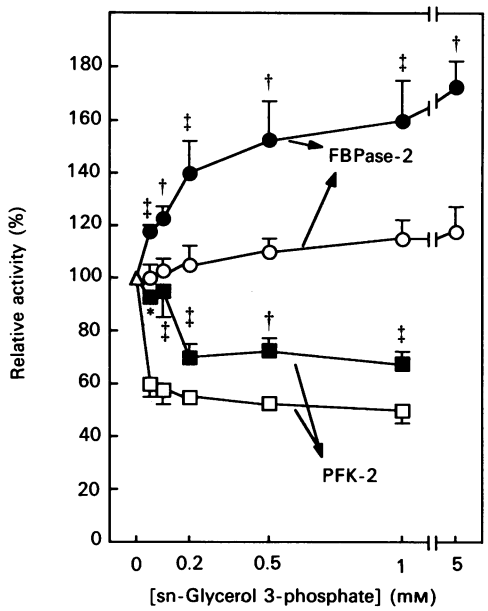

Fig. 4. Effect of *sn*-glycerol 3-phosphate on PFK-2/FBPase-2 activities

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^{2+}, 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 (△) 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 isoenzyme from adult liver is more sensitive than the isoenzyme 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 isoenzyme, 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

Partially purified PFK-2 from 96 h-hepatectomized and sham-operated animals were incubated with 50 m-units of catalytic subunit of protein kinase in a final volume of 0.1 ml containing 20 mM-Hepes buffer, pH 7.0, 100 mM-KCl, 5 mM-$MgCl_2$, 1 mM-ATP-Mg^{+2} and 1 mM-dithiothreitol at 30 °C for 10 min. Samples (40 μl) were taken to measure PFK-2 activity. Each value represents the mean ± S.E.M. for 3–5 rats. Statistically significant differences between the hepatectomized and sham-operated animals are indicated by * $P < 0.01$

Animals	Addition	PFK-2 activity (μ-units/mg of protein) pH 6.6	pH 8.5
Hepatectomized	None	43 ± 8	120 ± 31
	+catalytic subunit	42 ± 5	131 ± 13
Sham-operated	None	101 ± 5	144 ± 5
	+catalytic subunit	64 ± 8*	149 ± 4

protein kinase did not modify the 'active' PFK-2 activity from the hepatectomized animals, whereas it inhibited the enzyme from sham-operated animals. The 'total' PFK-2 was not affected in any case (Table 1). One could argue that the absence of inactivation of the regenerating PFK-2 by cyclic-AMP dependent protein kinase could result from the fact that the enzyme was already in a fully phosphorylated form, as suggested by Loiseau *et al.* (1988). However, no activation was observed when regenerating liver extracts were incubated with $MgCl_2$ (2 mM; 10 min at 30 °C) to stimulate phosphoprotein phosphatase activity (Pelech *et al.*, 1984).

Time course of glucokinase and hexokinase activities

Several enzymes undergo isoenzymic transitions during liver regeneration (Sato *et al.*, 1969; Bonney *et al.*, 1973). Therefore, we studied the change in the activity of the adult liver glucokinase and the less differentiated hexokinase after partial hepatectomy.

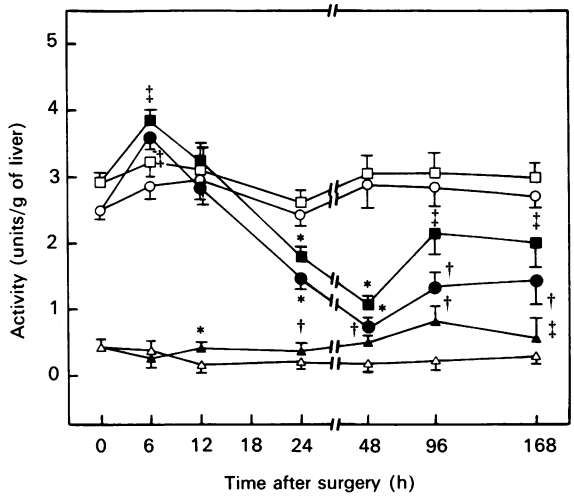

Fig. 5. Time course of glucokinase and hexokinase activities

Hexokinase (▲, △), glucokinase (●, ○) and total (glucokinase + hexokinase) activities (■, □) were measured in hepatectomized (▲, ●, ■) and sham-operated (△, ○, □) animals. Values at zero time represent control animals. Each point shows the mean ± S.E.M. for 4–6 rats. Statistically significant differences between hepatectomized and sham-operated animals are indicated by * $P < 0.001$, † $P < 0.01$ and ‡ $P < 0.05$.

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 hepatectomized 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; Petenusci *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. 1*b* and 1*c*), 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. 2*b*) 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-

fructo-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 glycogenolysis 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 enzymic cyclic AMP-dependent phosphorylations. As reported herein 'total' PFK-2 (Fig. 2*a*) 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 carboxykinase 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 (Petenusci *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 & McGarry, 1984; Kurland & Pilkis, 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 during the refeeding period (after 12 h) in the dark phase and in the presence of Fru-2,6-P_2. This would indicate that, in this experimental condition, either the direct pathway is prevailing or factors other than Fru-2,6-P_2 are influencing the glycogen deposition. A similar situation has been described after sucrose refeeding (Kuwajima *et al.*, 1986; McGarry *et al.*, 1987).

A shift from adult to foetal isoenzyme expression has been described for several glycolytic enzymes in hepatomas (Weinhouse, 1983) and during liver regeneration (Sato *et al.*, 1969; Bonney *et al.*, 1973; Garnett *et al.*, 1974). Recently, an isoenzymic transition has been found for PFK-2 in rat hepatoma cells, suggesting the attractive hypothesis that hepatoma-cell PFK-2 could be the foetal form of the enzyme (Loiseau *et al.*, 1988). The results now reported show that regenerating-liver PFK-2 was less inhibited and that regenerating FBPase-2 was more stimulated by *sn*-glycerol 3-phosphate than was the respective enzyme of normal liver. In addition, regenerating-liver PFK-2 could not be modified by the catalytic subunit of cyclic AMP-dependent protein kinase. Taken together, these results seem to indicate that regenerating-liver PFK/FBPase-2 is different from that of normal liver and possesses a kinetic behaviour similar to that of the foetal isoenzyme (Martín-Sanz *et al.*, 1987, 1989).

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