The mechanism by which ethanol decreases the concentration of fructose 2,6-bisphosphate in the liver

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The intragastric administration of ethanol to fed rats caused in their liver, within about 1 h, a 20-fold decrease in the concentration of fructose 2,6-bisphosphate, an activation of fructose 2,6-bisphosphatase, an inactivation of phosphofructo-2-kinase but no change in the concentration of cyclic AMP. Incubation of isolated hepatocytes in the presence of ethanol caused a rapid increase in the concentration of *sn*-glycerol 3-phosphate and a slower and continuous decrease in the concentration of fructose 2,6-bisphosphate with no change in that of hexose 6-phosphates. There was also a relatively slow activation of fructose 2,6-bisphosphatase and inactivation of phosphofructo-2-kinase. Glycerol and acetaldehyde had effects similar to those of ethanol on the concentration of phosphoric esters in the isolated liver cells. 4-Methylpyrazole cancelled the effect of ethanol but reinforced those of acetaldehyde. High concentrations of glucose or of dihydroxyacetone caused an increase in the concentration of hexose 6-phosphates and counteracted the effect of ethanol to decrease the concentration of fructose 2,6-bisphosphate. As a rule, hexose 6-phosphates had a positive effect and *sn*-glycerol 3-phosphate had a negative effect on the concentration of fructose 2,6-bisphosphate in the liver, so that, at a given concentration of hexose 6-phosphates, there was an inverse relationship between the concentration of fructose 2,6-bisphosphate and that of *sn*-glycerol 3-phosphate. These effects could be explained by the ability of *sn*-glycerol 3-phosphate to inhibit phosphofructo-2-kinase and to counteract the inhibition of fructose 2,6-bisphosphatase by fructose 6-phosphate. *sn*-Glycerol 3-phosphate had also the property to accelerate the inactivation of phosphofructo-2-kinase by cyclic AMP-dependent protein kinase whereas fructose 2,6-bisphosphate had the opposite effect. The changes in the activity of phosphofructo-2-kinase and fructose 2,6-bisphosphatase appear therefore to be the result rather than the cause of the decrease in the concentration of fructose 2,6-bisphosphate.

Ethanol has multiple effects on the rate of glycolysis and gluconeogenesis in the liver. In the presence of 10 mM-lactate, ethanol has an inhibitory effect on gluconeogenesis, which appears to result from a decrease in the concentration of pyruvate (Krebs *et al.*, 1969). When glucose is the

predominant substrate, glycolysis is in operation and is then inhibited by ethanol (Thurman & Scholz, 1977). It is generally assumed that this inhibition occurs at the level of triose phosphate dehydrogenase because of the increased NADH/NAD⁺ ratio. Another effect of ethanol, first reported by Rognstad & Katz (1976), is to decrease the flux through PFK, similarly to glucagon. More recently, this effect of glucagon has been explained by the property of the hormone to cause the disappearance of Fru-2,6-*P*₂, a potent activator of liver PFK. This effect of glucagon was itself explained by the property of cyclic AMP depen-

Abbreviations used: PFK, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK 2, 6-phosphofructo-2-kinase (EC 2.7.1.-); FBPase 2, fructose 2,6-bisphosphatase (EC 3.1.3.-); Fru-2,6-*P*₂, fructose 2,6-bisphosphate.

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dent protein kinase to inactivate PFK 2, the enzyme that forms Fru-2,6- P_2 , and to activate FBPase 2, the enzyme that destroys it (reviewed in Hers & Van Schaftingen, 1982). From the work of Rognstad & Katz (1976), one could therefore expect that ethanol also would presumably cause a decrease in Fru-2,6- P_2 in the liver, an observation that was actually reported by Claus *et al.* (1982) and was also independently made in this laboratory (Van Schaftingen *et al.*, 1982a). An extensive investigation of this effect of ethanol and of its mechanism is described in the present paper.

Methods

Materials

4-Methylpyrazole and cyanamide were from Janssen Chemica (Beerse, Belgium). The source of other materials has been described before (Bartrons *et al.*, 1983).

Preparation of hepatocytes

Hepatocytes were isolated from the liver of fed or overnight fasted rats either according to Hue *et al.* (1978) (the data reported in Figs. 2 and 3) or according to a modification (Bartrons *et al.*, 1983) of the method described by Seglen (1973). The hepatocytes were finally resuspended in a Krebs-Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) as gas phase and incubated in the presence of the indicated (usually 20 mM) concentration of glucose. Cyanamide and 4-methylpyrazole were added after 20 min of incubation; ethanol and other substrates were added after 30 min. Samples were taken for the measurement of enzymes, Fru-2,6- P_2 and other metabolites as described before (Bartrons *et al.*, 1983).

The wet weight of hepatocytes was determined on a pellet resulting from a 5 min $\times$ 1000 g centrifugation; 1 g of hepatocytes from fasted rats contains approx. 0.26 g of protein.

Some experiments were also performed on hepatocytes isolated and cultured for 24 h on floating collagen according to the procedure of Probst & Jungermann (1983) except for the gas phase, which consisted of CO_2 /air (1:9).

Assays of enzymes

These assays were performed at 30°C. The active forms of PFK 2 and FBPase 2 were measured at pH 6.6 and 7.1 respectively as described before (Bartrons *et al.*, 1983). The many-fold dilution of the cellular extracts in the assays prevented any interference of effectors present in the extracts on the enzymic activities. One unit is the amount of enzyme catalysing the formation of 1 μ mol of product/min.

Cyclic AMP-dependent inactivation of PFK 2 in a liver extract

A liver Sephadex filtrate, prepared according to Bartrons *et al.* (1983) and containing about 20 mg of protein/ml, was preincubated for 15 min at 20°C in the presence of 5 mM-MgCl₂; it was then diluted 4-fold and incubated in the presence of 2.5 mM-ATP, 3.5 mM-MgCl₂, 125 mM-KCl, 10 mM-theophylline, 40 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 5 mM-phosphate, 10 μ M-cyclic AMP and glycerol 3- P and Fru-2,6- P_2 at the concentrations indicated. At various times, aliquots were removed and frozen in a cooling mixture (solid CO_2 in acetone). These samples were subsequently thawed in 5 vol. of a buffer containing 20 mM- P_i , 10 mM-EDTA, 100 mM-KCl and adjusted to pH 7, and PFK 2 was measured.

Assays of metabolites

Fru-2,6- P_2 was assayed in alkaline extracts according to Van Schaftingen *et al.* (1982b) as modified by Van Schaftingen & Hers (1983). The hexose 6- P values obtained by the method of Hohorst (1963) were corrected for the Fru-6- P formed from Fru-2,6- P_2 by acid hydrolysis. Glycerol 3- P was measured according to Michal & Lang (1974). Cyclic AMP was determined by the method of Steiner *et al.* (1972).

Results

Experiments in vivo

When ethanol was given by stomach tube to normally fed rats, at a dose of 2 g/kg it caused a 10-fold decrease in the concentration of Fru-2,6- P_2 within 30 min and a 20-fold decrease within 1 h (Fig. 1). This effect persisted for 3–4 h, which is the estimated time required for the nearly complete consumption of that dose of ethanol by the intact rat (Makar & Mannering, 1977). There was also an activation of FBPase 2 and an inactivation of PFK 2; these changes also persisted for about 3.5 h. The concentration of cyclic AMP in the liver was not significantly affected by ethanol administration. In two similar experiments in which ethanol (5 g/kg) was given intragastrically but in association with glucose (2 g/kg), the decrease in the concentration of Fru-2,6- P_2 did not exceed 50% (results not shown).

For the sake of simplicity, further experiments on the mechanism of the ethanol effect were performed in isolated hepatocytes.

Effect of ethanol and of acetaldehyde on isolated hepatocytes

The incubation of hepatocytes obtained from either fed (Fig. 2) or fasted (Fig. 3) rats in the

Fig. 1. Effect of ethanol on the concentration of Fru-2,6- P_2 and cyclic AMP and on the activities of PFK 2 and FBPase 2 in the livers of fed rats

Ethanol (2g/kg as a 20% solution in saline) was administered by intragastric infusion. Data shown are means of two experiments.

presence of 10mM-ethanol caused a large decrease in the concentration of Fru-2,6- P_2 , an inactivation of PFK 2, an activation of FBPase 2 and, as already known, a several-fold increase in the concentration of glycerol 3- P (Nikkila & Ojala, 1963). The effects on the concentration of Fru-2,6- P_2 and of glycerol 3- P were more than half-maximal with 1 mM-ethanol and were prevented by 0.1 mM-4-methylpyrazole, a well-known inhibitor of alcohol dehydrogenase (Theorell *et al.*, 1969), (results not shown). The time-course of the effects indicates that the increase in glycerol 3- P concentration was an early event which preceded the change in Fru-2,6- P_2 concentration. As a rule, the interconversion of PFK 2 and FBPase 2 was relatively slow and reached completion after the decrease in Fru-2,6- P_2 had been initiated. Furthermore, comparison between Figs. 2 and 3 indicates that the concentration of glycerol 3- P increased faster and reached higher values in the cells from fed rats than in those from fasted rats, whereas the reverse was true for the decrease in Fru-2,6- P_2 concentration.

In the experiments described above, the concentration of glycerol 3- P and of Fru-2,6- P_2 always

Fig. 2. Effect of ethanol on the activities of PFK 2 and FBPase 2 and on the concentrations of Fru-2,6- P_2 and glycerol 3- P in hepatocytes from fed rats

The preincubation was performed in the presence of 10mM-glucose; ethanol (filled symbols) was added at a final concentration of 10mM. Data shown are the means $\pm$ S.E.M. of three experiments.

varied in the reverse direction, whereas they are known to be both increased in the presence of a high concentration of glucose. We therefore decided to investigate the combined effects of ethanol and glucose on the concentration of the two phosphoric esters in hepatocytes. As shown in Fig. 4, the concentration of Fru-2,6- P_2 was greatly increased in the presence of increasing concentrations of glucose in the incubation medium, and this occurred in the presence as well as in the absence of ethanol, with the main result that the relative effect of ethanol to decrease Fru-2,6- P_2 concentration was much smaller at high than at low glucose concentration. The complex shape of the control curve in Fig. 4(a) is similar to that observed in purified pancreatic B-cells (Sener *et al.*, 1984) and is presently poorly understood. The concentration of hexose 6- P was also greatly increased (7–8-fold) by glucose, but this concentration was unaffected by the presence of ethanol. The concentration of glycerol 3- P was 3–4-fold increased, both in the presence and the absence of ethanol.

Fig. 3. Effect of ethanol on the activities of PFK 2 and FBPase 2 and on the concentration of Fru-2,6- P_2 and glycerol-3- P in hepatocytes from fasted rats

The preincubation was performed in the presence of 20 mM-glucose; ethanol (filled symbols) was added at a final concentration of 10 mM. Data shown are means $\pm$ S.E.M. of three experiments.

Other precursors were also tested for their ability to affect the concentration of Fru-2,6- P_2 , glycerol 3- P and hexoses 6- P in isolated hepatocytes. We show in Table 1 that glycerol had an effect analogous to that of ethanol and that, when given together with ethanol, it greatly reinforced its effect; indeed, it decreased the concentration of Fru-2,6- P_2 and increased that of glycerol 3- P but did not affect so much that of hexose 6- P . Dihydroxyacetone and xylitol increased the concentration of glycerol 3- P but also that of hexose 6- P and had no (dihydroxyacetone) or little (xylitol) effect on the concentration of Fru-2,6- P_2 . The association of dihydroxyacetone and ethanol caused a now very large increase in the concentration of glycerol 3- P and only a moderate decrease (similar to that with ethanol alone) in that of Fru-2,6- P_2 , whereas the concentration of hexoses 6- P remained elevated. It should be noted that even in the presence of dihydroxyacetone, the concentration of dihydroxyacetone- P (another potent stimu-

Fig. 4. Effect of the concentration of glucose in the incubation medium on the concentration of Fru-2,6- P_2 , hexose 6- P and glycerol 3- P in hepatocytes isolated from fasted rats and incubated in the presence or in the absence of 10 mM-ethanol

Hepatocytes were incubated for 30 min in the presence of the indicated concentration of glucose. Ethanol was then added and the incubation was arrested 10 min thereafter.

lator of FBPase 2) never exceeded one-fifth that of glycerol 3- P .

Acetaldehyde (Table 2), butan-1-ol and crotyl alcohol, but not methanol (results not shown), shared with ethanol the effects of decreasing the concentration of Fru-2,6- P_2 and increasing that of glycerol 3- P . In the case of acetaldehyde these effects were reinforced by 4-methylpyrazole, which inhibits the back conversion to ethanol, and were decreased in the presence of cyanamide, an inhibitor of acetaldehyde dehydrogenase (Dietrich *et al.*, 1976). Fig. 5 shows that there exists an inverse relationship between the concentrations of Fru-2,6- P_2 and that of glycerol 3- P in hepatocytes with similar concentrations of hexose 6- P .

Experiments with hepatocytes in culture

Cultured hepatocytes contained approx. 60 pmol of Fru-2,6- P_2 /mg of protein and this value remained stable for at least 7 h. In the presence of 10 mM-ethanol this value was decreased 8-fold

Table 1. *Effect of ethanol and other additions on the concentration of several phosphate esters*

Hepatocytes isolated from fasted rats were preincubated for 30 min in the presence of 20 mM-glucose and incubated in the presence of other additions as indicated for a further 10 min. Values shown are means $\pm$ S.E.M. for the number of observations in parentheses.

Addition	Fru-2,6- P_2 (nmol/g)		Hexose 6- P (nmol/g)		Glycerol 3- P (μ mol/g)	
	– Ethanol	+ Ethanol	– Ethanol	+ Ethanol	– Ethanol	+ Ethanol
None	22.3 $\pm$ 2.1 (13)	6.1 $\pm$ 1.0† (10)	151 $\pm$ 11 (10)	165 $\pm$ 10 (10)	0.50 $\pm$ 0.04 (12)	1.44 $\pm$ 0.15† (12)
0.5 mM-Glycerol	6.2 $\pm$ 4.4* (3)	0.6 $\pm$ 0.1* (3)	199 $\pm$ 39 (3)	185 $\pm$ 44 (3)	2.06 $\pm$ 0.59 (3)	5.49 $\pm$ 1.59† (3)
1 mM-Glycerol	3.0 $\pm$ 1.8* (4)	0.5 $\pm$ 0.1* (4)	236 $\pm$ 36* (4)	184 $\pm$ 33 (3)	5.16 $\pm$ 0.89* (4)	7.91 $\pm$ 0.89*† (3)
10 mM-Dihydroxyacetone	21.4 $\pm$ 1.9 (5)	4.3 $\pm$ 1.5† (5)	286 $\pm$ 21* (4)	281 $\pm$ 35* (4)	1.39 $\pm$ 0.24* (5)	4.06 $\pm$ 0.74*† (5)
10 mM-Xylitol	14.0 $\pm$ 4.5 (3)	—	313 $\pm$ 21* (3)	—	2.29 $\pm$ 0.13* (3)	—

* Significantly different ($P < 0.05$ by paired t test) versus control without gluconeogenic substrate.

† Significantly different versus control without ethanol.

Table 2. *Effect of acetaldehyde and of cyanamide and methylpyrazole on the concentration of several phosphate esters*
Same experimental conditions as in Table 1 were used except that 4-methylpyrazole and cyanamide were added at 20 min.

Addition	Fru-2,6- P_2 (nmol/g)		Hexose 6- P (nmol/g)		Glycerol 3- P (μ mol/g)	
	–	+	–	+	–	+
10 mM-Acetaldehyde ...						
None	22.3 $\pm$ 2.1 (13)	6.6 $\pm$ 1.9* (4)	151 $\pm$ 11 (10)	184 $\pm$ 18 (3)	0.50 $\pm$ 0.04 (12)	0.83 $\pm$ 0.12* (4)
250 μ M-4-Methylpyrazole	27.6 $\pm$ 8.1 (3)	1.1 $\pm$ 0.2* (4)	148 $\pm$ 36 (4)	145 $\pm$ 20 (3)	0.46 $\pm$ 0.12 (4)	2.50 $\pm$ 0.49* (4)
100 μ M-Cyanamide	24.1 $\pm$ 3.6 (4)	14.1 $\pm$ 3.0* (4)	193 $\pm$ 31 (4)	165 $\pm$ 25 (3)	0.49 $\pm$ 0.07 (4)	0.57 $\pm$ 0.04 (4)
250 μ M-Methylpyrazole + 100 μ M-cyanamide	27.9 $\pm$ 7.6 (3)	2.4 $\pm$ 1.4* (4)	145 $\pm$ 28 (3)	137 $\pm$ 25 (3)	0.46 $\pm$ 0.10 (3)	1.15 $\pm$ 0.20 (4)

* Significantly different ($P < 0.05$ by paired t test) versus control without acetaldehyde.

within 30 min and 20–30-fold after 1 h and remained at this low level for at least an additional 6 h (results not shown).

Effect of glycerol 3-P on the activities of PFK 2 and FBPase 2

The inverse relationship between the concentrations of Fru-2,6- P_2 and glycerol 3- P could result from an effect of the latter ester to inhibit PFK 2 (Claus *et al.*, 1982) or to stimulate FBPase 2 (Van Schaftingen *et al.*, 1982a) or of both effects. Since the two enzymes appear to be part of a single protein (Van Schaftingen *et al.*, 1982a; El-Maghribi *et al.*, 1982a,b) and are therefore always present simultaneously in the assay, the decreased production of Fru-2,6- P_2 in the presence of glycerol 3- P , reported by Claus *et al.* (1982), could be explained by its faster hydrolysis by a more active FBPase 2. We have checked that this was not the case since we observed a 50% inhibition of

PFK 2, measured by the production of ADP at 50 μ M-Fru-6- P due to the presence of 2.5 mM-glycerol 3- P (results not shown). We also report in Fig. 6 an investigation of the antagonistic effects of glycerol 3- P and of Fru-6- P on the activity of FBPase 2. It appears that glycerol 3- P has little effect in the absence of Fru-6- P and that its action is essentially to counteract the inhibition by Fru-6- P . However, this effect rapidly reaches a plateau, indicating that even at high concentrations, glycerol 3- P can never completely overcome the inhibition by Fru-6- P .

Effect of glycerol 3-P and Fru-2,6- P_2 on the inactivation of PFK 2 in a liver extract

Since the inactivation of PFK 2 by ethanol, both *in vivo* and in isolated hepatocytes was slower than the changes in the concentration of Fru-2,6- P_2 and glycerol-3- P , we decided to investigate the effects of these two phosphoric esters on the cyclic AMP-

Fig. 5. Correlation between the concentrations of Fru-2,6- P_2 and glycerol 3-P in hepatocytes

Data shown concern hepatocytes with a concentration of hexose 6-P in the indicated range. They were taken from Fig. 4 and Tables 1 and 2. Glucose was 40mM, without (∇) or with ($\blacktriangledown$) 10mM-ethanol or was 20mM (other symbols). Other additions were none ($\circ$), 10mM-ethanol ($\bullet$), 10mM-dihydroxyacetone without ($\square$) or with ($\blacksquare$) 10mM-ethanol, 10mM-xylitol ($\diamond$), 10mM-acetaldehyde either alone ($\triangle$), with 100 μ M-cyanamide ($\blacktriangle$), with 250 μ M-4-methylpyrazole ($\blacktriangle$) or with both inhibitors ($\blacktriangle$).

Fig. 6. Effect of glycerol 3-P and of Fru-6-P on the activity of FBPase 2

0.03m-unit of FBPase 2 purified according to Van Schaftingen *et al.* (1982a) were incubated for 10min at 30°C in a final volume of 0.25ml and in the presence of 5 μ M [32 P]Fru-2,6- P_2 , 0.4mM-GTP, 2.4mM-MgCl₂, 5mM-phosphate, 100mM-KCl, 40mM-Hepes, pH 7.1, and Fru-6-P and glycerol 3-P as indicated.

Fig. 7. Effect of 1mM-glycerol 3-P and of 1 μ M-Fru-2,6- P_2 on the cyclic AMP-dependent inactivation of PFK 2 in a liver Sephadex filtrate

No inactivation occurred when cyclic AMP was omitted (results not shown).

Fig. 8. Concentration dependency of the effects of Fru-2,6- P_2 and glycerol 3-P on the cyclic AMP-dependent inactivation of PFK 2 in a liver Sephadex filtrate
PFK 2 activity was measured after a 3min incubation in the inactivation mixture. The initial activity was 220 μ -unit/ml of incubation mixture.

dependent inactivation of PFK 2 in a liver extract. We show in Fig. 7 that $1\text{ }\mu\text{M}$ -Fru-2,6- P_2 inhibited and 1 mM -glycerol 3- P enhanced this inactivation. The dose-dependency of these effects is shown in Fig. 8. Half-maximal effects were obtained with approx. $0.5\text{ }\mu\text{M}$ -Fru-2,6- P_2 and 0.1 mM -glycerol 3- P . No such effect of Fru-2,6- P_2 was observed on the inactivation of pyruvate kinase under similar conditions (results not shown).

Discussion

The concentration of Fru-2,6- P_2 in the liver can be decreased by glucagon (Van Schaftingen *et al.*, 1980), adenosine (Bartrons *et al.*, 1984) and ethanol (Claus *et al.*, 1982; the present paper). The effect of glucagon and that of adenosine have been explained by the binding of these effectors to membranous receptors, activation of adenylate cyclase and of cyclic AMP-dependent protein kinase and phosphorylation of the PFK 2/FBPase 2 protein. In contrast, ethanol does not increase cyclic AMP concentration in the liver. It can therefore be used as a tool to study the effect of a Fru-2,6- P_2 deprivation in the liver, *in vivo*, as well as in hepatocytes in suspensions or in culture, independently of the presence of cyclic AMP.

The fact that the effect of ethanol is shared by other substrates of alcohol dehydrogenase and by acetaldehyde, and is inhibited by methylpyrazole, indicates that the decrease in the concentration of Fru-2,6- P_2 in the liver is mediated by the reduction of NAD. Such a decrease can result from a decreased activity of PFK 2 or an increased activity of FBPase 2 or from both. The activities of these two enzymes are controlled by two mechanisms: (1) stimulation or inhibition by a series of metabolites, and (2) phosphorylation by cyclic AMP-dependent protein kinase and dephosphorylation. These two control mechanisms will be considered separately.

Control by metabolites

Since an early and striking effect of ethanol on the liver is to increase its concentration of glycerol 3- P , we must consider the possibility that, as already proposed by Claus *et al.* (1982), this change could explain the decrease in Fru-2,6- P_2 concentration. Indeed, glycerol 3- P is a potent stimulator of FBPase 2 and its action on that enzyme is to antagonize the inhibitory effect of Fru-6- P (Van Schaftingen *et al.*, 1982a; Fig. 6). It is also an inhibitor of the production of Fru-2,6- P_2 (Claus *et al.*, 1982) and of ADP (the present paper) by PFK 2.

All our data are in agreement with this simple hypothesis with an emphasis on the antagonistic action of glycerol 3- P and Fru-6- P . As a rule, a rela-

tively low dose of ethanol would cause the virtual disappearance of Fru-2,6- P_2 from the liver of fed rats, in proportion with the rise in glycerol 3- P and with no change in the concentration of hexose 6- P . The effect on Fru-2,6- P_2 concentration is attenuated if the concentration of glucose is increased. The data in Fig. 4 clearly indicate that the effect of glucose is mediated by an increased concentration of Fru-6- P which is, however, accompanied by a somewhat smaller increase in glycerol 3- P . That the effect of these two opposite effectors was an increase in the concentration of Fru-2,6- P_2 is in agreement with the fact that glycerol 3- P is unable to relieve completely the inhibition exerted by Fru-6- P on FBPase 2 (Fig. 6). The fact that glycerol, as already shown by Hue *et al.* (1982), can also decrease the concentration of Fru-2,6- P_2 is in agreement with the general hypothesis. Finally, the absence of effect of dihydroxyacetone cannot be taken as an objection since, in the presence of this triose, the concentration of glycerol 3- P reached lower values and that of hexose 6- P higher values than in the presence of glycerol.

Interconversion of PFK 2/FBPase 2

As shown in Figs. 2 and 3, these interconversions are relatively slow events which appear to be the consequence rather than the cause of the disappearance of Fru-2,6- P_2 . Since ethanol does not affect the concentration of cyclic AMP, the only explanation that we can propose for the inactivation of PFK 2 and activation of FBPase 2 is the ability of Fru-2,6- P_2 to prevent, and that of glycerol 3- P to enhance, the inactivation of PFK 2. An inhibitory effect of Fru-2,6- P_2 had already been shown by El Maghrabi *et al.* (1982a), who reported an approx. 60% inhibition of the phosphorylation of PFK 2/FBPase 2 in the presence of $80\text{ }\mu\text{M}$ -Fru-2,6- P_2 but only 12% inhibition in the presence of $8\text{ }\mu\text{M}$ Fru-2,6- P_2 . The weakness of this effect is presumably explained by the high concentration of enzymes used by these authors, sufficient to hydrolyse more than 10 nmol of Fru-2,6- P_2 /ml in 1 min. The complete inhibition observed at $2\text{ }\mu\text{M}$ -Fru-2,6- P_2 (Fig. 7), together with the stimulation of the same reaction by glycerol 3- P , offers an explanation for the progressive inactivation of PFK 2 in the presence of ethanol. The fact that, in hepatocytes, the inactivation of PFK 2 proceeds in the presence of concentrations of Fru-2,6- P_2 higher than $2\text{ }\mu\text{M}$ indicates, however, that this inhibition is counteracted by the presence of glycerol 3- P or of other ligands. We have also good indications that in the hepatocytes, a large part of Fru-2,6- P_2 is bound to fructose 1,6-bisphosphatase (E. Van Schaftingen & H.-G. Hers, unpublished work).

Knowing that PFK 2 and FBPase 2 are part of a

single protein (reviewed in Bartrons *et al.*, 1983), one must assume that the activation of FBPase 2 is parallel with the inactivation of PFK 2. A potential effect of other ligands of the two enzymes, such as Fru-6-P and P-enolpyruvate, on their interconversion cannot be discarded but has not yet been investigated.

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