Biochem. J. (1989) 257, 795–799 (Printed in Great Britain)

Glucagon-induced changes in fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase in cultured rat foetal hepatocytes

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The sensitivity of 6-phosphofructo-2-kinase to glucagon and cyclic AMP was studied during the perinatal period. In liver homogenates from foetal and neonatal rats, incubation with cyclic AMP produced inactivation of 6-phosphofructo-2-kinase 3 h after birth. The maximal effect was obtained 12 h after birth. In primary cultures of hepatocytes from 22-day-old foetuses, glucagon induced an inhibition of 6-phosphofructo-2-kinase that required 45 min to reach the half-maximal effect. Cycloheximide prevented the glucagon-induced changes in this activity from cultured foetal hepatocytes. These results suggest that the adult form of 6-phosphofructo-2-kinase is rapidly induced after birth, probably by the hormonal changes that occur in this period.

INTRODUCTION

The transition from the foetal to the neonatal state is accompanied by important changes in liver carbohydrate metabolism that allow this organ to play its physiological roles in the neonatal situation. These changes are mainly elicited by modifications in the expression pattern of enzymes and even of tissue-specific isoenzymes [1,2].

The hypoglycaemic phase that occurs in the newborn as a result of the fall in the maternal glucose supply is decreased by different mechanisms, among them glycogen mobilization [3], a decrease in the glycolytic capacity of the hepatocytes, and the progressive development of gluconeogenesis. This situation is favoured by hormonal and nutritional factors, such as the inversion in the insulin/glucagon ratio in newborns [4].

One of the factors that plays an important role in the control of the glycolytic and gluconeogenic pathway in liver is the change in fructose 2,6-bisphosphate (Fru-2,6-P_2) concentration [5]. It has been reported that foetal hepatocytes contain a form of 6-phosphofructo-2-kinase (PFK-2) that lacks the well-established glucagon-dependent inactivation of the enzyme that is exhibited by the adult liver. In foetal hepatocytes glucagon produces a rise in the concentration of cyclic AMP, and the activity of the cyclic AMP-dependent protein kinase is even higher than that found in the adult liver [6]. Moreover, foetal PFK-2 exhibits some kinetic properties similar to those reported for the bovine heart isoenzyme [7].

Since foetal PFK-2 did not respond to glucagon, it was decided to investigate the time course of the expression of PFK-2, regulated by this hormone, during the perinatal period *in vivo*. In addition, primary cultures of foetal hepatocytes were used to study the factors relevant to the regulation of this enzyme activity.

EXPERIMENTAL

Animals

Pregnant Albino Wistar rats (300–350 g) fed on a standard laboratory diet were killed for the experiments between 09:00 and 10:00 h. Gestational age was confirmed by standard criteria [8]. Newborn rats were delivered by Caesarean section in the morning of day 21 or 22 of gestation and were immediately used. Livers of newborns were removed at appropriate times and stored in liquid N_2.

Materials

Substrates, antibiotics, hormones, coenzymes and enzymes were obtained from Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, U.S.A.). Standard analytical-grade laboratory reagents were purchased from Merck (Darmstadt, Germany). Tissue-culture dishes were from Costar (Cambridge, MA, U.S.A.). Foetal-calf serum was from Biochrom (Berlin, Germany). Leibovitz L-15 and F-10 media were from GIBCO (Hounslow, Middx., U.K.). Radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.).

Isolation of foetal hepatocytes

Hepatocytes from foetal-rat liver were prepared by a non-perfusion collagenase dispersion method that involved incubation in a rotary shaking bath (40 cycles/min) with Ca^{2+}-free Krebs bicarbonate buffer [6] containing 0.5 mM-EGTA, for 30 min at 37 °C under continuous gassing (O_2/CO_2, 19:1). The resulting cell suspension was centrifuged (50 *g* for 2 min), and the cells were resuspended and incubated for 60 min in the presence of 2.5 mM-Ca^{2+} and 0.5 mg of collagenase/ml. At the end of this incubation period, the cells were centrifuged (35 *g* for 4 min) and filtered through nylon membranes of different meshes (500–50 μm). Separation of parenchymal from haematopoietic cells was carried out by washing the suspension by centrifugation at controlled very low speed (35 *g*). Haematopoetic-cell contamination was determined by microscopic observation to be less than 5%. Cells were obtained (approx. 1.5×10^7 cells/g of liver) with about 15% recovery. Cell viability (Trypan Blue exclusion and lactate dehydrogenase activity) was always higher than 90%. Finally, the hepatocytes were

Abbreviations used: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

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washed twice with sterile Leibovitz L-15 medium and then suspended in this medium supplemented with 10 mM-Hepes adjusted to pH 7.4 with NaOH, 10 mM-glucose, 50 μg of gentamycin/ml, 50 μg of streptomycin/ml and 100 units of penicillin G/ml.

Hepatocytes [$(2–3) \times 10^6$] were dispensed into 60 mm tissue-culture dishes that had been previously coated with collagen and which contained a final volume of 2 ml of modified Leibovitz L-15 medium (as above) supplemented with 2% (v/v) foetal-calf serum. The dishes were incubated for 2 h at 37 °C in air, and the unattached cells were removed together with the medium. The medium was then replaced by 2 ml of Leibovitz L-15 medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin.

Additions were made so that the total volume of the medium changed by less than 2%. Hormones were prepared under sterile conditions (passed through a 0.22 μm-pore-size filter). At the indicated times, the cells were washed once with 4 ml of Leibovitz L-15 medium without serum or albumin and then scraped from the plates in 1 ml of ice-cold buffer A (100 mM-KCl/1 mM-EDTA/1 mM-dithiothreitol/20 mM-potassium phosphate, pH 7.4). The suspensions were homogenized at 4 °C in a Teflon/glass homogenizer, then sonicated for 10 s at 22 kHz, and centrifuged at 12000 *g* for 15 min, and the supernatants were stored at −20 °C. For the measurement of Fru-2,6-P_2 concentration the medium was aspirated and the dishes were scraped in 0.5 ml of 50 mM-NaOH, followed by heating at 80 °C for 10 min.

Preparation of liver homogenates

Foetal and neonatal livers of different ages were homogenized in 3 vol. of ice-cold buffer A. The homogenates were then centrifuged at 4 °C for 30 min at 20000 *g*, and 250 μl portions of the supernatants were incubated at 30 °C for 5 min in a medium containing 100 mM-KCl, 5 mM-$MgCl_2$, 2 mM-MgATP, 50 mM-Hepes, pH 7.4, with or without 1 mM-isobutylmethylxanthine and 50 μM of 8-(4-chlorophenylthio) cyclic AMP in a total volume of 0.5 ml. The incubation was stopped by adding 1 vol. of 200 mM-NaF, and the tubes were chilled on ice to prevent any further change in the phosphorylation state of PFK-2. Samples of these incubations (0.4 ml) were processed for PFK-2 activity measurements.

To determine the concentration of Fru-2,6-P_2 in livers, small portions of tissue were immediately homogenized with 50 mM-NaOH (1:20, w/v).

Measurements of metabolites and enzymes

Fru-2,6-P_2 was determined as described by Van Schaftingen *et al.* [9]. Cyclic AMP was measured by using the binding-protein assay with the Amersham kit, as follows. The cells were incubated with 1 mM-isobutylmethylxanthine for 5 min before addition of glucagon, and at appropriate times the medium was aspirated and the dishes were scraped with 0.5 ml of 0.5 M-$HClO_4$. After centrifugation in a Eppendorff centrifuge, the supernatants were neutralized with a saturated K_2CO_3 solution and the pH was adjusted to 7.0 with 0.5 M-Tris/HCl. These supernatants were maintained for 15 min in an ice-cold bath and, after centrifugation (Eppendorff), cyclic AMP was assayed.

For the measurement of enzyme activities, the cells or liver supernatants were fractionated after precipitation with 15% (cells) or 6–15% (liver homogenates) (v/v) poly(ethylene glycol) [6]. After resuspension of the pellets in the extraction medium, PFK-2 activity was assayed. PFK-2 was measured at pH 6.6 in the presence of 5 mM-phosphate, 5 mM-MgATP and 1 mM-fructose 6-phosphate, and at pH 8.5 with 1 mM-phosphate, 5 mM-MgATP and 5 mM-fructose 6-phosphate [10]. Fructose 6-phosphate was in a 1:3 ratio with glucose 6-phosphate in order to prevent changes in its concentration by phosphoglucose isomerase. The sensitivity of PFK-2 activity to *sn*-glycerol 3-phosphate inhibition was determined at pH 7.1 and in the presence of 0.1 mM-fructose 6-phosphate and 0.5 mM-MgATP [7]. One unit of PFK-2 activity is the amount of enzyme that catalyses the formation of 1 pmol of Fru-2,6-P_2/min.

To measure the rates of RNA and protein synthesis, 5 μCi of [^{3}H]uridine or 50 μCi of [^{3}H]leucine respectively was added to the plates in a final volume of 1 ml. After incubation for 3 h, the cells were scraped in 1 ml of an ice-cold solution containing 1% SDS and 0.5% Nonidet P-40, followed by 5 ml of 10% trichloroacetic acid. The radioactivity retained on GF/C glass-fibre filters after filtration was determined by counting the oven-dried filters in 2 ml of scintillation liquid.

Protein concentration was determined by the method of Bradford [11], with bovine serum albumin as standard.

RESULTS

PFK-2 in liver homogenates

Liver homogenates were prepared from foetal and neonatal rats in order to determine the developmental changes in PFK-2 activity during the perinatal period. In these homogenates two parameters were measured: the total activity of PFK-2, assayed at pH 8.5 in the presence of saturating concentrations of substrates, and the ability of the cyclic AMP-dependent protein kinase activated by added cyclic AMP to induce changes in PFK-2 activity. As shown in Fig. 1(*a*), PFK-2 activity in foetal liver hepatocytes was approx. 120 units/mg of protein when measured at pH 8.5, and exhibited a transient increase (40%) in the enzyme activity 12 h after birth, followed by a decrease, and reached stability 2 days later. The activity measured at this pH represents the total activity of PFK-2, which is independent of the phosphorylation state of the enzyme, at least in adult liver. Accordingly, the activity was similar for each incubation, regardless of the presence or absence of 8-(4-chlorophenylthio) cyclic AMP. However, when the activity was measured at pH 6.6 in order to exhibit differences depending on the phosphorylation state of PFK-2 [10], a clear inhibition was observed in post-natal liver homogenates incubated with 8-(4-chlorophenylthio) cyclic AMP. When the activity of PFK-2 is expressed as the ratio of the activity at pH 8.5 to that at pH 6.6 of liver homogenates incubated in the presence of 8-(4-chlorophenylthio) cyclic AMP, a progressive increase in the ratio values appared 3 h after birth (Fig. 1*b*). It is also noteworthy that during the foetal period (days 20–22 of gestation) no changes in the PFK-2 activity ratio were observed in the presence of 8-(4-chlorophenylthio) cyclic AMP. Taken together, these results suggest that the foetal isoenzyme is progressively replaced after birth by a form that exhibits kinetic changes in response to the cyclic AMP-dependent protein kinase. This enzyme is presumably similar to that present in adult liver. Moreover, a progressive increase in the sensitivity to *sn*-glycerol 3-phosphate inhibition was

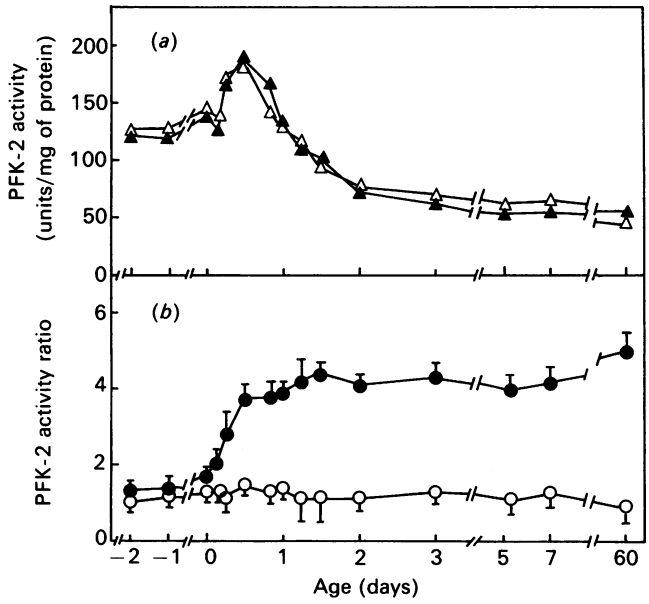

Fig. 1. PFK-2 activity during the perinatal period

Liver homogenates were prepared from foetal and neonatal rats and incubated in the absence or the presence of 50 μM-cyclic AMP derivative. PFK-2 was fractionated with poly(ethylene glycol) (6–15%) before the assay. (*a*) PFK-2 activity at pH 8.5; (*b*) PFK-2 activity ratio (pH 8.5/pH 6.6). White and black symbols respectively represent incubations in the absence or in the presence of the cyclic AMP derivative. Results are means ± S.E.M. for five or six different determinations.

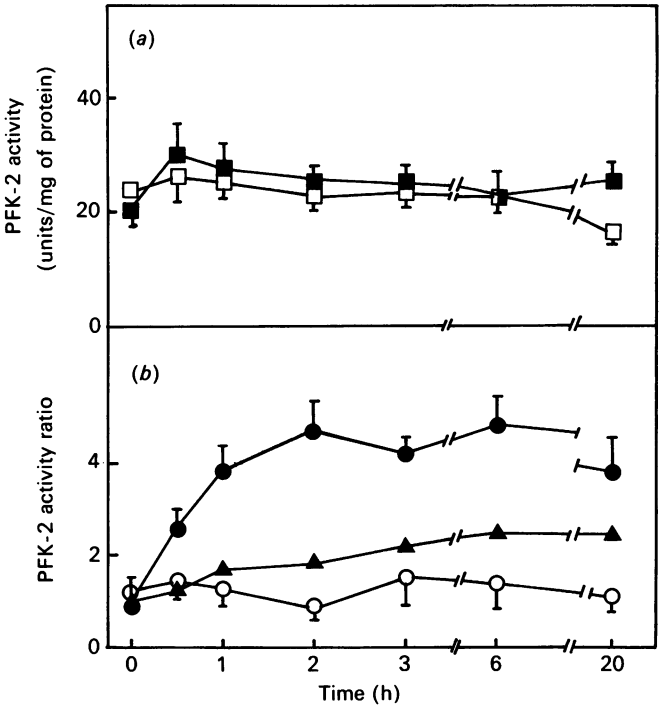

Fig. 2. Glucagon-induced changes in PFK-2 activity in primary cultures of foetal hepatocytes

Hepatocytes were prepared from 22-day foetuses and incubated for 2 h with Leibovitz L-15 medium supplemented with 2% foetal-calf serum, followed by a 30 min preincubation period with Leibovitz L-15 medium containing 2 mg of fatty-acid poor bovine serum albumin/ml before addition of 0.1 μM-glucagon. PFK-2 was fractionated with poly(ethylene glycol) (0–15%) before the assay. (*a*) PFK-2 activity at pH 8.5; (*b*) PFK-2 activity ratio (pH 8.5/pH 6.6). White symbols, controls; black symbols, in the presence of glucagon. When cycloheximide (1 μM) was present in the culture medium, it was added 30 min before glucagon (▲). Each point was obtained by combining cells from two dishes. Results are means ± S.E.M. for three different experiments, except for those with cycloheximide, which are means of two experiments.

observed in liver homogenates from neonatal rats. At birth the inhibition of PFK-2 activity by 1 mM-glycerol 3-phosphate was 37% of control value, and increased up to 68% in liver homogenates from 5-day-old newborns.

Glucagon-induced changes in PFK-2

Since the response of PFK-2 activity to the activation of the cyclic AMP-dependent protein kinase in liver homogenates appears during the first 24 h of life, we decided to investigate whether this effect could be reproduced in primary cultures of hepatocytes from 22-day foetuses. As shown in Fig. 2(*a*), the PFK-2 activity from cultured foetal hepatocytes incubated in the absence or presence of 0.1 μM-glucagon remained unchanged when assayed at pH 8.5 (total activity).

Fig. 2(*b*) shows the PFK-2 activity ratio (pH 8.5/pH 6.6) of the enzyme at different times after exposure of the hepatocytes to glucagon. When PFK-2 activity was measured at pH 6.6, a time-dependent inhibition of the enzyme activity was evident in hepatocytes exposed to glucagon. The half-maximal inhibitory effect was observed about 45 min after exposure to the hormone. Moreover, when the hepatocytes were preincubated with 1 μM-cycloheximide 30 min before addition of glucagon, the inhibitory effect of the hormone on PFK-2 activity was partially prevented (about 75%). To ensure that cycloheximide used at 1 μM concentration did not interfere in the cellular response to glucagon, a parallel experiment was carried out using isolated adult hepatocytes. In this system glucagon produced the expected inhibition of PFK-2 activity and a decrease in the intracellular concentration of Fru-2,6-P_2 regardless of the preincubation with cycloheximide (results not shown). The maximal effect on PFK-2 inhibition in cultured foetal hepatocytes was obtained 2 h after exposure to glucagon. These experiments suggest that protein synthesis is required for the observation of glucagon-induced changes in PFK-2. No significant differences in the [^{3}H]uridine or [^{3}H]leucine incorporation in the macromolecular fraction were observed between control and glucagon-incubated hepatocytes (results not shown).

When isolated foetal hepatocytes were maintained in culture for long periods (more than 1 day in Leibovitz L-15 medium), and the cells were exposed for 15 min to glucagon, a rapid inhibition of PFK-2 activity measured at pH 6.6 was observed (after incubation for 48 h the pH 8.5/pH 6.6 activity was 3.8), suggesting that the culture conditions were sufficient to induce progressively a glucagon-responsive form of PFK-2. However, this process was accelerated by the presence of glucagon in the culture medium.

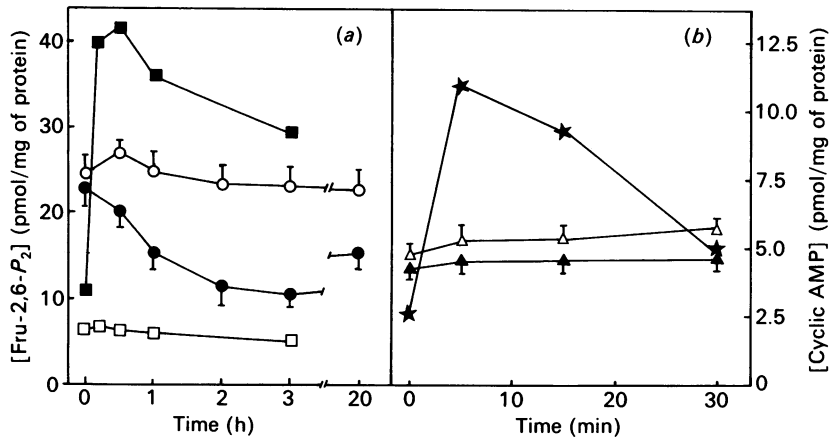

Fig. 3. Effect of gluagon on Fru-2,6-P_2 and cyclic AMP concentrations in foetal hepatocytes

Cultured (*a*) or isolated (*b*) foetal hepatocytes were incubated in the absence (white symbols) or in the presence (black symbols) of 0.1 μM-glucagon, and at the indicated times samples were collected to determine the Fru-2,6-P_2 (○, ●, △, ▲) or cyclic AMP (□, ■, ★) concentrations. Results are means ± S.E.M. for three experiments (Fru-2,6-P_2) or means of two experiments (cyclic AMP).

Table 1. Effect of several hormones on PFK-2 activity and on Fru-2,6-P_2 concentration in primary cultures of foetal hepatocytes

Liver cells were isolated from 22-day foetuses and cultured with Leibovitz L-15 medium with 2% foetal-calf serum. At 2 h after seeding (2×10^6 cells; 6 cm plates) the medium was aspirated and replaced by 2 ml of Leibovitz L-15 medium supplemented with 2 mg of fatty-acid-poor bovine serum albumin/ml. After preincubation for 30 min, hormones were added for 3 h. Samples were taken to determine PFK-2 activity and Fru-2,6-P_2. Results are means ± S.E.M. for three different experiments. Significance of differences with respect to control values is given by: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (NS, not significant).

Addition	PFK-2 activity		Fru-2,6-P_2
	pH 8.5/pH 6.6 ratio	(units/mg of protein)	(pmol/mg of protein)
None	1.4±0.2	24±2	21.2±0.8
Glucagon (0.1 μM)	4.3±0.3***	29±3 NS	10.8±1.2**
8-(4-Chlorophenylthio) cyclic AMP (50 μM)	4.2±0.1***	24±1 NS	9.6±1.2**
8-(4-Chlorophenylthio) cyclic AMP (50 μM)+ cycloheximide (1 μM)	2.8±0.2*	19±3 NS	15.2±1.6*
Insulin (10 nM)	2.2±0.4 NS	24±3 NS	18.4±0.8*
Glucagon (0.1 μM)+insulin (10 nM)	2.9±0.6*	23±4 NS	14.8±0.8*
Dexamethasone (1 μM)	2.7±0.2*	18±2 NS	18.0±1.6 NS
Dexamethasone (1 μM)+glucagon (0.1 μM)	2.4±0.3 NS	19±3 NS	14.8±1.6*

Fru-2,6-P_2 in foetal hepatocytes

To investigate the effect of the inactivation of PFK-2 by glucagon on Fru-2,6-P_2, primary cultures of foetal hepatocytes were exposed to the hormone (0.1 μM) and samples were collected to determine Fru-2,6-P_2 concentration. As shown in Fig. 3(*a*), glucagon decreased the concentration of this metabolite, although a long period of exposure (45 min) was required to produce the half-maximal effect. This is in agreement with the inactivation of PFK-2 activity reported in Fig. 2(*b*), but in striking contrast with the reported effect of glucagon on both PFK-2 and Fru-2,6-P_2 in isolated adult hepatocytes [12].

To ascertain that the lag period observed in the inactivation of PFK-2 and in the fall in Fru-2,6-P_2 concentration was not due to damage to the glucagon receptors or to the mechanism of generation of cyclic AMP as a result of cell preparation, the changes in the intracellular concentration of cyclic AMP were measured. Fig. 3(*a*) shows that cultured hepatocytes effectively responded to the hormone by increasing the concentration of cyclic AMP up to 5 times the value of controls.

When freshly isolated foetal hepatocytes were used to test rapid effects of glucagon on both cyclic AMP and Fru-2,6-P_2 concentrations, these cells lacked the effect on Fru-2,6-P_2 (exposed to the hormone for up to 30 min), whereas the changes in cyclic AMP were rapid (maximal effect obtained in less than 5 min) and qualitatively similar to those reported for the cultured hepatocytes (Fig. 3*a*).

The glucagon-induced changes in PFK-2 activity and Fru-2,6-P_2 in cultured hepatocytes were also obtained when the cells were incubated with a permeant cyclic AMP derivative as shown in Table 1. Moreover, when cycloheximide was present in the medium in addition to cyclic AMP, the changes in PFK-2 and Fru-2,6-P_2 were decreased by 50%, in agreement with the results obtained

with glucagon. The action of different hormones in the activity of PFK-2 from cultured hepatocytes was also tested. In cells incubated with both insulin and glucagon, at saturating concentrations of these hormones, the effect of glucagon on PFK-2 was decreased to about 20% the value of controls. A similar situation was observed when the hepatocytes were incubated in the presence of dexamethasone and glucagon. The total activity of the enzyme remained unchanged in all cases. Moreover, a good correlation was observed between Fru-2,6-P_2 content and the value of the PFK-2 activity ratio.

DISCUSSION

In the present paper we have investigated the time course for the expression of glucagon-induced changes in PFK-2 activity and Fru-2,6-P_2 during the perinatal period. Foetal hepatocytes contain a PFK-2 isoenzyme, which activity is not affected by either the cyclic AMP-dependent protein kinase or glucagon [6]. This occurs despite the increase in cyclic AMP metabolism on the last day of pregnancy [13]. The results reported clearly show that a PFK-2 activity regulated by glucagon is expressed after birth. Presumably the adult form of PFK-2 would follow an expression pattern similar to that reported for the hepatic isoenzyme of pyruvate kinase [14,15]. Both enzymes, PFK-2 and pryuvate kinase, play an important role in the control of the glycolytic and gluconeogenic fluxes in liver through its action on the rates of the substrate futile cycles. In addition, in the adult liver a co-ordinate response to glucagon appears between both enzymes [16]. However, there is a lack of parallelism between the time course for glucagon-induced changes in PFK-2 in the early postnatal period (hours) if compared with the time required for the induction of pyruvate kinase L-form (days) [14]. Moreover, 12 h after birth the total PFK-2 enzyme activity was increased, followed by a decrease, which supports the expected high dynamism in the rates of synthesis and degradation of the enzyme. Thus the evolution of the total activity is in agreement with the changes in the PFK-2 activity ratio. The use of liver extracts to test the response of PFK-2 to cyclic AMP has some additional advantages over the use of hepatocytes and glucagon as effector, because on the last day of pregnancy a maturation in the adenylate cyclase system has been described [17,18]. Our experimental system by-passes these steps in the activation of the cyclic AMP-dependent protein kinase.

Attempts to elucidate the factors that bring about the response of PFK-2 to glucagon were made by using cultures of hepatocytes from 22-day-foetuses. Cells attached to a collagen substratum have been reported to retain most of the properties of hepatocytes [19]. Leibovitz L-15 and F-10 media were used for the culture, and both gave the same results with respect to PFK-2 changes. The exposure of the 22-day-foetal hepatocytes to glucagon produced a progressive inhibition of PFK-2 activity at pH 6.6. To obtain this effect, protein synthesis is required as deduced from the lack of response to glucagon when the foetal cells were preincubated with cycloheximide. However, in isolated adult hepatocytes cycloheximide did not alter the response to glucagon. The mechanism involved in the inhibition of PFK-2 by glucagon in foetal hepatocytes is not clear. One possibility may be that the synthesis of the adult enzyme is stimulated by glucagon. Work is needed to ascertain whether the rate of the synthesis of the adult liver enzyme could be affected by glucagon, by the use of antibodies raised against this isoenzyme. Experiments on incorporation of [^{3}H]uridine or [^{3}H]leucine in the macromolecular fraction in hepatocytes incubated in the absence or in the presence of glucagon did not show significant differences in the rate of RNA or protein synthesis.

Hepatocytes from 21-day foetuses were also used to test whether these cells had the same capacity to exhibit glucagon-induced changes in PFK-2 activity as did hepatocytes from 22-day foetuses. The pattern of response to glucagon was the same, regardless of the day of pregnancy (results not shown). These results suggest the involvement of this hormone in the observed changes in PFK-2 activity and Fru-2,6-P_2 concentration.

We thank Mrs. Dolores Velasco for valuable technical assistance, and Erik Lundin for helpful criticism in manuscript preparation. This work was supported by grants from Fondo de Investigaciones Sanitarias and Comisión Asesora de Investigación Cientifica y Técnica.

REFERENCES

1. El-Manoubi, L., Callikan, S., Duee, P. H., Ferre, P. & Girard, J. (1983) Am. J. Physiol. **244**, E24–E30
2. Hanson, R. W., Reshef, L. & Ballard, F. J. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. **34**, 166–171
3. Devos, P. & Hers, H. G. (1974) Biochem. J. **140**, 331–340
4. Blázquez, E., Sugase, T., Glázquez, M. & Foa, P. P. (1974) J. Lab. Clin. Med. **83**, 957–967
5. Hue, L. & Rider, M. H. (1987) Biochem. J. **245**, 313–324
6. Martin-Sanz, P., Cascales, M. & Boscá, L. (1987) FEBS Lett. **225**, 37–42
7. Rider, M. H., Foret, D. & Hue, L. (1985) Biochem. J. **231**, 193–196
8. Di Marco, P. N., Ghisalverti, A. V., Pearce, P. H. & Oliver, I. T. (1976) Biol. Neonate **30**, 205–215
9. Van Schaftingen, E. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 3rd edn., pp. 335–341, Verlag Chemie, Weinheim
10. Bartrons, R., Hue, L., Van Schaftingen, E. & Hers, H. G. (1983) Biochem. J. **214**, 829–837
11. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
12. Hers, H. G. & Van Schaftingen, E. (1982) Biochem. J. **206**, 1–12
13. Christoffersen, T., Mørland, J., Osues, J. B. & Øye, I. (1973) Biochim. Biophys. Acta **313**, 338–349
14. Poole, G. P., Postle, A. D. & Bloxham, D. P. (1982) Biochem. J. **204**, 81–87
15. Blair, J. B., Sattsangi, S. & Hartwell, R. (1986) J. Biol. Chem. **261**, 2425–2433
16. Hue, L. & Bartrons, R. (1985) in Regulation of Carbohydrate Metabolism (Beitner, R., ed.), pp. 29–44, CRC Press, New York
17. Bázquez, E., Rubalcaba, B., Montesano, R., Orci, L. & Unger, R. H. (1976) Endocrinology (Baltimore) **98**, 1014–1023
18. Di Marco, P. N. & Oliver, I. T. (1978) Eur. J. Biochem. **87**, 235–241
19. Ichihara, A., Nakamura, T., Noda, C. & Tanaka, K. (1986) in Isolated and Cultured Hepatocytes (Guillouzo, A. & Guguen-Guillouzo, C., eds.), pp. 187–208, John Libbey Eurotext Ltd./INSERM, London and Paris

Received 6 July 1988/21 September 1988; accepted 5 October 1988