Biochem. J. (1983) **214**, 829–837
Printed in Great Britain

Hormonal control of fructose 2,6-bisphosphate concentration in isolated rat hepatocytes

Ramon BARTRONS, Louis HUE,* Emile VAN SCHAFTINGEN and Henri-Géry HERS
Laboratoire de Chimie Physiologique, Université de Louvain, and International Institute of Cellular and Molecular Pathology, UCL 75.39, Avenue Hippocrate 75, 1200 Brussels, Belgium

(*Received 7 April 1983/Accepted 1 June 1983*)

The ability of glucagon and of adrenaline to affect the concentration of fructose 2,6-bisphosphate in isolated hepatocytes was re-investigated because of important discrepancies existing in the literature. We were unable to detect a significant difference in the sensitivity of the hepatocytes with regard to the effect of glucagon to initiate the interconversion of phosphorylase, pyruvate kinase, 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase, and also to cause the disappearance of fructose 2,6-bisphosphate. In contrast, we have observed differences in the time-course of these various changes, since the interconversions of phosphorylase and of pyruvate kinase were at least twice as fast as those of 6-phosphofructo-2-kinase and of fructose 2,6-bisphosphatase. When measured in a cell-free system in the presence of MgATP, the cyclic AMP-dependent interconversion of pyruvate kinase was 5–10-fold more rapid than those of 6-phosphofructo-2-kinase and of fructose 2,6-bisphosphatase. These data indicate that 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase are relatively poor substrates for cyclic AMP-dependent protein kinase; they also support the hypothesis that the two catalytic activities belong to a single protein. Adrenaline had only a slight effect on the several parameters under investigation, except for the activation of phosphorylase. In the absence of Ca^{2+} ions from the incubation medium, however, adrenaline had an effect similar to that of glucagon.

Fru-2,6-P_2 was discovered (Van Schaftingen *et al.*, 1980*a*,*b*) because of its property of stimulating liver PFK 1 and of being destroyed on addition of glucagon to isolated hepatocytes. It also inhibits fructose 1,6-bisphosphatase (EC 3.1.3.11) (Van Schaftingen & Hers, 1981*a*; Pilkis *et al.*, 1981). The effect of glucagon has been explained by the property of cyclic AMP-dependent protein kinase of inactivating PFK 2 and activating FBPase 2, the enzymes that respectively synthesize and degrade Fru-2,6-P_2 (reviewed by Hers & Van Schaftingen, 1982). It is by this mechanism that glucagon controls glycolysis and gluconeogenesis at the level of the Fru-6-*P*/Fru-1,6-P_2 interconversion. However, glucagon, via cyclic AMP-dependent protein kinase, has other actions which affect directly or indirectly the rates of glycolysis and gluconeogenesis. First, it inactivates pyruvate kinase (Engström, 1978), and by doing so favours the conversion of phosphoenolpyruvate into glucose rather than into pyruvate. Second, by the activation of glycogen phosphorylase, it initiates glycogenolysis and causes the accumulation of hexose 6-phosphates, which in turn favours the formation of Fru-2,6-P_2 and glycolysis (Hue *et al.*, 1981*b*). It was therefore desirable to know if the effects of glucagon to cause the interconversion of the four enzymes involved in the control of glycolysis and gluconeogenesis, namely PFK 2, FBPase 2, pyruvate kinase and phosphorylase, occur simultaneously and at similar doses of the hormone. This problem has been studied by two other groups of investigators, who reported a 10- (El-Maghrabi *et al.*, 1982*a*) to 1000-fold (Richards & Uyeda, 1982; Richards *et al.*, 1981, 1982) greater sensitivity for the

Abbreviations used: Fru-6-*P*, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Glc-6-*P*, glucose 6-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; PFK 1, 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.–).

* Present address: Hormone and Metabolic Research Unit, UCL 75.29, Avenue Hippocrate 75, 1200 Brussels, Belgium.

interconversion of the two first enzymes, compared with that of pyruvate kinase (El-Maghrabi *et al.*, 1982*a*) or with cyclic AMP formation (Richards & Uyeda, 1982; Richards *et al.*, 1981, 1982). By contrast, Hue *et al.* (1981*b*) found the same sensitivity for the conversion of phosphorylase and PFK 2.

α-Adrenergic agents have in common with glucagon the property of activating phosphorylase and of increasing the concentration of hexose 6-phosphates in the liver cell. Through this mechanism, they also increase the concentration of Fru-2,6-P_2 and the rate of glycolysis in hepatocytes obtained from fed rats (Hue *et al.*, 1981*a*,*b*). Their action is normally not mediated by cyclic AMP, but depends on the presence of Ca^{2+} in the incubation medium [reviewed by Exton (1981) and Williamson *et al.* (1981)]. In the absence of Ca^{2+}, α-adrenergic agents cause an activation of cyclic AMP-dependent protein kinase, with a secondary inactivation of pyruvate kinase and inhibition of glycolysis (Hue *et al.*, 1978; Chan & Exton, 1978). The action of adrenaline on the liver is mainly α-adrenergic, but a small activation of cyclic AMP-dependent protein kinase is observed in the presence of inhibitors of phosphodiesterase (reviewed by Exton, 1981). The subsequent report (Richards *et al.*, 1982; Richards & Uyeda, 1982) that adrenaline caused a Ca^{2+}-dependent inactivation of PFK 2 and activation of FBPase 2 was therefore unexpected.

The present work was undertaken to investigate these various discrepancies.

Materials and methods

Chemicals and enzymes

Glucagon was from Novo-Industri (Copenhagen, Denmark), adrenaline from Parke–Davis (Brussels), L-phenylephrine from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and bacitracin from Aldrich–Europe (Steinheim/Albuch, West Germany). Enzymes and other biochemical reagents were obtained either from Boehringer G.m.b.h. (Mannheim, Germany) or from Sigma. All chemicals were of analytical grade. Fru-2,6-P_2 (Van Schaftingen & Hers, 1981*b*) and Fru-2,6-[2-^{32}P]P_2 (Van Schaftingen *et al.*, 1982*a*) were prepared as described in the indicated references.

Preparation and incubation of isolated hepatocytes

In most experiments we used male fed or overnight-starved Wistar rats (local strain; 200–250g). In one experiment a fed (CD-strain) rat from Charles River France was also used. Hepatocytes were isolated by the following procedure, derived from that of Seglen (1973); the liver was perfused at 37°C, without recirculation, with 200ml of a Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) gas phase but devoid of Ca^{2+}. It was then perfused by recirculating 100ml of the same buffer containing 2.5mM-$CaCl_2$ and 50mg of collagenase. After disruption of the liver, the cells were washed twice with 80ml of a Krebs–Henseleit buffer containing 2.5mM-$CaCl_2$. Some experiments were also performed with hepatocytes prepared as described by Hue *et al.* (1978). Both methods gave similar results.

Hepatocytes were incubated at 37°C as described by Hue *et al.* (1978). Except where otherwise indicated, final concentrations of glucose and bacitracin were 20mM and 0.5% respectively. Hormones were added after 30min of incubation. For the measurement of enzymes and of Fru-2,6-P_2, samples (0.1ml) were withdrawn at the appropriate times and immediately frozen in tubes kept in a cooling mixture (solid CO_2 in acetone). For the measurement of other metabolites, the cell suspension was mixed with 0.25vol. of 20% (w/v) $HClO_4$. These samples were kept at −20°C for no more than 2 days until further processing.

Preparation of a liver Sephadex filtrate

The liver of a fed rat was homogenized in a Potter–Elvehjem tube in the cold with 2vol. of ice-cold 100mM-KCl/25mM-Hepes, pH7.1. The homogenate was centrifuged for 30min at 100000*g* and the resulting supernatant filtered through 20vol. of Sephadex G-25 (fine grade) equilibrated with the homogenization buffer.

Measurements of enzymes and metabolites

The active form of phosphorylase was assayed as described by Hue *et al.* (1975) in extracts prepared by thawing the cellular suspension in 3vol. of a solution containing 0.1M-NaF, 0.5% glycogen, 10mM-EDTA and 10mM-glycylglycine and adjusted to pH7.4. The procedure used for the assay of pyruvate kinase was described by Feliu *et al.* (1977), except that the homogenization buffer was supplemented with 5mM-P_i. The pyruvate kinase activity ratio ($v_{0.15}/V$) is the ratio of the rates measured at 0.15mM- and 5mM-phosphoenolpyruvate respectively.

For the determination of PFK 2 and of FBPase 2, the cell suspensions were thawed in 1vol. of a buffer containing 20mM-P_i (PFK 2) or 20mM-glycerol 2-phosphate (FBPase 2), as well as 10mM-EDTA and 100mM-KCl and adjusted to pH7. The extracts were centrifuged at 3000*g* for 5min at 0°C. For both assays, 10–20μl portions of the resulting supernatants were incubated for 10min at 30°C in the presence of the constituents described below, in a final volume of 0.25ml. PFK 2 was assayed in the presence of 5mM-ATP, 7mM-$MgCl_2$ and 100mM-KCl. Furthermore, 50mM-Tris, 1mM-P_i, 5mM-Fru-6-*P* and 17.5mM-Glc-6-*P* were also present and the pH was adjusted to 8.5 for determination of the

'total' activity; 50mM-Mes, 5mM-P_i, 1mM-Fru-6-*P* and 3.5mM-Glc-6-*P* were present and the pH was adjusted to 6.6 for the determination of the 'active' form of enzyme (Van Schaftingen *et al.*, 1981). The assays were stopped by the addition of 1vol. of 0.1M-NaOH, and the Fru-2,6-P_2 formed was determined in the resulting mixture. A blank was subtracted for the presence of Fru-2,6-P_2 at zero time. FBPase 2 was assayed in the presence of 5mM-glycerol 2-phosphate, 1mM-ATP, 3mM-$MgCl_2$, 100mM-KCl, 1μM-Fru-2,6-[2-^{32}P]P_2 (5×10^4c.p.m.), either with 50mM-Hepes at pH7.1 (active form) or with 50mM-Mes at pH5.8 (total activity) (Van Schaftingen *et al.*, 1982*a*). A blank was incubated in the absence of enzyme. The incubations were stopped and [^{32}P]P_i was isolated and counted for radioactivity as described by Van Schaftingen *et al.* (1982*a*).

Concentrations of Glc-6-*P* and Fru-6-*P* (Hohorst, 1963), phosphoenolpyruvate (Czok & Lamprecht, 1974) and Fru-1,6-P_2 (Hohorst, 1963) were measured enzymically in neutralized $HClO_4$ extracts as indicated. Fru-2,6-P_2 was extracted and measured as described by Van Schaftingen *et al.* (1982*b*).

Results

Our results concern the effect of glucagon and adrenergic agents on the activity of four interconvertible enzymes of carbohydrate metabolism, namely pyruvate kinase, PFK 2, FBPase 2 and phosphorylase, in isolated hepatocytes. The changes in the concentrations of Fru-2,6-P_2 and of several other phosphate esters were also recorded. Furthermore, the effect of cyclic AMP on the interconversion of enzymes in a cell-free system was investigated. Results shown are illustrative of a series of experiments which gave consistently the same type of results.

Effect of glucagon on isolated hepatocytes

The effects of glucagon were investigated in hepatocytes obtained from both fed and starved rats.

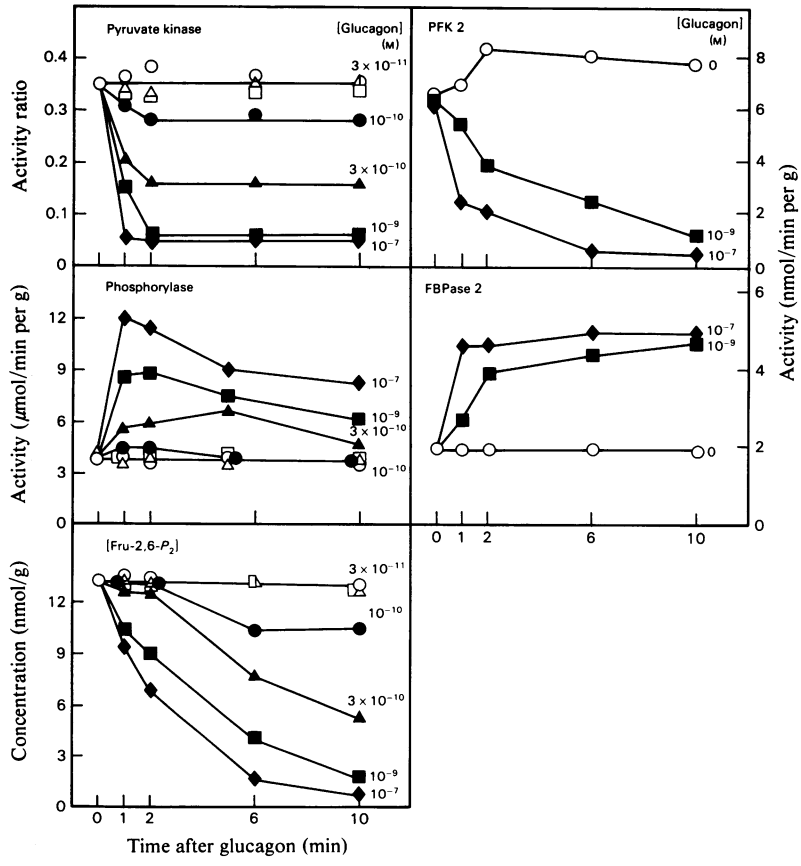

Fig. 1. *Effect of glucagon on the interconversion of enzymes and on the concentration of Fru-2,6-P_2 in isolated hepatocytes*

Hepatocytes were obtained from a starved rat. Minimal effects were observed at 3×10^{-11}M-(□) and 10^{-11}M-(△) glucagon. The active forms of PFK 2 and FBPase 2 were measured.

In the latter case, preincubation of the cells in the presence of 20mM-glucose allowed them to resynthesize a relatively large amount of Fru-2,6-P_2, and in general the behaviour of these cells was very similar to that of cells obtained from fed rats. The two factors investigated were the concentration of glucagon in the incubation medium and the time-course of its effect. Fig. 1 gives the full details of such an experiment performed with cells from a starved rat; it reports the changes induced by a large range of glucagon concentrations during the first 10min after the hormonal addition. Experiments shown in Figs. 2 and 3 were performed with hepatocytes obtained from fed rats and incubated in the presence of 1nM-glucagon for various periods of time. In Fig. 2, the results were normalized in order to facilitate the comparison between the relative velocities of the several hormonal effects. Fig. 3 shows the changes in the concentrations of several phosphate esters. Finally, on the basis of the previous experiments, a 6min period of incubation was chosen to establish the dose–response curve for the various effects. An example of such an experiment performed with hepatocytes from fed rats is shown in Fig. 4, and data from a larger number of similar experiments are collected in Table 1.

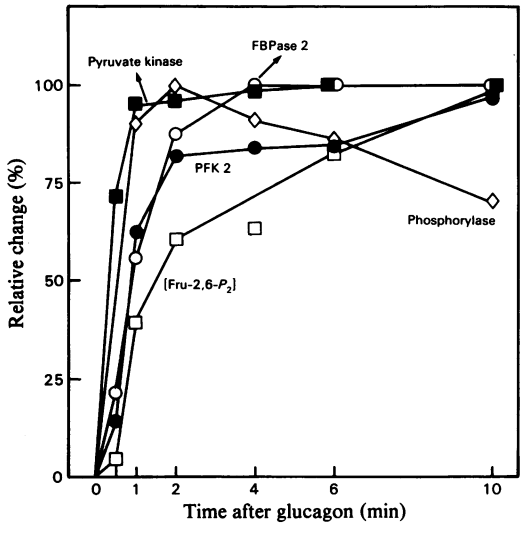

Fig. 2. *Time-course of the relative changes in the enzymic activities and Fru-2,6-P_2 concentration after the addition of 1nM-glucagon to isolated hepatocytes*

The hepatocytes were obtained from fed rats. Data shown are the means of two experiments. The results are expressed as percentages of the maximal change. Similar results were obtained in two other experiments performed with different timing. ■, Pyruvate kinase; ◇, phosphorylase; ●, PFK 2; ○, FBPase 2; □, Fru-2,6-P_2.

The main conclusions from this series of experiments can be summarized as follows. A half-maximal effect of glucagon was usually obtained at 0.2–0.4nM, with variations within the limits of the experimental errors, but not systematically in favour of one particular parameter. By contrast, systematic differences in time-course were observed. The changes in the activity of phosphorylase and pyruvate kinase were early events that were usually terminated in 1–2min and were accompanied by a rise in the concentrations of hexose 6-phosphates and of phosphoenolpyruvate. The inactivation of PFK 2 and activation of FBPase 2 occurred more slowly, reaching half-maximal values after a time that was 2–3-fold longer than for phosphorylase and pyruvate kinase. The decrease in the concentration of Fru-2,6-P_2 took yet a longer time, requiring about

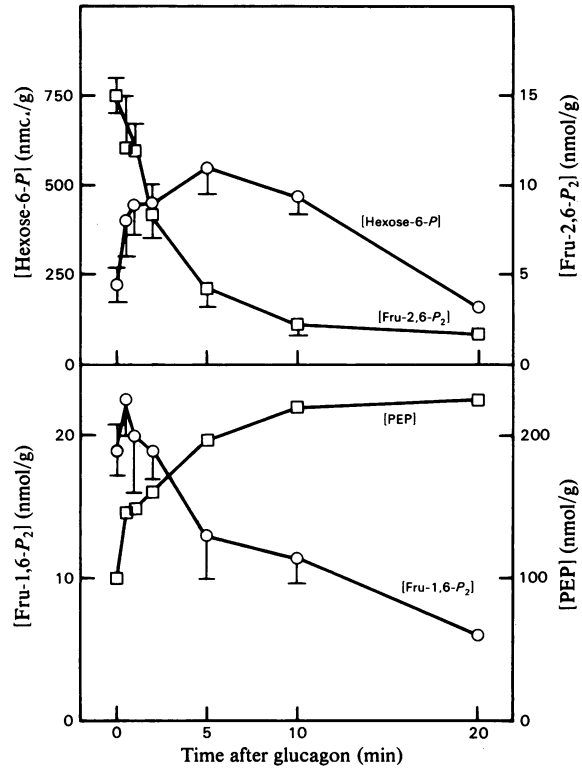

Fig. 3. *Effect of 1nM-glucagon on the concentration of hexose phosphates and of phosphoenolpyruvate (PEP) in isolated hepatocytes*

The hepatocytes were obtained from fed rats. The concentrations of hexose phosphates are the means ± S.E.M. for six experiments. The concentrations of phosphoenolpyruvate were obtained in a single experiment, but similar data at different timings were obtained in two other experiments.

Table 1. *Concentrations of glucagon required to obtain half-maximal effect on the activity of several enzymes and on the concentration of Fru-2,6-P_2 in hepatocytes*

Hepatocytes were incubated as in Fig. 1, for the time indicated in parentheses.

		[Glucagon] causing a half-maximal effect (nM)				
Nutritional state	Expt. no.	Pyruvate kinase inactivation	Phosphorylase activation	[Fru-2,6-P_2]	PFK 2 inactivation	FBPase 2 activation
Fed	1 (6 min)	0.12	0.63	0.45		
	2 (6 min)	0.45	0.20			
	3 (6 min)	0.08	0.45	0.40		
	4 (6 min)†	0.08	0.11	0.16	0.11	0.12
	Mean ± S.E.M.	0.18 ± 0.09 ($n = 4$)	0.35 ± 0.12 ($n = 4$)	0.33 ± 0.09 ($n = 3$)		
Starved	1 (10 min)	0.28	0.13	0.16	0.15	
	2 (10 min)	0.14		0.19		
	3 (6 min)	0.14	0.20	0.32	0.15	
	4 (10 min)	0.25		0.35		
	5* (2 min)‡	0.20	0.56	0.63	0.50	0.40
	(10 min)‡	0.20	0.70	0.25		
	Mean ± S.E.M.	0.20 ± 0.03 ($n = 5$)	0.32 ± 0.16 ($n = 3$)	0.29 ± 0.05 ($n = 5$)	0.27 ± 0.12 ($n = 3$)	

* Hepatocytes were prepared by the procedure of Hue *et al.* (1978).
† Results shown in Fig. 4.
‡ Results partially shown in Fig. 1.

10 min to be complete, whereas the decrease in the concentration of Fru-1,6-P_2 was a slow event that was not yet complete after 20 min. Differences were also observed in the time required by the different systems to return to their initial value. Phosphorylase was at least twice as fast as pyruvate kinase (Fig. 1), which was itself faster than Fru-2,6-P_2 concentration (results not shown).

Since bacitracin was present in all the experiments described in Figs. 1–4 to prevent the degradation of glucagon, we have checked the possible interference of this peptide with the hormonal effect. In the absence of bacitracin, the sensitivity to glucagon was the same as in its presence. As a rule, however, all events were faster in the absence of bacitracin, and in particular the increase in phosphoenolpyruvate was already maximal after 1 min (results not shown).

Effect of cyclic AMP on enzyme interconversion in a cell-free system.

The slower interconversion of PFK 2 and FBPase 2 as compared with that of pyruvate kinase and phosphorylase, observed in isolated hepatocytes, suggested that the two former enzymes could be rather poor substrates for cyclic AMP-dependent protein kinase. Accordingly, we show in Fig. 5 that when a liver Sephadex filtrate was incubated in the presence of MgATP and of two different concentrations of cyclic AMP, the inactivation of PFK 2 and activation of FBPase 2 occurred in parallel and that the time required to reach a half-maximal modification was 5–10-fold longer than for the inactivation of pyruvate kinase. The system was entirely cyclic AMP-dependent and, as apparent from several other experiments (results not shown), its sensitivity to the cyclic nucleotide was indistinguishable from that for pyruvate kinase interconversion.

Effect of adrenergic agents

These effects were investigated in hepatocytes obtained from fed rats, as in the experiments by Richards *et al.* (1982) that we wanted to reproduce. Furthermore, it is known that Fru-2,6-P_2 concentration is not affected by phenylephrine and vasopressin in hepatocytes obtained from starved rats (Hue *et al.*, 1981*a*,*b*). The effect of Ca^{2+} removal was investigated because the Ca^{2+}-dependence of the action of adrenaline reported by Richards & Uyeda (1982) and Richards *et al.* (1982) was in direct contradiction with the glucagon-like effect of α-adrenergic agents in the absence of Ca^{2+} (Hue *et al.*, 1978; Chan & Exton, 1978).

We show in Fig. 6 the effect of 10 μM-adrenaline on the activity of the four interconvertible enzymes and on the concentration of Fru-2,6-P_2 in hepatocytes incubated with or without Ca^{2+}. In the presence of Ca^{2+}, phosphorylase was clearly activated, but all the other changes were either slight or transient. It is only in the absence of Ca^{2+} that a series of other effects were observed, which were all of the β-adrenergic type. This includes the inactivation of pyruvate kinase and PFK 2, the activation of FBPase 2 and a decrease in the

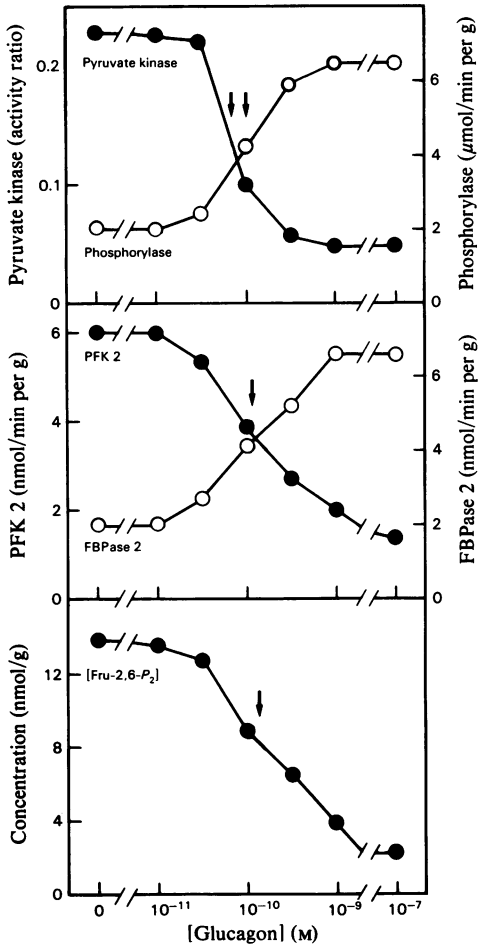

Fig. 4. *Dose–response curves of the glucagon effect on the interconversion of enzymes and on the concentration of Fru-2,6-P_2*

Hepatocytes isolated from a fed rat were incubated for 6 min in the presence of various concentrations of glucagon. Arrows indicate concentrations of glucagon causing a half-maximal effect. The active forms of PFK 2 and FBPase 2 were measured.

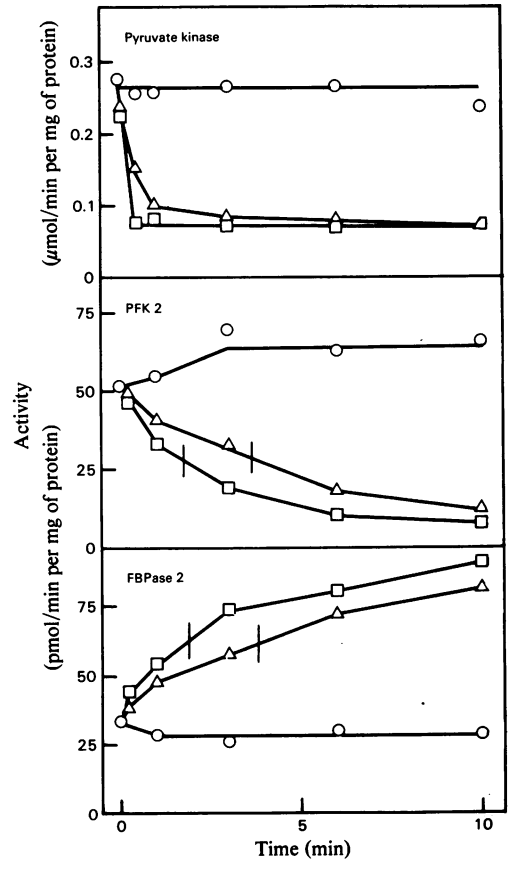

Fig. 5. *Enzyme interconversion in a cell-free system*

A liver Sephadex filtrate, containing 18 mg of protein/ml, was preincubated for 5 min at 25°C in the presence of 5 mM-$MgCl_2$; it was then diluted 4-fold and incubated in the presence of 2.5 mM-ATP, 3.5 mM-$MgCl_2$, 125 mM-KCl, 10 mM-theophylline, 40 mM-Hepes and 5 mM-P_i at pH 7.1 and without (○) or with 0.25 μM-(△) or 1 μM-(□) cyclic AMP. At various times, samples were removed and frozen in a cooling mixture (acetone/solid CO_2) and further analysed. Pyruvate kinase was measured at 0.15 mM-phosphoenolpyruvate, PFK 2 at pH 6.6 and FBPase 2 at pH 7.1. Vertical bars represent the time required for a half-maximal effect.

concentration of Fru-2,6-P_2. Results similar to those described in this paragraph were also obtained with 10 μM-phenylephrine.

The experiment shown in Fig. 6 was also repeated with hepatocytes obtained from a Charles River rat, since this was the strain of rats used in the experiments by Richards & Uyeda (1982). Several differences were then observed, as compared with Fig. 6. First, the inactivation of pyruvate kinase and of PFK 2, as well as the activation of FBPase 2 in the presence of Ca^{2+}, were greatly increased, although still less than in the absence of Ca^{2+}. Second, the initial concentration of Fru-2,6-P_2 in these cells was nearly twice that measured in hepatocytes from the local strain, reaching 30 nmol/g at the beginning of the experiment. In the presence of adrenaline there was a slight and transient (lasting less than 5 min) increase in Fru-2,6-P_2 concentration, followed by a decrease. This decrease was, however, much larger in the absence than in the presence of Ca^{2+}, indicating that our disagreement with Richards & Uyeda (1982) does not result from a difference in the strain of rats used.

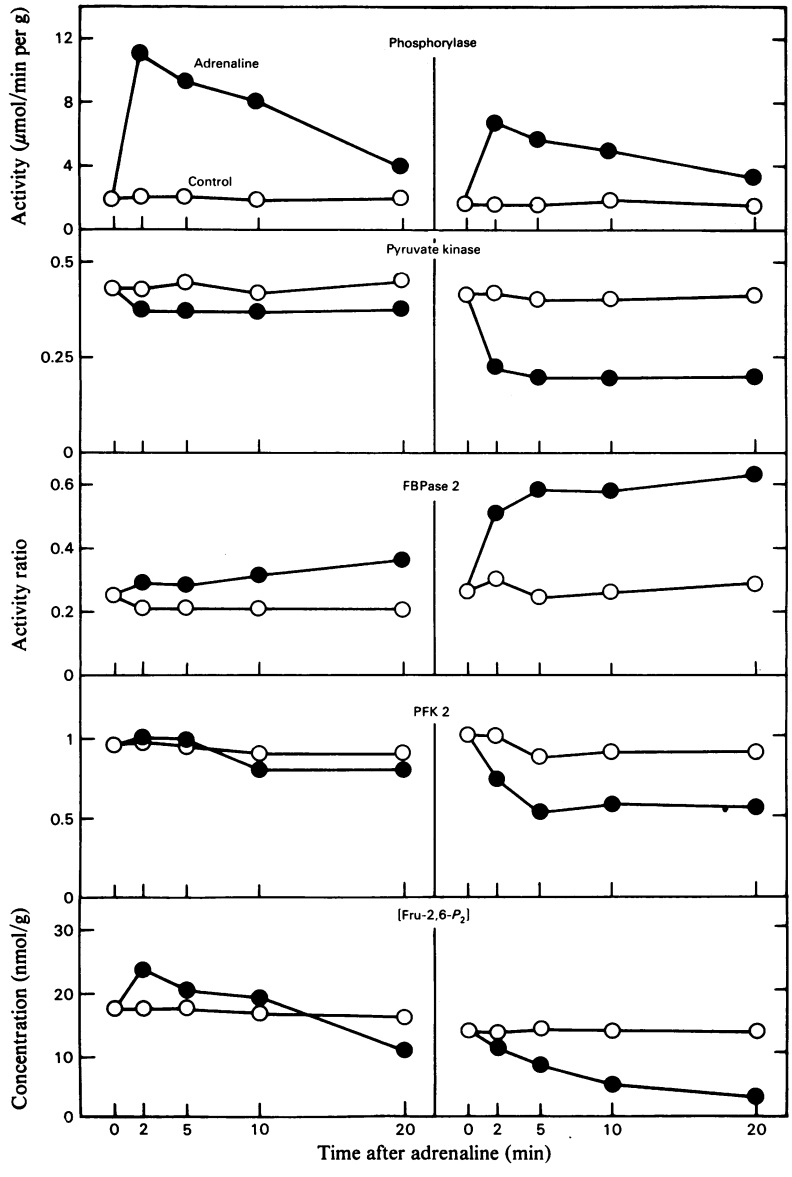

Fig. 6. *Effect of 10 μM-adrenaline on the interconversion of enzymes and on Fru-2,6-P_2 concentration in isolated hepatocytes*

Hepatocytes were obtained from fed rats and incubated in the presence of 10 mM-glucose. The experiments shown in the right-hand panels were performed without addition of Ca^{2+} to the incubation medium and in the presence of 1 mM-EGTA. The activity ratios for PFK 2 and FBPase 2 are the ratios of the active to the total form of enzyme as defined in the Materials and methods section. ○, Control; ●, adrenaline.

Discussion

Sensitivity to glucagon

It has been known for a long time that the effect of glucagon to stimulate gluconeogenesis could be mimicked by cyclic AMP. Like the stimulation of glycogenolysis, the activation of phosphorylase and the inactivation of pyruvate kinase, this effect was half-maximal at 0.2–0.4 nM-glucagon (Exton *et al.*, 1970; Pilkis *et al.*, 1975; Feliu *et al.*, 1976; Van de Werve *et al.*, 1977; Hue *et al.*, 1978; Blackmore *et al.*, 1978; Claus *et al.*, 1980; Hue *et al.*, 1981*b*). If the interconversion of the enzymes that control the concentration of Fru-2,6-P_2 displayed a greatly

different sensitivity, it would mean that Fru-2,6-P_2 concentration does not play an important role in the control of gluconeogenesis by glucagon. This problem was therefore of great importance, and our answer is unambiguous, since we could not detect any significant difference in the hormonal sensitivity of the four interconvertible enzymes.

The discrepancy between our results and those obtained by two other groups of workers is difficult to explain, particularly since these two groups disagree deeply. Their results will therefore be considered separately. However, it must first be recalled that in the present work Fru-2,6-P_2 was measured by a very simple and accurate procedure which allowed numerous determinations and also greatly facilitated the measure of PFK 2.

El-Maghrabi *et al.* (1982*a*) found different half-saturating concentrations of glucagon for the interconversion of FBPase 2 (0.01 nM), PFK 2 (0.04 nM) and pyruvate kinase (0.25 nM). They tried to explain these differences by assuming that FBPase 2 and PFK 2 were better substrates for cyclic AMP-dependent protein kinase than was pyruvate kinase. This assumption was, however, not supported by any comparative experimental data, whereas we have now found that the contrary is true (Fig. 5). Furthermore, we obtained no indication that the interconversion of PFK 2 and of FBPase 2 could occur at lower concentrations of cyclic AMP than that of pyruvate kinase.

Richards & Uyeda (1982) and Richards *et al.* (1982) have reported that the activities not only of PFK 2 and FBPase 2 but also of phosphorylase were half-maximally affected by 0.1 pM-glucagon. This is obviously in deep disagreement with our data and with those of El-Maghrabi *et al.* (1982*a*), as well as with all previous work on the activation of phosphorylase by glucagon in the isolated liver. A word of caution should be added concerning the use of extremely low concentrations of glucagon; in fact, when 100 mg of hepatocytes/ml are incubated with 0.01 pM-glucagon (Richards *et al.*, 1981), there are more hepatocytes than molecules of hormone.

Differences in time-course

An unexpected observation made in the course of the present work was that the interconversions of PFK 2 and of FBPase 2 were noticeably slower than those of phosphorylase and pyruvate kinase. This difference does not exceed a few minutes and presumably has little physiological implication if the changes in hormone concentration in the blood are relatively slow. As said above, it indicates that the two first enzymes are relatively poor substrates for cyclic AMP-dependent protein kinase. The difference in velocity was even more important in a cell-free system incubated in the presence of Mg-ATP and cyclic AMP. This may be related to the removal of phosphoenolpyruvate and of Fru-1,6-P_2, two known inhibitors of the inactivation of pyruvate kinase (Feliu *et al.*, 1977). The relatively slow decrease in Fru-2,6-P_2 is in agreement with our earlier work on that subject (see Fig. 9 in Van Schaftingen *et al.*, 1980*a*), but is again in deep contradiction with the report by Pilkis *et al.* (1983) that full effect was obtained within 1 min.

It is also noticeable that the concentration of Fru-1,6-P_2 decreased more slowly than that of Fru-2,6-P_2, indicating a causal relationship between these two modifications. This slow change in Fru-1,6-P_2 concentration is also against the hypothesis (Pilkis *et al.*, 1983) that its disappearance is required for the inactivation of pyruvate kinase.

Finally, the relatively slow return of Fru-2,6-P_2 concentration, as well as of pyruvate kinase activity, as compared with phosphorylase activity is in agreement with the previous proposal (Jett *et al.*, 1981) that pyruvate kinase phosphatase is different from phosphorylase phosphatase. Nothing is known of the phosphatase(s) that act on the phospho forms of PFK 2 and FBPase 2.

Are PFK 2 and FBPase 2 a single bifunctional protein?

The fact, reported by Van Schaftingen *et al.* (1981), that PFK 2 and FBPase 2 co-purify in the various systems of separation used has since been confirmed and amplified, and the possibility that the two enzymes could be activities of a single bifunctional peptide has received serious consideration (Van Schaftingen *et al.*, 1982*a*; El-Maghrabi *et al.*, 1982*a*,*b*). However, co-purification, even if it reaches near homogeneity, indicates very great similarity of structure, but is no proof of identity. El-Maghrabi *et al.* (1982*b*) have claimed that the interconversion of the two enzymes by cyclic AMP-dependent protein kinase occurred in parallel, but they did not show any intermediary values. We show in Fig. 5 that this parallelism actually occurs, and this fact must be taken as an additional argument in favour of the existence of a bifunctional protein. It is also consistent with a single site of phosphorylation controlling the two activities. Finally, if the two enzymes are catalytic activities of the same protein, it also implies their identical sensitivity to glucagon, as was reported in the present work, but in contrast with others (El-Maghrabi *et al.*, 1982*a*; see above).

Ca^{2+}-dependency of the adrenaline effect

The data of Fig. 6 show that adrenaline has minimal effects on the Fru-2,6-P_2 system except in the absence of Ca^{2+}, the effects becoming then similar to those of glucagon. Again, we have no explanation to offer for the discrepancy between our data and those of Richards *et al.* (1982), which

have also been criticized by Pilkis *et al.* (1983). We have verified that the discrepancy could not be explained by a difference in the strain of rats used. This gave us the opportunity to observe that with hepatocytes obtained from another strain of rats, adrenaline, even in the presence of Ca^{2+}, had a pronounced β-adrenergic type of effect that was not clearly apparent with our local strain of rats. The response of rat hepatocytes to adrenaline is also known to depend on the age and the sex of the rats (Blair *et al.*, 1979; Studer & Borle, 1982).

As a conclusion, we have no reason to believe that the hormonally induced decrease in the concentration of Fru-2,6-P_2 is mediated by anything else than cyclic AMP.

This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale and by the U.S. Public Health Service (Grant AM 9235). R. B. is recipient of a Long-Term European Molecular Biology Organization Fellowship. L. H. is Maître de Recherches and E. V. S. is Chargé de Recherches du Fonds National de la Recherche Scientifique.

References

Blackmore, P. F., Brumley, F. T., Marks, J. L. & Exton, J. H. (1978) *J. Biol. Chem.* **253**, 4851–4858

Blair, J. B., James, M. E. & Foster, J. L. (1979) *J. Biol. Chem.* **254**, 7579–7584

Chan, T. M. & Exton, J. H. (1978) *J. Biol. Chem.* **253**, 6393–6400

Claus, T. H., Schlumpf, J. R., El-Maghrabi, M. R., Pilkis, J. & Pilkis, S. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6501–6505

Czok, R. & Lamprecht, W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 1446–1451, Academic Press, New York

El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E. & Pilkis, S. J. (1982*a*) *J. Biol. Chem.* **257**, 7603–7607

El-Maghrabi, M. R., Fox, E., Pilkis, J. & Pilkis, S. J. (1982*b*) *Biochem. Biophys. Res. Commun.* **106**, 794–802

Engström, L. (1978) *Curr. Top. Cell. Regul.* **13**, 29–51

Exton, J. H. (1981) *Mol. Cell. Endocrinol.* **23**, 233–264

Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., Miller, T. B., Jr. & Park, C. R. (1970) *Recent Prog. Horm. Res.* **26**, 411–461

Felíu, J. E., Hue, L. & Hers, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2762–2766

Felíu, J. E., Hue, L. & Hers, H. G. (1977) *Eur. J. Biochem.* **81**, 609–617

Hers, H. G. & Van Schaftingen, E. (1982) *Biochem. J.* **206**, 1–12

Hohorst, H. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 134–138, Academic Press, New York

Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114

Hue, L., Felíu, J. E. & Hers, H. G. (1978) *Biochem. J.* **176**, 791–797

Hue, L., Van Schaftingen, E. & Blackmore, P. F. (1981*a*) *Biochem. J.* **194**, 1023–1026

Hue, L., Blackmore, P. F. & Exton, J. H. (1981*b*) *J. Biol. Chem.* **256**, 8900–8903

Jett, M.-F., Hue, L. & Hers, H. G. (1981) *FEBS Lett.* **132**, 183–186

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Pilkis, S. J., Claus, T. H., Johnson, R. A. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 6328–6336

Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J. & Claus, T. H. (1981) *J. Biol. Chem.* **256**, 3619–3622

Pilkis, S. J., Chrisman, T. D., El-Maghrabi, M. R., Colosia, A., Fox, E., Pilkis, J. & Claus, T. H. (1983) *J. Biol. Chem.* **258**, 1495–1503

Richards, C. S. & Uyeda, K. (1982) *J. Biol. Chem.* **257**, 8854–8861

Richards, C. S., Furuya, E. & Uyeda, K. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1673–1679

Richards, C. S., Yokoyama, M., Furuya, E. & Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1073–1079

Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398

Studer, R. K. & Borle, A. B. (1982) *J. Biol. Chem.* **257**, 7987–7993

Van de Werve, G., Hue, L. & Hers, H. G. (1977) *Biochem. J.* **162**, 135–142

Van Schaftingen, E. & Hers, H. G. (1981*a*) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2861–2863

Van Schaftingen, E. & Hers, H. G. (1981*b*) *Eur. J. Biochem.* **117**, 319–323

Van Schaftingen, E., Hue, L. & Hers, H. G. (1980*a*) *Biochem. J.* **192**, 887–895

Van Schaftingen, E., Hue, L. & Hers, H. G. (1980*b*) *Biochem. J.* **192**, 897–901

Van Schaftingen, E., Davies, D. R. & Hers, H. G. (1981) *Biochem. Biophys. Res. Commun.* **103**, 362–368

Van Schaftingen, E., Davies, D. R. & Hers, H. G. (1982*a*) *Eur. J. Biochem.* **124**, 143–149

Van Schaftingen, E., Lederer, B., Bartrons, R. & Hers, H. G. (1982*b*) *Eur. J. Biochem.* **129**, 191–195

Williamson, J. R., Cooper, R. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295