*Proc. Natl. Acad. Sci. USA*
Vol. 73, No. 8, pp. 2762–2766, August 1976
Biochemistry

# Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes

(glucagon/epinephrine/insulin/cyclic AMP/glucose)

JUAN E. FELÍU, LOUIS HUE, AND HENRI-GÉRY HERS

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 75.39, B—1200 Brussels, Belgium

*Communicated by Christian de Duve, June 9, 1976*

ABSTRACT **Treatment of isolated rat hepatocytes with saturating concentrations of glucagon caused several modifications in the kinetic properties of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40): $S_{0.5}$ (substrate concentration at half maximum velocity) for phospho*enol*pyruvate was about doubled, whereas $V_{max}$ was not changed; the activity measured at 0.15 mM phospho*enol*pyruvate (physiological concentration) was reduced 65–80%; and there was also an increase in the Hill coefficient and in the affinity of the enzyme for the inhibitors Mg-ATP and alanine. Glucagon, 3′:5′-cyclic AMP, and epinephrine caused an inactivation of pyruvate kinase together with a stimulation of gluconeogenesis. Insulin (10 nM) antagonized the effect of suboptimal doses of glucagon or cyclic AMP and of even maximal doses of epinephrine, on both pyruvate kinase activity and on gluconeogenesis. These observations can be explained by a phosphorylation of pyruvate kinase by cyclic-AMP-dependent protein kinase, as described by Ljungström *et al.* [(1974) *Biochim. Biophys. Acta* 358, 289–298] in a reconstructed system. They offer a molecular explanation for the hormonal control of gluconeogenesis. Glucose caused an inhibition of gluconeogenesis with no corresponding change in pyruvate kinase activity.**

The stimulation of gluconeogenesis by glucagon, 3′:5′-cyclic AMP, and catecholamines, as well as the antagonistic effect of insulin against small doses of the hyperglycemic agents, has been described by several groups of investigators (for a review, see ref. 1). However, according to recent papers (2, 3), the sites of action of glucagon and catecholamines on hepatic gluconeogenesis are still unknown. A possible site of regulation of gluconeogenesis is at the level of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) and it has indeed been shown by Krebs and Eggleston (4) that the activity of this enzyme was greatly increased in the livers of rats put on a high carbohydrate diet and was decreased upon fasting, when gluconeogenesis is enhanced. More recently, a rapid inactivation of pyruvate kinase by glucagon in isolated liver preparations has also been briefly reported (5, 6). The activity of pyruvate kinase when gluconeogenesis is in progress should cause a recycling of pyruvate via oxalacetate and phospho*enol*pyruvate (*P-enol*pyruvate). Accordingly, Friedmann *et al.* (7) had shown that such a recycling occurs in perfused rat liver and increases when gluconeogenesis is reduced.

The demonstration that purified liver pyruvate kinase can be phosphorylated and simultaneously inactivated by cyclic-AMP-dependent protein kinase (8) and can be dephosphorylated and reactivated by histone phosphatase (9) offers a molecular explanation to the known stimulation of gluconeogenesis by glucagon. The purpose of the present work was to study in a parallel manner the action of several hormones on the activity of pyruvate kinase and on the rate of gluconeogenesis in isolated liver cells.

Abbreviation: *P-enol*pyruvate, phospho*enol*pyruvate.

## MATERIAL AND METHODS

**Reagents.** Type I collagenase (Sigma), bacitracin (Aldrich), ion exchange resin AG 501-X8 (Bio-Rad), [*U*-$^{14}$C]pyruvate (Radiochemical Centre), glucagon, insulin (Novo), and other biochemical reagents (Sigma or Boehringer) were purchased as indicated.

**Isolation and Incubation of Hepatocytes.** Hepatocytes were isolated from livers of fed Wistar rats, as previously described (10), except that a Krebs–Henseleit bicarbonate buffer (11) was used as suspension medium. One gram of packed cells corresponded to 220 mg of protein (10), measured according to Lowry *et al.* (12). Two milliliters of cell suspension containing about 100 mg of cells were shaken (120 strokes/min) in stoppered 20 ml-vials at 37° in the presence of glucose (10 mM, unless otherwise stated), 0.1% bacitracin, and, when added, 10 nM insulin. Bacitracin was added to prevent degradation of the hormones (13). The gas phase was 95% $O_2$ and 5% $CO_2$. Glucagon, epinephrine, or cyclic AMP and 2 mM [*U*-$^{14}$C]pyruvate, dissolved in isotonic NaCl, were added after 30 min of incubation. Five to 10 min later, 0.1 ml aliquots of the cell suspension were frozen at the temperature of solid $CO_2$ in acetone for the determination of enzyme activities. Twenty minutes after the addition of the labeled precursor, 0.5 ml aliquots of cell suspension were taken in duplicate for the determination of radioactive glucose and glycogen formed from pyruvate.

**Measurement of Enzyme Activities.** Frozen aliquots of cells were thawed by shaking them in 5 volumes of an ice-cold medium; further homogenization of the cells was not necessary and did not increase the enzymatic activities. For the determination of pyruvate kinase, the suspending medium contained 0.1 M KF, 15 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 50 mM glycylglycine at pH 7.4. Pyruvate kinase was assayed at room temperature (22–24°) by recording at 340 nm the oxidation of NADH. The incubation mixture contained 50 mM glycylglycine at pH 7.4, 0.1 M KCl, 10 mM $MgCl_2$, 1.25 mM Mg-ADP, 0.15 mM NADH, and 2 units of lactate dehydrogenase in a total volume of 1 ml. Aliquots (25–50 μl) of the homogenate were added to the mixture and allowed to stand for 10 min at 22–24°; the reaction was started by the addition of 0.15 mM *P-enol*pyruvate. The method used for the determination of glycogen synthetase *a* and the homogenization medium in which the frozen cells were thawed were previously described (10). One unit of pyruvate kinase or synthetase *a* is the amount of enzyme that converts 1 μmol of substrate per min under the conditions of the assay.

**Measurement of Gluconeogenesis.** Gluconeogenesis was measured by the conversion of 2 mM [*U*-$^{14}$C]pyruvate into glucose and glycogen. Although this method underestimates the actual gluconeogenic flux, it has been accepted that the

Table 1. Kinetic parameters of pyruvate kinase from hepatocytes incubated with or without 100 nM glucagon

| | $V_{max}$ (units/g of cells) | *P-enol*pyruvate | | L-Alanine $K_i$ app. (mM) | Mg-ATP $K_i$ app. (mM) | Fru-1,6-$P_2$ $K_a$ app. ($\mu$M) |
|---|---|---|---|---|---|---|
| | | $S_{0.5}$ (mM) | Hill coefficient | | | |
| Control hepatocytes | 46 ± 1.8 | 0.17 ± 0.03 | 1.18 ± 0.05 | 1.08 | 1.22 | <0.5 |
| Glucagon-treated hepatocytes | 47 ± 2 | 0.37 ± 0.03 | 1.82 ± 0.07 | 0.52 | 0.32 | <0.5 |

Values shown are means ± SEM of five to seven determinations made on different batches of cells.

incorporation data are representative enough of the overall phenomenon and of the hormonal influence on this process (2, 14). Aliquots (0.5 ml) of cell suspension were pipetted into conical tubes containing 2 ml of ice-cold 0.9% NaCl and immediately centrifuged for 1 min at 3000 × *g*. The supernatant was treated with 0.5 ml of 0.15 M $ZnSO_4$ and 0.5 ml of 0.15 M $Ba(OH)_2$ and vigorously shaken. The precipitate was removed by centrifugation and ionized compounds were eliminated from the supernatant by shaking it for 30 min at room temperature with 0.5 g (wet weight) of a mixed-bed resin (AG 501-X8). The radioactivity of glucose was measured in the remaining solution with the scintillating mixture of Patterson and Greene (15). The cell pellet was suspended in 1 ml of 10% KOH containing 10 mg of carrier glycogen and heated for 20 min at 80°; after cooling, the radioactivity of glycogen was measured by spotting 0.2 ml of the mixture on a filter paper which was processed as recommended by Thomas *et al.* (16). The radioactivity of glycogen varied from 10 to 30% of that of glucose depending upon the concentration of glucagon.

## RESULTS

### Kinetic properties of pyruvate kinase of hepatocytes incubated with or without a saturating concentration of glucagon

Table 1 shows that several known kinetic parameters of liver pyruvate kinase (17, 18) were modified upon incubation of hepatocytes in the presence of a saturating concentration of glucagon. Whereas $V_{max}$ was not changed, $S_{0.5}$ (substrate concentration at half-maximum velocity) for *P-enol*pyruvate was about doubled and the Hill coefficient was increased from 1.18 to 1.82; the inhibitory action of Mg-ATP and of alanine was reinforced. The affinity for fructose 1,6-bisphosphate could not be measured because concentrations below 0.5 $\mu$M could not be ascertained in a crude liver preparation. The activity measured at 0.15 mM *P-enol*pyruvate [physiological concentration (19, 20)] and in the absence of inhibitors and fructose 1,6-bisphosphate was decreased 65–80%. It has been verified that these modifications persisted after filtration of a cell extract on Sephadex G-25 and also after ammonium sulfate precipitation (not shown).

### Effect of various concentrations of glucagon on the activity of pyruvate kinase and on gluconeogenesis and its reversal by insulin

In a series of experiments summarized in Table 2 and illustrated by Fig. 1, we have investigated the effect of various concentrations of glucagon on the activity of pyruvate kinase and on the rate of gluconeogenesis of hepatocytes incubated with or without 10 nM insulin. It is apparent that approximately the same doses of glucagon caused the inactivation of pyruvate kinase and the stimulation of gluconeogenesis. The dose causing a half-maximal effect was variable from one preparation to another and was a little more than doubled by the presence of 10 nM insulin; in some experiments, this dose was also increased in the presence of 40 mM glucose (not shown). These variations always affected in a similar manner the inactivation of pyruvate kinase and the stimulation of gluconeogenesis. Furthermore, in a series of experiments in which glycogen synthetase had been activated by incubation of the cells in the presence of 40 mM glucose, the same doses of glucagon caused the inactivation of pyruvate kinase and of glycogen synthetase (not shown).

Fig. 2 shows that the effect of 0.3 nM glucagon on the activity of pyruvate kinase lasted for about 20 min and that, after that time, the activity of the enzyme returned to its initial value within the next 40 min. However, the addition of 10 nM insulin caused a rapid reactivation that was apparent after a short latency and was complete within a few minutes. Fig. 3 illustrates the dose dependency of the insulin effect on the activity of pyruvate kinase and the rate of gluconeogenesis.

Table 2. Effect of glucagon on the activity of pyruvate kinase and on the rate of gluconeogenesis of hepatocytes incubated with or without 10 nM insulin

| Experiment | Pyruvate kinase activity | | | Gluconeogenesis | | |
|---|---|---|---|---|---|---|
| | Maximal effect of glucagon* | [Glucagon] giving a half-maximal effect (nM) | | Maximal effect of glucagon* | [Glucagon] giving a half-maximal effect (nM) | |
| | | − insulin | + insulin | | − insulin | + insulin |
| 1 | 5.2 | 0.43 | 0.94 | 3.4 | 0.56 | 0.94 |
| 2 | 5.5 | 0.05 | 0.20 | 3.9 | 0.16 | 0.31 |
| 3 | 5.1 | 0.57 | 0.8 | 3.75 | 1.4 | 1.90 |
| Mean ± SEM | 5.26 ± 0.12 | 0.35 ± 0.15 | 0.64 ± 0.22 | 3.68 ± 0.14 | 0.70 ± 0.36 | 1.05 ± 0.46 |

General procedure is as in Fig. 1.

* Expressed as ratio of pyruvate kinase activity without and with glucagon and the reverse for gluconeogenesis.

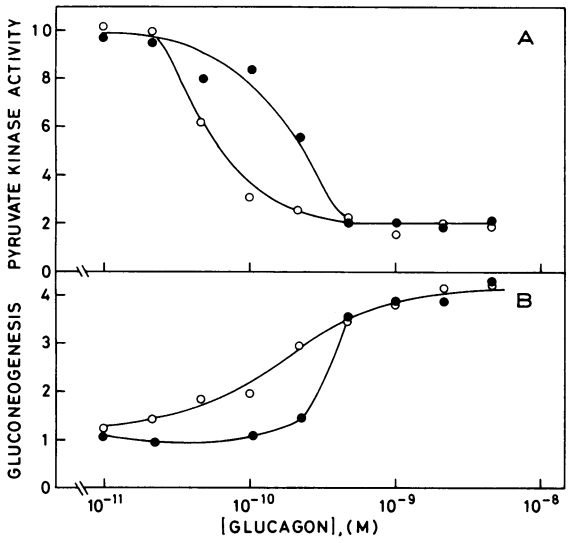


FIG. 1. Effect of various concentrations of glucagon on the activity of pyruvate kinase (A) and on the rate of gluconeogenesis (B) of hepatocytes incubated with (●) or without (○) 10 nM insulin. Pyruvate kinase was measured at 0.15 mM *P-enol*pyruvate in aliquots of cells taken 5 min after the addition of glucagon and is expressed in units/g of cells. Gluconeogenesis was measured by the conversion of [$^{14}$C]pyruvate into glucose and glycogen over a period of 20 min and is expressed in μmol of pyruvate converted per 20 min/g of cells.

## The effect of epinephrine and its reversal by insulin

It has recently been shown (3) that the stimulation of gluconeogenesis by even maximally effective concentrations of epinephrine can be suppressed, at least in part, by insulin. To this respect, the epinephrine effect is qualitatively different from that of glucagon, of which only submaximal concentrations can be antagonized by insulin. The effect of epinephrine is also markedly smaller than that of glucagon. Fig. 4 shows that insulin suppressed the major part of the effect of even very high concentrations of epinephrine (0.3 mM) not only on the stimulation of gluconeogenesis, but also on the inactivation of pyruvate kinase. Both effects are smaller than those of glucagon.

## The effect of cyclic AMP

Fig. 5 shows that, in this case again, inactivation of pyruvate kinase and stimulation of gluconeogenesis were obtained with

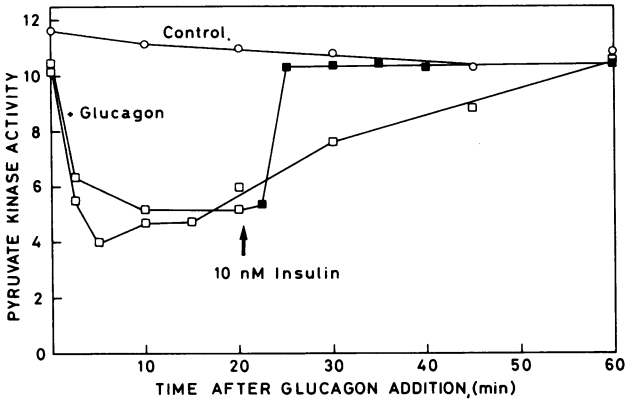


FIG. 2. Time-course of the effect of 0.3 nM glucagon on the activity of pyruvate kinase and of its reversal by 10 nM insulin. Glucagon (□, ■) and insulin (■) were added as indicated. Pyruvate kinase activity is expressed in units/g of cells.

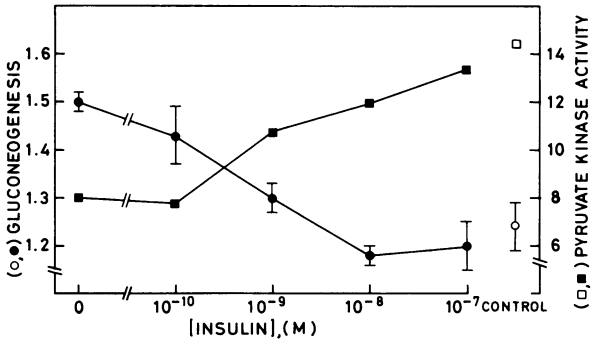


FIG. 3. Effect of various concentrations of insulin on the activity of pyruvate kinase (■) and on the rate of gluconeogenesis (●) of hepatocytes incubated in the presence of 0.3 nM glucagon. Insulin and glucagon were added after 20 and 30 min of incubation, respectively. Aliquots of cells were taken 10 min after the addition of glucagon for the measurement of pyruvate kinase activity. Gluconeogenesis was measured over a period of 20 min. Controls (□, ○) refer to hepatocytes incubated for the same period of time without any hormone. Values shown are means of two and four measurements for pyruvate kinase and gluconeogenesis, respectively. Expression of results is as in Fig. 1.

about the same concentrations of cyclic AMP and that insulin decreased the sensitivity of the cells by a factor of about 2 for both effects.

## The effect of glucose

Ruderman and Herrera (21) have shown that a high concentration of glucose inhibits gluconeogenesis in the isolated perfused liver. More recently, Claus *et al.* (2) have made a similar observation on isolated liver cells; these authors reported, however, that glucose had no effect in the presence of glucagon and they concluded that glucagon and glucose exert their effects on the same site.

It is apparent from Fig. 5 that, in our hands, glucose did inhibit gluconeogenesis, whatever the concentration of cyclic AMP. It also appears from this experiment and from another one performed with various amounts of glucagon at 10 and 40 mM glucose (not shown), that the effect of a high concentration

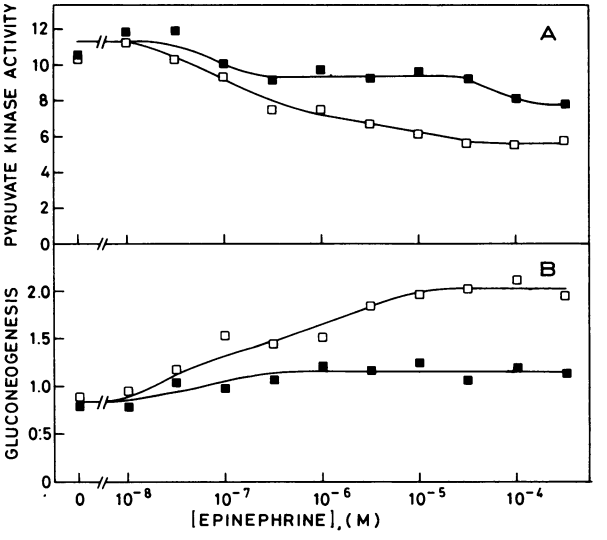


FIG. 4. Effect of various concentrations of epinephrine on the activity of pyruvate kinase (A) and on the rate of gluconeogenesis (B) of hepatocytes incubated with (■) or without (□) 10 nM insulin. General procedure and expression of results are as in Fig. 1.

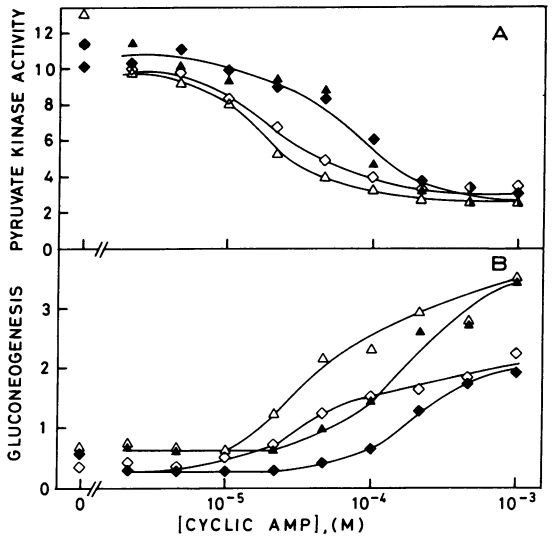


FIG. 5. Effect of various concentrations of cyclic AMP on the activity of pyruvate kinase (A) and on the rate of gluconeogenesis (B) of hepatocytes incubated with (▲, ♦) or without (△, ◇) 10 nM insulin. Isolated hepatocytes were incubated in the presence of 10 (△, ▲) or 40 (◇, ♦) nM glucose. General procedure and expression of results are as in Fig. 1, except that gluconeogenesis was measured by conversion of pyruvate to glucose only.

of glucose is to lower the rate of gluconeogenesis for a same activity of pyruvate kinase.

A second and irregular effect of glucose was to lower the sensitivity of the cells to glucagon, mimicking to some respect the effect of insulin. This effect was not statistically significant and may be due to seasonal factors.

### Relationship between pyruvate kinase activity and gluconeogenesis

The relationship between these two parameters is shown in Fig. 6.

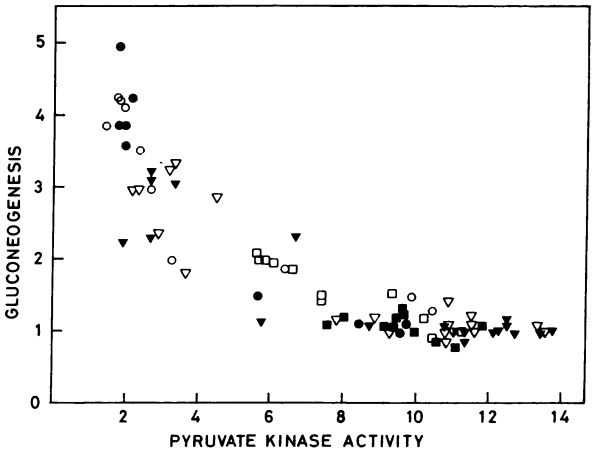


FIG. 6. Relationship between the rate of gluconeogenesis and the activity of pyruvate kinase of hepatocytes incubated in different conditions. Data and symbols are those of Figs. 1 and 4 and of two other experiments (▽, ▼) similar to the one represented in Fig. 1. Expression of results is as in Fig. 1.

## DISCUSSION

**Pyruvate Kinase as an Interconvertible Enzyme.** The working hypothesis that initiated the present work was that the phosphorylation of pyruvate kinase by protein kinase, observed in a reconstructed system by Ljungström *et al.* (8), also occurs in intact liver cells under the action of glucagon, epinephrine, and cyclic AMP. The facts presented in this paper are in accordance with this hypothesis. Indeed, all the changes in the kinetic properties of pyruvate kinase brought about by glucagon are very similar to those occurring upon phosphorylation of purified liver pyruvate kinase by protein kinase (22). The observed effect is, however, apparently not identical with a similar, although much slower, inactivation of pyruvate kinase reported by Taunton *et al.* (23) to occur *in vivo*, since under the assay conditions used by these authors (0.8 mM *P-enol*pyruvate) the change in activity would be barely detectable.

The parallel inactivation of pyruvate kinase and of glycogen synthetase in hepatocytes incubated with various amounts of glucagon is also best explained by a phosphorylation of both enzymes by cyclic-AMP-dependent protein kinase. It is worthwhile to recall that the pyruvate kinase activities shown in Figs. 1–6 were measured in the absence of inhibitors and that, because of the increased affinity for Mg-ATP and alanine, the actual diminution of activity brought about by glucagon should be larger than what was measured in our assay conditions.

**The Control of Gluconeogenesis by Pyruvate Kinase Activity.** The inverse effect of all hormonal effectors on the activity of pyruvate kinase and on gluconeogenesis makes it difficult to escape the conclusion that the large variations in the activity of pyruvate kinase that we have observed were at least in part responsible for the changes in the rate of gluconeogenesis. The main discrepancies between the two hormonal actions were in the values of the half maximally effective doses of glucagon, at least in some experiments (see Table 2). It must be emphasized that a precise determination of these parameters is not easy to make. For instance, in the experiment reported in Fig. 1, concentrations of glucagon higher than 1 nM still stimulated gluconeogenesis, although they did not further inactivate pyruvate kinase; this badly understood effect of very high doses of glucagon was responsible for the high value of the half maximal effective dose of glucagon. With regards to pyruvate kinase, one must remember that the activity measured 5 or 10 min after the addition of small doses of glucagon may not be identical to the mean value of this activity over the whole 20-min period during which gluconeogenesis was measured. The inactivation of pyruvate kinase also offers an adequate explanation for the inhibition of lactate formation from low concentrations of fructose (24, 25) associated with an increased conversion of fructose into glucose.

The control of gluconeogenesis by the activity of pyruvate kinase is necessarily a push mechanism in which the effect of pyruvate kinase is mediated by a change in the concentration of *P-enol*pyruvate. The steady-state level of this metabolite in the cell when gluconeogenesis predominates over glycolysis is the result of a balance between an input via the activity of *P-enol*pyruvate carboxykinase and an output via enolase and pyruvate kinase. The concentration of *P-enol*pyruvate is expected to be elevated when pyruvate kinase is poorly active and this is indeed the case under the action of glucagon (1). If one increases the activity of pyruvate kinase, the concentration of *P-enol*pyruvate and, secondarily, the rate of gluconeogenesis, are expected to decrease. However, because of the sigmoid saturation curve [Hill coefficient = 3 in the presence of ATP at physiological concentrations (18)] the kinetics of this effect are complex; it is expected that at low concentration of substrate the activity of the enzyme would be greatly decreased and that even the fully activated form (the dephospho form) of pyruvate kinase would not overcome the continuous input via *P-enol*-pyruvate carboxykinase. The right part of Fig. 6 indicates that

even at the highest activity of pyruvate kinase registered in our experimental conditions gluconeogenesis was not cancelled and one could even get the impression that a plateau was reached. This impression is due to the dispersion of the results obtained in different experiments; indeed in one experiment (Fig. 3), in which gluconeogenesis was measured in quadruplicate, changes in pyruvate kinase in this range of activity were still efficient in changing the rate of gluconeogenesis. One can therefore assume that the very high activity of pyruvate kinase observed by Krebs and Eggleston (4) in the liver of rats receiving a high-carbohydrate diet could play a role in further decreasing gluconeogenesis, without presumably suppressing it completely.

Phosphorylation of pyruvate kinase is likely to account, in part at least, for the action of glucagon on the rate of gluconeogenesis. It appears, however, that this inactivation of pyruvate kinase is not the only regulatory mechanism of the pathway, since the inhibition of gluconeogenesis by glucose is not related to a stable activation of this enzyme. It has recently been proposed that glucose may act through an inhibition of pyruvate carboxylase (26).

This work was supported by the Fonds de la Recherche Médicale and by National Institutes of Health Grant AM 9235. J.E.F. is recipient of a Long-Term European Molecular Biology Organization Fellowship. L.H. is Chargé de Recherches du Fonds National de la Recherche Scientifique.

1. Exton, J. H. & Park, C. R. (1972) in *Handbook of Physiology*, eds. Steiner, D. F. & Freinkel, N. (The American Physiological Society, Washington D.C.), pp. 431–455.
2. Claus, T. H., Pilkis, S. J. & Park, C. R. (1975) *Biochim. Biophys. Acta* **404**, 110–123.
3. Claus, T. H. & Pilkis, S. J. (1976) *Biochim. Biophys. Acta* **421**, 246–262.
4. Krebs, H. A. & Eggleston, L. V. (1965) *Biochem. J.* **94**, 3C.
5. Cimbala, M. & Blair, J. B. (1975) *Fed. Proc.* **34**, 618.
6. Friedrichs, D. (1975) *10th FEBS Meeting, Paris*, abstr. 1455.
7. Friedmann, B., Goodman, E. H., Jr., Saunders, H. L., Kostos, V. & Weinhouse, S. (1971) *Arch. Biochem. Biophys.* **143**, 566–578.
8. Ljungström, O., Hjhelmquist, G. & Engström, L. (1974) *Biochim. Biophys. Acta* **358**, 289–298.
9. Titanji, V. P. K., Zetterqvist, O. & Engström, L. (1976) *Biochim. Biophys. Acta* **422**, 98–108.
10. Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114.
11. Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120.
14. Krebs, H. A., Hems, R., Weideman, M. J. & Speake, R. N. (1966) *Biochem. J.* **101**, 242–249.
15. Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857.
16. Thomas, J. A., Schlender, K. K. & Larner, J. (1968) *Anal. Biochem.* **25**, 486–499.
17. Tanaka, T., Sue, F. & Morimura, H. (1967) *Biochem. Biophys. Res. Commun.* **29**, 444–449.
18. Carbonell, J., Felíu, J. E., Marco, R. & Sols, A. (1973) *Eur. J. Biochem.* **37**, 148–156.
19. Faupel, R. P., Seitz, H. J., Tarnowski, W., Thiemann, V. & Weiss, C. H. (1972) *Arch. Biochem. Biophys.* **148**, 509–522.
20. Flory, W., Peczon, B. D., Koeppe, R. E. & Spivey, H. O. (1974) *Biochem. J.* **141**, 127–131.
21. Ruderman, N. B. & Herrera, M. G. (1968) *Am. J. Physiol.* **214**, 1346–1351.
22. Ekman, P., Dahlqvist, U., Humble, E. & Engström, L. (1976) *Biochim. Biophys. Acta* **429**, 374–382.
23. Taunton, O. D., Stifel, F. B., Greene, H. L. & Herman, R. H. (1974) *J. Biol. Chem.* **249**, 7228–7239.
24. Veneziale, C. M. (1971) *Biochemistry* **10**, 3443–3447.
25. Clark, M. G., Kneer, N. M., Bosch, A. L. & Lardy, H. A. (1974) *J. Biol. Chem.* **249**, 5695–5703.
26. McDaniel, H. G. (1975) *Am. J. Physiol.* **229**, 1569–1575.