Control of Gluconeogenesis in Rat Liver Cells

I. KINETICS OF THE INDIVIDUAL ENZYMES AND THE EFFECT OF GLUCAGON*

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Control of gluconeogenesis from lactate was studied by titrating rat liver cells with lactate and pyruvate in a ratio of 10:1 in a perfusion system. At different steady states of glucose formation, the concentration of key gluconeogenic intermediates was measured and plotted against gluconeogenic flux ($J_{\text{glucose}}$). Complete saturation was observed only in the plot relating $J_{\text{glucose}}$ to the extracellular pyruvate concentration. Measurement of pyruvate distribution in the cell showed that the mitochondrial pyruvate translocator operates close to equilibrium at high lactate and pyruvate concentrations. It can therefore be concluded that pyruvate carboxylase limits maximal gluconeogenic flux. Addition of glucagon did not cause a shift in the plots relating $J_{\text{glucose}}$ to glucose 6-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, and phosphoenolpyruvate. It can thus be concluded that glucagon does not affect the kinetic parameters of the enzymes involved in the conversion of phosphoenolpyruvate to glucose. Addition of glucagon led to a shift in the curves relating $J_{\text{glucose}}$ to the concentration of cytosolic oxalacetate and extracellular pyruvate. The shift in the curve relating $J_{\text{glucose}}$ to oxalacetate is due to glucagon-induced inhibition of pyruvate kinase. The stimulation of gluconeogenesis by glucagon can be accounted for almost completely by inhibition of pyruvate kinase. There was almost no stimulation by glucagon of pyruvate carboxylation.

In the absence of glucagon, control on gluconeogenesis from lactate is distributed among different steps including pyruvate carboxylase and pyruvate kinase. Assuming that in the presence of glucagon all pyruvate kinase flux is inhibited, the control of gluconeogenesis in the presence of the hormone is confined exclusively to pyruvate carboxylase.

In mammals, hepatic gluconeogenesis plays an important role in glucose homeostasis. In order to maintain a constant level of glucose and also of lactate (1) in the blood, strict regulation of flux through the pathway is essential. It is therefore not surprising that a number of pathway enzymes are under allosteric control (reviewed in Refs. 2–4). The characteristics of the individual enzymes participating in gluconeogenesis from lactate are now well established. Surprisingly little is known, however, of how flux through these enzymes is controlled under intracellular conditions (discussed in Ref. 5). Even the most fundamental question, i.e. which is the enzyme with the lowest capacity under intracellular conditions, has as yet not been answered. The main candidates suggested in the literature are pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose 1,6-bisphosphatase (2, 3). Recently, the mitochondrial translocator for pyruvate has been added to this list (6).

The rate of gluconeogenesis from lactate can, in principle, also be controlled at the three points at which substrate cycling can occur (2–5). Especially the phosphoenolpyruvate-pyruvate cycle has been suggested in numerous reports to be a major control point (reviewed in Refs. 4, 5, 7, and 8). Flux through pyruvate kinase during gluconeogenesis from lactate has, indeed, been demonstrated in several laboratories (7–14), and it has been suggested that stimulation of gluconeogenesis by glucagon is brought about through inhibition of pyruvate kinase (14, 15). However, glucagon also has other effects which could lead to stimulation of gluconeogenesis. For instance, it has been reported that glucagon inhibits phosphofructokinase (16–19) and stimulates fructose 1,6-bisphosphatase (20–22), pyruvate carboxylase (23), and the transport of pyruvate across the mitochondrial membrane (24–28), although conflicting reports have also appeared (29, 30). Evaluation of the importance of the different effects is difficult.

Until now, most quantitative studies on control of gluconeogenesis have been carried out with radioactively labeled substrates so that flux through the different enzymes could be estimated from label redistribution in the different gluconeogenic intermediates (reviewed in Refs. 5 and 7). Katz and Rognstad (7) were the first to discuss the problems that are encountered when an attempt is made to quantify fluxes in such studies.

In the present investigation, we have used another approach to study control of gluconeogenesis. By using a perfusion system (31) and titrating substrates at subsaturating concentrations, different steady state rates of gluconeogenesis could be obtained. The steady state concentrations of intermediates were measured at the different rates of glucose formation so that the kinetics of the individual enzymes under intracellular conditions could be ascertained. Furthermore, with this approach it was possible to quantify the effect of glucagon on flux through the individual enzymes in the pathway.

MATERIALS AND METHODS

Liver parenchymal cells were isolated from 20–24-h starved Wistar rats (200–300 g) by the method of Berry and Friend (32), modified as described in Ref. 33.
Liver cells were perifused by the method of Van der Meer and Tager (31) with the modifications described in Ref. 33. The perfusion fluid was Krebs' bicarbonate buffer containing 1 mM Ca\(^2+\). Intracellular metabolites in each steady state were determined as follows. A 0.7-ml sample was taken from the perifusion chamber, the cells were centrifuged through silicone oil (Wacker AR 200/Wacker AR 20, 3:2 (v/v) into HCIO, (final concentration, 14% (w/v)), and metabolites were measured in the neutralized protein-free extract of the cells. The concentration of cytosolic malate in each steady state was measured after fractionation of the cells by the digitonin technique (34), modified as described in Ref. 33. The concentration of cytosolic oxalacetate was subsequently calculated from the cytosolic malate concentration and the lactate/pyruvate ratio, assuming equilibrium in the lactate dehydrogenase and cytosolic malate dehydrogenase reactions (35). When distribution of pyruvate was studied, cells (25 mg/ml) were incubated in 25-ml Erlenmeyer flasks in a Krebs' bicarbonate buffer supplemented with 2% (w/v) bovine serum albumin and 2.5 mM Ca\(^2+\). After 15 min of incubation, a 0.5-ml sample of the cell suspension was taken and either immediately centrifuged through silicone oil or fractionated by the digitonin technique essentially as described in Ref. 33, with the following minor modifications. In order to decrease the amount of pyruvate present in the adhering water in the pellet fraction, the dilution of the cell sample in the digitonin fractionation medium was increased by a factor of 12. All metabolites were measured spectrophotometrically or fluorometrically using standard enzymic methods (36-38).

Collagenase (type IV), glucagon, and lactate were obtained from Sigma. All other enzymes and biochemicals were obtained from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany).

RESULTS

In order to study the overall kinetics of gluconeogenesis from lactate, rat liver cells were titrated with lactate and pyruvate in a fixed ratio of 10:1 in the presence of oleate. As shown in Fig. 1, there was good agreement between the amount of lactate removed and the amount of glucose formed. When the reciprocal of the rate of lactate concentration in the perfusate, a linear relationship was obtained (Fig. 2). Thus, surprisingly, the complex pathway of gluconeogenesis behaves like a single enzyme with simple Michaelis-Menten kinetics. The question arises of which enzyme in the gluconeogenic pathway is responsible for this behavior.

![Fig. 1. Titration of rat liver parenchymal cells with lactate and pyruvate in the presence of oleate.](image)

![Fig. 2. Lineweaver-Burk plot for the rate of glucose formation as a function of the lactate concentration in the perfusate.](image)

Also shown in Fig. 2 is the effect of omitting oleate. Again, apparent Michaelis-Menten kinetics were observed (Fig. 2), but both the \(K_m\) and the \(V_{max}\) were changed. In the absence of oleate, the \(K_m\) for lactate was decreased from 260 to 60 \(\mu\)M and the \(V_{max}\) was decreased from 4.7 to 2.1 \(\mu\)mol/min/g, dry weight. It is generally accepted that oleate stimulates gluconeogenesis by providing acetyl-CoA, the allosteric activator of pyruvate carboxylase.

One explanation for the Michaelis-Menten kinetics and the stimulatory effect of oleate could be that pyruvate carboxylase is the enzyme that limits maximal gluconeogenic flux from lactate. However, the question arises of whether the approach used is sensitive enough to draw this conclusion. In order to obtain information about the possible existence of additional rate-controlling steps in gluconeogenesis from lactate, we studied the kinetics of the individual enzymes in the pathway. If, in addition to pyruvate carboxylase, other steps are rate-controlling, the enzymes catalyzing such steps will become saturated with their substrates at high gluconeogenic fluxes. Accordingly, in experiments of the type shown in Fig. 1, cell samples were taken at each steady state rate of glucose formation and the amounts of the following intermediates were measured: glucose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, phosphoenolpyruvate, cytosolic oxaloacetate, and extracellular pyruvate.

In Fig. 3, the steady state rate of glucose formation (\(J_{\text{glucose}}\)) is plotted as a function of the concentration of each of these metabolites. The final enzyme in the pathway, glucose 6-phosphatase, exhibits first order kinetics, which is in accordance with the relatively high \(K_m\) for glucose 6-phosphate (1.8 mM; Ref. 39) of the enzyme. The relationship between the rate of glucose formation and the concentration of phosphoenolpyruvate, 3-phosphoglycerate, dihydroxyacetone phosphate, and fructose 1,6-bisphosphate is clearly of a non-Michaelis-Menten type. With regard to fructose 1,6-bisphosphate, this was no surprise. Because of binding compartmentation, the measured amount of fructose 1,6-bisphosphate is probably not directly related to the free concentration of the metabolite in the cell (40), so that the observed relationship probably does not reflect the actual situation in the cell (see below).

In Fig. 3D, \(J_{\text{glucose}}\) is plotted as a function of the intracellular...
phosphoenolpyruvate concentration. The curve is very similar to that obtained when \( J_{\text{glucose}} \) is plotted versus the 3-phosphoglycerate concentration. Since both enolase and phosphoglycerate mutase are in near-equilibrium under these conditions (41-43), the similarity of the two curves suggests that even though 40% of the phosphoenolpyruvate is located in the mitochondrial matrix (44), the cytosolic/mitochondrial phosphoenolpyruvate ratio remains constant.

The 3-phosphoglycerate concentration is also geared to the dihydroxyacetone phosphate concentration, because the glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase couple is in near-equilibrium under these conditions (45), while changing the lactate and pyruvate concentrations has little effect on the cytosolic redox state and the [ATP]/[ADP][Pi] ratio (data not shown).

The relationship between cytosolic oxalacetate and \( J_{\text{glucose}} \) is, like that between extracellular pyruvate and \( J_{\text{glucose}} \) in hyperbolic (Fig. 3, E and F). A double reciprocal plot of the data in Fig. 3, E and F, yielded straight lines. Extrapolation of the data in this way leads to a \( V_{\text{max}} \) value of 8.1 \( \mu \text{mol/min} \cdot \text{g, dry weight} \), at saturating oxalacetate concentrations and a value of 5.0 \( \mu \text{mol/min} \cdot \text{g, dry weight} \), at saturating pyruvate concentrations. At the highest concentration of lactate + pyruvate infused, the steady state concentration of pyruvate was approximately 30 times the \( K_m \) for pyruvate, whereas the steady state concentration of oxalacetate was 2 times the \( K_m \) for oxalacetate. Thus, full saturation was achieved only with respect to pyruvate. Hence, the step(s) that limit maximal gluconeogenic flux must be located between pyruvate and oxalacetate. Since transport of pyruvate across the plasma membrane and transport of oxalacetate (in the form of aspartate) to the cytosol have high capacities (Refs. 46 and 47; see also “Discussion”), either transport of pyruvate across the mitochondrial membrane or pyruvate carboxylase determines maximal gluconeogenic flux.

In the experiments shown in Fig. 3, we were unable to study the kinetics of mitochondrial pyruvate transport and pyruvate carboxylase separately because of the low concentration of pyruvate present in the cytosol at subsaturating lactate concentrations, which makes accurate measurement of pyruvate difficult. Furthermore, binding compartmentation of pyruvate in the mitochondria may occur (48, 49). The pyruvate distribution in the cells was therefore measured in separate experiments at saturating concentrations of lactate. Table 1 shows that under conditions of maximal gluconeogenic flux there is still an increase in the mitochondrial pyruvate concentration when the extracellular lactate and pyruvate concentrations are increased. At low pyruvate concentrations, there is an apparent accumulation of pyruvate in the mitochondrial matrix with respect to the extracellular concentration. This is probably caused by binding compartmentation. However, at higher pyruvate concentrations, the gradient of the metabolite across the mitochondrial membrane is about 2.5, which is in accordance with the pH difference between mitochondria and cytosol that has been reported for isolated rat liver cells (pH 7.0 in the cytosol and pH 7.4 in the mitochondria (50–53)).
Control of Gluconeogenesis in Rat Liver Cells

Cells (25 mg/ml) were incubated with lactate (Lac) and pyruvate (Pyr) as indicated in the table in the presence of 2 mM oleate and in the presence or absence of 5 mM 3-mercaptopicolinic acid (3-MPA). After 15 min of incubation, cell samples were taken and processed as described under "Materials and Methods." The cytosolic concentration of pyruvate ([Pyr]cyt) was calculated from the difference between intracellular and mitochondrial ([Pyr]mt) contents. The results of a representative experiment are presented; similar results were obtained in other experiments.

Table I

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The question arises of what the reason is for the saturation effect observed in the plot relating Jglucose to cytosolic oxalacetate. An obvious possibility is that phosphoenolpyruvate carboxykinase becomes saturated with its substrate. However, a second possibility is that part of the phosphoenolpyruvate formed is converted back to pyruvate by pyruvate kinase. In order to distinguish between these two possibilities, glucagon was used to inhibit pyruvate kinase. The experiments of Fig. 1 were repeated, but now in the presence of glucagon. As shown in Fig. 4, glucagon stimulates gluconeogenesis over the whole range of lactate and pyruvate concentrations used. However, the percentage stimulation increases progressively with the lactate and pyruvate concentrations.

In contrast to the report of Thomas and Halestrap (6) but in agreement with the data of Siess et al. (44) and Tischler et al. (50), these results indicate that the mitochondrial pyruvate translocator is in near-equilibrium when gluconeogenesis from lactate and pyruvate occurs. That is, this is no artifact caused by binding compartmentation follows from the fact that inhibition of gluconeogenic flux with mercaptopicolinic acid has no effect on the direction of pyruvate (Table 1), although under these conditions the flux through the pyruvate translocator is greatly diminished. We can therefore conclude that it is pyruvate carboxylase itself that determines maximal gluconeogenic flux.

The question arises of what the reason is for the saturation effect observed in the plot relating Jglucose to cytosolic oxalacetate. An obvious possibility is that phosphoenolpyruvate carboxykinase becomes saturated with its substrate. However, a second possibility is that part of the phosphoenolpyruvate formed is converted back to pyruvate by pyruvate kinase. In order to distinguish between these two possibilities, glucagon was used to inhibit pyruvate kinase. The experiments of Fig. 1 were repeated, but now in the presence of glucagon. As shown in Fig. 4, glucagon stimulates gluconeogenesis over the whole range of lactate and pyruvate concentrations used. However, the percentage stimulation increases progressively with the lactate and pyruvate concentrations.
Control of Gluconeogenesis in Rat Liver Cells

**FIG. 5.** Relationship between the concentration of gluconeogenic intermediates and the steady state rate of gluconeogenesis from lactate and pyruvate in the presence and absence of glucagon. Cells (280–300 mg, dry weight) were perifused as described in the legend to Fig. 4. In each steady state of glucose formation, 0.7-ml samples of the cell suspension were taken and processed as described under "Materials and Methods." The gluconeogenic intermediates were determined as described in the legend to Fig. 3. The data for the condition without glucagon (○) were taken from Fig. 3. The data are given as means ± S.E. of three separate cell preparations. The standard error in Jglucose was <5% in all steady states. Glucose 6-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, and phosphoenolpyruvate are given in nanomoles/g, dry weight. The concentrations of cytosolic oxalacetate and extracellular pyruvate are given in micromolar.

**FIG. 6 (left).** Flux through pyruvate kinase in relation to the rate of gluconeogenesis from lactate and pyruvate and the intracellular phosphoenolpyruvate concentration. A minimal estimate of the pyruvate kinase flux (JPK) was made by subtraction of the two curves in Fig. 5E relating Jglucose to oxalacetate, and multiplication of the difference by 2. JPK + ΔJPC was calculated similarly from the difference between the two curves in Fig. 5F. JPC, flux through pyruvate carboxylase.

**FIG. 7 (right).** Relationship between the calculated free fructose 1,6-bisphosphate concentration and the rate of glucose formation. On the assumption that there is equilibrium in the triosephosphate isomerase and aldolase reactions the free fructose 1,6-bisphosphate concentration was calculated by using the following equation:

\[
[\text{Fructose-1,6-P}_2] = \frac{[\text{dihydroxyacetone-P}]^2}{K_{\text{al}}K_{\text{TIM}}}
\]

where \(K_{\text{al}}\) is the apparent equilibrium constant for aldolase \((85 \times 10^{-4} \text{ M}^{-1}; \text{Ref. 72})\) and \(K_{\text{TIM}}\) is the apparent equilibrium constant for triosephosphate isomerase \((22; \text{Refs. 73 and 74})\). The values for dihydroxyacetone-P and the rate of glucose formation were taken from Fig. 5. The open circles refer to the condition plus glucagon, the closed circles to the control experiments.
ruvate carboxykinase have not been reported in the literature, the second explanation is the most likely one and is, indeed, in accordance with literature data (9–14).

Thus, the difference between the two curves in Fig. 5E can be considered due to pyruvate kinase activity and can, in fact, be used to make a minimum estimate of flux through the enzyme in the absence of the hormone. In Fig. 6A (solid line), the values for minimum flux through pyruvate kinase obtained in this way are plotted against the rate of glucose formation. The estimated pyruvate kinase flux in the absence of glucagon is almost zero at the low rates of gluconeogenesis but increases sharply when the rate of glucose formation increases. Fig. 6B provides the explanation for this phenomenon. When the calculated flux through pyruvate kinase is plotted against the concentration of phosphoenolpyruvate, positive cooperativity with respect to phosphoenolpyruvate is observed, in agreement with studies on the isolated enzyme (15).

As indicated above, the difference between the two curves obtained in the absence and presence of glucagon in Fig. 5E is most probably caused by pyruvate kinase. The difference in the two curves in Fig. 5F relating \( V_{\text{glucose}} \) to the pyruvate concentration is, therefore, at least in part also due to pyruvate kinase. However, possible changes induced by glucagon in the activity of pyruvate carboxylase or any of the other steps involved in the conversion of pyruvate to cytosolic oxalacetate will also be reflected by a difference in the curves in Fig. 5F. Hence, if glucagon activates pyruvate carboxylase in addition to the inhibition of pyruvate kinase, the difference between the curves in Fig. 5F will be greater than the difference between the curves in Fig. 5E. The difference between the two curves in Fig. 5F is given in Fig. 6A (dashed line). At high gluconeogenic fluxes, the lines in Fig. 6A almost coincide, so that under these conditions the stimulation of gluconeogenesis by glucagon can be explained almost quantitatively by inhibition of pyruvate kinase. The slight stimulation (<10%) by glucagon of pyruvate carboxylation observed at low rates of gluconeogenesis is probably not significant.

**DISCUSSION**

It has been suggested (28, 46) that transport of pyruvate across the mitochondrial membrane, one of the initial steps in gluconeogenesis from lactate, takes part in control of flux through this pathway. Later, Thomas and Hales (6) concluded, on the basis of titrations with 4-hydroxycyanocinnamate, an inhibitor of the pyruvate translocator, that the transport of pyruvate is, indeed, rate-controlling for gluconeogenesis. They observed inhibition of gluconeogenesis already at the lowest concentration of inhibitor used. However, a quantification of the extent of control (control strength; Ref. 54) of the mitochondrial pyruvate translocator in their experiments is not possible since the \( K_i \), for 4-hydroxycyanocinnamate under intracellular conditions is unknown (6). Tischler et al. (50) have reported that the distribution of pyruvate across the mitochondrial membrane follows the pH difference, which indicates that the transport step is in near-equilibrium. A similar conclusion can be drawn from experiments of Siess et al. (44). However, artifacts caused by binding compartmentation cannot be excluded in the experiments of Tischler et al. (50) and Siess et al. (44). The data in Table I suggest that binding of pyruvate does, indeed, occur at low pyruvate concentrations. At the higher concentrations, the contribution of binding is masked (e.g. Ref. 49) and pyruvate appears to be distributed according to the pH difference. This was also observed in the absence of oleate (data not shown). Since there is still a proportional increase in the mitochondrial pyruvate concentration on increasing the extracellular lactate and pyruvate concentrations while the pathway flux is maximal, it can be concluded that the transport of pyruvate across the mitochondrial membrane is not the step with the lowest capacity. Thus, the 2-fold increase in \( V_{\text{max}} \) of gluconeogenesis brought about by addition of oleate cannot be explained by activation of pyruvate transport. Similar arguments can be used to show that stimulation of gluconeogenesis by glucagon is not mediated by activation of the mitochondrial pyruvate translocator (contrast Ref. 6).

The next step in the gluconeogenic pathway, pyruvate carboxylase, is essentially irreversible under intracellular conditions (55, 56). It is therefore a good candidate for exerting control on flux. The concentration of the product of the reaction, oxalacetate, can be estimated to be 2 orders of magnitude lower than the inhibition constant for oxalacetate (55). Since the concentrations of the other substrates and products of the enzyme remain almost constant under our experimental conditions (data not shown), Michaelis-Menten type kinetics of the enzyme with respect to pyruvate could be expected (56). Thus, on theoretical grounds the hyperbolic kinetics for gluconeogenic flux with respect to pyruvate could be explained. The observed kinetic parameters should reflect the kinetic parameters of pyruvate carboxylase and may also include the pitfalls of drawing such conclusions from linear Lineweaver-Burk plots. Flux through pyruvate kinase, which would be expected to lead to a deviation from linearity, is not reflected in the plot. The observed kinetic parameters in the absence of glucagon are therefore underestimated. In the presence of glucagon, the observed \( V_{\text{max}} \) of 7.9 \( \mu \text{mol/min.g} \), dry weight, corresponds to a pyruvate carboxylase flux of 15.8 \( \mu \text{mol/min.g} \), dry weight. This value is close to the \( V_{\text{max}} \) of 15 \( \mu \text{mol/min.g} \), dry weight, for pyruvate carboxylase in isolated rat liver mitochondria (57, 58). Still, it cannot be excluded that some activity of pyruvate kinase occurs in the presence of glucagon.

The steps involved in the transport of oxalacetate from the mitochondria to the cytosol have not been considered in the present study. Theoretically, these steps could influence gluconeogenic flux via product control by oxalacetate of pyruvate carboxylase. Since the mitochondrial oxalacetate concentration is extremely low under all conditions (e.g. Ref. 59), this control obviously does not occur. The same considerations hold for phosphoenolpyruvate carboxykinase. Rognstad (60) reported that phosphoenolpyruvate carboxykinase may be the rate-limiting enzyme in gluconeogenesis from lactate. Inhibitor titration experiments with mercaptopicolinate, a noncompetitive inhibitor of phosphoenolpyruvate carboxykinase (61), he observed no threshold in the inhibition of gluconeogenesis from lactate, even at the lowest concentrations used (60). According to Rognstad, this suggests that a step may be rate-limiting. However, we have recently shown that the shape of an inhibition curve gives no quantitative information about the control strength of a step on pathway flux (62). Indeed, assuming equilibration of 3-mercaptopicolinic acid across the plasma membrane, a very low control strength of phosphoenolpyruvate carboxykinase could be calculated from the data of Rognstad (60) and also of Akerboom (63).

Since pyruvate kinase is active in the absence of glucagon, all steps which are able to influence the phosphoenolpyruvate concentration can exert control on gluconeogenic flux. Theoretically, this involves all steps that participate in the conversion of phosphoenolpyruvate to glucose, although the degree to which these enzymes are able to influence the phosphoenolpyruvate concentration of course differs. The first two
steps involved, enolase and phosphoglycerate mutase, are near to equilibrium and thus can exert only little control on the phosphoenolpyruvate concentration (64). However, the reactions catalyzed by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase, although also in near-equilibrium, can influence the phosphoenolpyruvate concentration by a mass action effect via the cytosolic ATP/ADP-Pi ratio and/or the cytosolic redox state. This could be the reason why gluconeogenesis is so sensitive to inhibition of the adenine nucleotide translocator by atracysolide (65, 66). A decrease in the cytosolic ATP/ADP-Pi ratio is immediately translated into an increase in the phosphoenolpyruvate concentration and consequently an increase in pyruvate kinase flux.

The next enzyme in the gluconeogenic pathway, aldolase, is generally thought to catalyze a near-equilibrium reaction (2). This assumption is difficult to verify because the free fructose 1,6-bisphosphate concentration cannot be measured. The maximal activity of the enzyme when measured in a homogenate is, however, low (about 40 μmol/min·g, dry weight; Ref. 2). Thus, certainly at high gluconeogenic fluxes, the enzyme is probably not in equilibrium. Aldolase plays a special role in gluconeogenesis since both its substrates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, are pathway intermediates. It can be calculated that even if the aldolase reaction is in equilibrium the enzyme could cause the sigmoidicity observed for dihydroxyacetone phosphate, 3-phosphoglycerate, and phosphoenolpyruvate (Fig. 5). This is demonstrated in Fig. 7, where the free fructose 1,6-bisphosphate concentration was calculated from the dihydroxyacetone phosphate concentration assuming equilibrium in the aldolase and triosephosphate isomerase reactions. The sigmoidicity observed for dihydroxyacetone phosphate has now disappeared, and the curve relating $\gamma$ to the concentration of free fructose 1,6-bisphosphate tends to show hyperbolic type kinetics. This is, in fact, to be expected since isolated fructose 1,6-bisphosphatase shows hyperbolic kinetics with respect to fructose 1,6-bisphosphate (reviewed in Ref. 67).

It is clear from Fig. 7 that glucagon has no effect on the kinetics of fructose 1,6-bisphosphate conversion to glucose. Phosphofructokinase is apparently not active under these conditions, which is in accordance with the prediction of Reinhart and Lardy (68). However, there is also no activation of fructose 1,6-bisphosphatase visible, suggesting that the activation of the enzyme by glucagon either directly (20-22) or via fructose 2,6-bisphosphate (69, 70) is not expressed under our experimental conditions. The fructose 1,6-bisphosphatase reaction is far from equilibrium under intracellular conditions. Theoretically, changes in the activity of the enzyme, for instance by an allosteric activator or inhibitor, could lead to changes in the phosphoenolpyruvate concentration and thus gluconeogenic flux. Therefore, fructose 1,6-bisphosphatase could be rate-controlling for gluconeogenesis. Studies to quantify the extent of control which the enzyme exerts under the different conditions are now in progress.

The contribution of the steps after fructose 1,6-bisphosphatase to control of flux depends on the ability of these steps to influence the rate through fructose 1,6-bisphosphatase. Since the enzyme is not very sensitive to its product fructose 6-phosphate (71), the control strength of the steps after fructose 1,6-bisphosphatase will be low. Thus, even if there should be activity in the glucose 5-phosphate-glucose cycle, there will be little influence of this cycle on gluconeogenic flux as long as glucose 6-phosphatase does not reach its maximal activity (e.g. Ref. 54).

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