Coordinate Control of Intermediary Metabolism in Rat Liver by the 
Insulin/Glucagon Ratio during Starvation and after Glucose 
Refeeding

Regulatory Significance of Long-Chain Acyl-CoA and Cyclic AMP

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The levels of serum insulin, glucagon, and free fatty acids (FFA) and the tissue concentrations of hepatic cyclic AMP, long-chain acyl-CoA (LCA), adenine nucleotides, inorganic phosphate, the intermediates of the Embden-Meyerhof pathway, the citric acid cycle (including acetyl-CoA and free CoA), and the cytoplasmic and mitochondrial redox couples were determined in the rat 12, 24, and 48 h after food withdrawal and 5, 10, 20, 40, 60, and 120 min after the refeeding of glucose. Using the measured metabolite contents in the liver, the alterations in the concentration of malate, oxaloacetate, citrate, and α-ketoglutarate and the changes in the energy state of the adenine nucleotide system and the redox state of the NAD system were attributed to the cytoplasmic and mitochondrial compartments by applying established calculation methods. Glucose refeeding provoked significant alterations in all parameters investigated. These changes occurred within minutes, reversing the hormone and metabolite pattern which had developed within 24 h in response to food withdrawal. Particularly, glucose refeeding resulted in a drastic increase in the insulin/gluca
gon ratio. Simultaneously, the level of serum FFA and the concentration of LCA in the liver declined. The latter alteration was accompanied by an increase in the cytoplasmic and a decrease in the mitochondrial ATP/ADP × P ratios. Moreover, the redox state of the cytoplasmic NAD system was shifted toward the oxidized state. When the appropriate data were plotted against each other, highly significant correlations were obtained (i) between the insulin/gluca
gon ratio and the serum FFA concentration, (ii) between the serum FFA concentration and the concentration of hepatic LCA, (iii) between the hepatic LCA concentration and the cytoplasmic energy state, and (iv) between the cytoplasmic energy state and the redox state of the cytoplasmic NAD system. These findings are interpreted to support the hypothesis derived from experiments carried out in vitro that the insulin/gluca
gon ratio via the FFA-dependent concentration of hepatic LCA might affect the translocation of adenine nucleotides between the cytoplasmic and the mitochondrial compartment, thereby regulating the cytoplasmic energy state and the redox state of the cytoplasmic NAD system, consequently. Glucose refeeding provoked rapid coordinate changes in the concentration of the intermediates of both the citric acid cycle and the Embden-Meyerhof chain, indicating the altered substrate flow through these pathways. Those metabolites, known to modulate the activity of key regulatory enzymes in vitro, were analyzed with respect to their suggested regulatory function. As to the established shift from pyruvate carboxylation to pyruvate de
carboxylation after glucose refeeding, the data revealed that the decrease in pyruvate carboxylase activity can be attributed to the decrease in the intramitochondrial ATP/ADP ratio and the simultaneous fall in acetyl-CoA concentration, while the coordinate increase in pyruvate dehydrogenase activity can be ascribed to the decline in the concentration of LCA and, consequently, in the ratios of ATP/ADP, NADH/NAD, and
acetyl-CoA/CoA within the mitochondria. As for the citric acid cycle, increased citrate synthesis from acetyl-CoA and oxaloacetate was supported by the rapid drop in the concentration of the established inhibitor of citrate synthesis, LCA. In contrast, the concentration of succinyl-CoA, an inhibitor of the enzyme in vitro, remained practically constant, questioning its regulatory function under the present experimental conditions. In addition to the activation of citrate synthase, the coordinate activation of isocitrate dehydrogenase was indicated by the LCA-mediated decline in both the mitochondrial ATP/ADP and the NADH/NAD ratios. Glucose refeeding immediately reduced urea excretion to basal values. This alteration was preceded by a drastic fall in the tissue concentration of cyclic AMP, supporting the physiological role of the nucleotide in the control of hepatic gluconeogenesis. In contrast, the observed changes in the concentration of the effector acting metabolites (ATP, AMP, fructose 1,6-diphosphate, citrate, and alanine) were incompatible with the suggested function of these intermediates in switching over the substrate flow through the Embden-Meyerhof pathway from gluconeogenesis to glycolysis. The results are discussed in reference to the known rapid stimulation of fatty acid biosynthesis in the liver and to the transfer of reducing equivalents by the different shuttles of the inner mitochondrial membrane. In summary, it can be concluded that the insulin/glucagon ratio in a moment-to-moment fashion controls the glucose balance across the liver by regulating hepatic intermediary metabolism via the concentration of both LCA and cyclic AMP.

Felig et al. (1) have recently demonstrated that oral intake of glucose in starved men resulted in the switching over of hepatic intermediary metabolism from glucose production to glucose utilization within minutes. This rapid adaptation is due to coordinate changes in the activity of the major pathways of glucose metabolism. It has therefore been anticipated "that common factors exist which would affect all of these pathways in some interlinked fashion" (2).

One of these factors might be cyclic AMP. In fact, the second messenger has been demonstrated in different liver systems to stimulate glycogenolysis, gluconeogenesis, ureogenesis, and ketone body production (for a review, see Ref. 3). Inversely, the metabolite flux through these sequences is reduced immediately when hepatic cyclic AMP concentration decreases (4).

Another common factor integrating the control of hepatic intermediary metabolism may possibly be the tissue concentration of long-chain acyl-CoA. The following findings are in favor of such an assumption. (i) The concentration of long-chain acyl-CoA in the liver coincides with different states of intermediary glucose metabolism. It is increased in diabetes and after prolonged starvation and decreased several days after restoration of insulin or glucose refeeding, respectively (5, 6). (ii) In isolated rat liver mitochondria, several long-chain acyl-CoA derivatives effectively inhibit translocation of adenine nucleotides through the inner mitochondrial membrane (7), thus affecting the energy state (ATP/ADP x P) in the cytoplasmic and mitochondrial compartments. The latter have been demonstrated to exert a coordinate control of gluconeogenesis (8, 9), pyruvate conversion to acetyl-CoA (9, 10), and citric acid cycle activity (11). (iii) Long-chain acyl-CoA derivatives have been shown to modulate the activity of different key enzymes of hepatic intermediary metabolism in a variety of rat liver preparations (cf. Discussion). The proposed regulatory role of both cyclic AMP and long-chain acyl-CoA in the rapid adaptation of hepatic glucose metabolism would entail the following presumptions:

Cyclic AMP and long-chain acyl-CoA concentrations should change in the direction expected from a series of published in vitro findings. These alterations should occur rapidly enough to explain switching over of hepatic intermediary metabolism from glucose production to glucose utilization within minutes.

Within the postulated sequences of metabolic events, the changes in the concentration of regulatory acting intermediates should be closely correlated.
In the present study, these presumptions have been examined under appropriate in vivo conditions, i.e., during a starvation-refeeding cycle. Our results suggest that in the liver, besides the level of cyclic AMP, the concentration of long-chain acyl-CoA, depending on the insulin/glucagon ratio in the blood, might actually account for the moment-to-moment control of the balance of glucose across the liver.

MATERIALS AND METHODS

Experimental animals and treatments. Male Wistar rats, weighing 180-200 g, were housed at 23°C under controlled conditions providing light from 6 AM to 6 PM. In starvation experiments, the animals were prefed a protein-free, carbohydrate-rich diet (Altromin GmbH, Lage/Lippe, C-1004) for 3 days prior to the experiments. Such a diet lowers the serum insulin concentration to the starvation level and suppresses the unsatisfactory synchronous circadian rhythm of the insulin/glucagon ratio in the blood (12). It thereby standardized the hormone and hepatic cyclic AMP values during the early phase of starvation.

In refeeding experiments, normal fed animals were starved for 48 h and refed with glucose ad libitum for a maximum of 1 h using pieces prepared by desiccating a glucose pap.

Determination of hormones, glucose, and free fatty acids in the blood. For hormone determination, rats were decapitated and plasma samples were prepared by centrifugation and stored deep-frozen for up to several weeks. Insulin was measured according to Meade and Klitgaard (13), using the radioimmunoassay kit obtained from Hoechst AG, Frankfurt/Main. For the preparation of the standard curve, rat insulin was used.

For glucagon determination, the collection, preparation, and storage of the plasma samples as well as the immunoassay were performed according to Falloona and Unger (14) using [125I]iodo-glucagon purchased from Cambridge Nuclear Radiochemical Corp., Billerica, Massachusetts. Pancreatic glucagon antiserum 30 K (prepared in Dr. Unger’s laboratory) was obtained from the Diabetes Research Fund, University of Texas, Dallas.

The plasma free fatty acid concentration was measured according to Duncombe (15).

Blood glucose was determined from the HCIO4 extract of whole blood, using the hexokinase/glucose 6-phosphate dehydrogenase method.

Determination of metabolites in the liver. Rats were killed by the double hatchet method and liver samples were deep-frozen within 2-3 s between aluminum blocks precooled in liquid nitrogen (16). Cyclic AMP was determined from an aliquot of the frozen tissue after deproteinization with trichloroacetic acid according to Gilman (17). Another aliquot was deproteinized with HClO4 as described previously (16). From the acid-insoluble sediment of the latter extract, long-chain acyl-CoA derivatives were estimated after alkaline hydrolysis (18) by measurement of the CoA liberated (19). All other metabolites were determined enzymatically by spectrophotometry from the neutralized supernatant of the HCIO4 extract. Measurements of ATP, ADP, AMP, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, glycerol 3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA, citrate, and oxaloacetate have been described previously (16). For the remaining metabolites, the following methods cited in the respective references were applied: inorganic phosphate (20), lactate (21), α-ketoglutarate (22), succinate (23), malate (24), acetoadetate and β-hydroxybutyrate (25), glutamate (26), aspartate (27), and NH₃ (28). Succinyl-CoA was measured combining the succinic thiokinase, hexokinase, and glucose 6-phosphate dehydrogenase reactions.

Determination of enzyme activities. After killing the animals by decapitation, liver tissue was immediately homogenized at 1000 rpm and 2°C for 45 s in a Potter-Elvehjem homogenizer, using different buffer solutions. The homogenates were centrifuged within 20 min at 150,000 g for 30 min, and assays for enzyme activities were performed within 2 h. The extraction procedures and established methods for the determination of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose 1,6-diphosphatase activities have been described in detail (12). The activity of glucokinase was estimated according to Dipietro et al. (29) using the extraction buffer of Grossman et al. (30). Extraction and measurement of phosphofructokinase activity were performed according to Underwood and Newsholme (31). Pyruvate kinase activity was measured from a KCl (0.15 M) extract using a final concentration of fructose 1,6-diphosphate of 0.1 mM in the assay (32).

Determination of nitrogen excretion in the urine. This was quantitated by separate NII₄ (28) and urea (33) measurements from the urine collected from each rat.

Reagents. Buffer substances, EDTA, salts, and organic solvents were purchased from E. Merck A. G., Darmstadt; substrates, cosubstrates, and coupling enzymes were from C. F. Buehringer Mannheim GmbH, Mannheim; and succinic thiokinase was from Sigma Chemical Co., St. Louis, Missouri.

Statistical analysis. Differences of means were established by Student’s t test.

Calculation of the distribution of metabolites between the mitochondrial and cytoplasmic compart-
ments. All calculations were based upon the fact that a series of defined—single or coupled—reactions, particularly catalyzed by certain dehydrogenases, kinases, and transaminases, operates near equilibrium independently of the metabolic state of the liver cell. The underlying assumptions for the different calculation procedures and the validity of the respective approach have been described in detail by Krebs and Veech (34).

The following procedures were applied: ATP/ADP \times P ratio in the cytoplasm according to Veech et al. (35); ATP/ADP \times P ratio in the mitochondria according to Krebs and Veech (36); GTP/GDP ratio in the cytoplasm according to Gumaa et al. (37) (including the equilibrium constant); malate and oxaloacetate in the cytoplasm and mitochondria according to Williamson (38); glutamate and aspartate in the cytoplasm and mitochondria according to Gumaa et al. (37); \( \alpha \)-ketoglutarate in the cytoplasm and mitochondria according to Krebs and Veech using the glutamate oxaloacetate transaminase reaction (34); citrate in the cytoplasm and mitochondria according to Williamson (38) (equilibrium constant of the aconitase reaction taken from Veloso et al. (39)). If not otherwise stated, the equilibrium constants of the respective reactions were taken from the review article of Krebs and Veech (36).

RESULTS

The present results indicate that after food withdrawal the characteristic changes in hormone and metabolite concentrations developed gradually within 1–2 days, whereas after the refeeding of glucose reversed changes occurred within 20–40 min. The latter observation is more informative, since it demonstrates a coordinated moment-to-moment control of hepatic intermediary metabolism independent of the more delayed process of enzyme induction/repression. Accordingly, the following description is confined to the results obtained after glucose refeeding, although the slow developing inverse changes during starvation are shown in the figures and tables.

Starved rats responded to glucose refeeding with a drastic increase in the previously low insulin/glucagon ratio (Fig. 1) and with a drop in the hepatic cyclic AMP concentration (Fig. 2). Moreover, glucose refeeding induced a rapid decline in the level of serum free fatty acids (Fig. 3), which was inversely correlated to the rising insulin/glucagon ratio \((r = -0.81, P < 0.025)\). Simultaneously, the concentration of hepatic long-chain acyl-CoA decreased (Fig. 3).

Glucose refeeding induced a rapid increase in the ATP/ADP \times P ratio in the liver within minutes (Fig. 4), due to the drastic elevation in the concentration of ATP, and a simultaneous drop in ADP tissue level, while the concentration of inorganic phosphate remained practically constant (Table I). The changes in the ATP/ADP \times P ratio were inversely corre-

![Fig. 1. Alteration in the level of serum insulin (▲, ●) and serum glucagon (△, ○) during starvation and after glucose refeeding. Data are given as means ± SEM (n = 6-8).](image-url)
Fig. 2. Alteration in the concentration of hepatic cyclic AMP during starvation and after glucose refeeding. Data are given as means ± SEM (n = 6–8).

Fig. 3. Alteration in the concentration of serum free fatty acids (Δ, ○) and hepatic long-chain acyl-CoA (▲, ●) during starvation and after glucose refeeding. Data are given as means ± SEM (n = 6–8).

The increase in the ATP/ADP × P ratio in the total liver tissues essentially represented the increase of this ratio in the cytoplasmic compartment (Fig. 4). In contrast, the energy state in the mitochondria declined by about 75% in comparison with the starved control (Fig. 4). To assure that the overall ATP/ADP ratio actually represented the ATP/ADP ratio in the cytoplasm of the liver cell, the cytoplasmic GTP/GDP ratio, which is known to be in equilibrium with the cytoplasmic adenine nucleotide system (40), was calculated from the measured oxaloacetate and phosphoenolpyruvate concentrations.\(^1\) In fact, during starvation as well as after glucose refeeding, the calculated cytoplasmic GTP/GDP ratio was found to be closely

\(^1\) The calculation is based on the fact that, in rat liver, 99% of phosphoenolpyruvate carboxykinase is located in the cytoplasm (41), enzyme activity being submaximally elevated as early as 12 h after food withdrawal.
correlated with the measured ATP/ADP overall ratio (starvation, $r = 0.64$, $P < 0.01$; glucose refeeding, $r = 0.61$, $P < 0.0005$).

Following glucose refeeding, the lactate/pyruvate ratio in the liver was diminished rapidly (Fig. 6). Every change in the lactate/pyruvate ratio was accompanied by synchronous changes in both the malate/oxaloacetate ($r = 0.94$, $P < 0.0025$) and the glycerol 3-phosphate/dihydroxyacetone phosphate ($r = 0.89$, $P < 0.005$) ratios. Thus, it is apparent that the latter redox parameters, like the lactate/pyruvate ratio, represented predominantly the redox

**TABLE I**

**ALTERATION IN THE CONCENTRATION OF ATP, ADP, AMP, AND INORGANIC PHOSPHATE IN RAT LIVER DURING STARVATION AND AFTER GLUCOSE REFEEDING**

<table>
<thead>
<tr>
<th>Metabolic condition</th>
<th>Metabolite concentration in the liver (nmol/g wet weight)$^a$</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starvation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>2970 ± 110</td>
<td>580 ± 60</td>
<td>89 ± 11</td>
<td>2970 ± 140</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>3100 ± 170</td>
<td>730 ± 25</td>
<td>104 ± 7</td>
<td>2800 ± 80</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>2950 ± 30</td>
<td>1040 ± 50</td>
<td>131 ± 7</td>
<td>3140 ± 120</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>2940 ± 90</td>
<td>980 ± 60</td>
<td>183 ± 14</td>
<td>3030 ± 150</td>
<td></td>
</tr>
<tr>
<td>Glucose refeeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>2590 ± 180</td>
<td>1070 ± 20</td>
<td>212 ± 6</td>
<td>3100 ± 80</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>3020 ± 70</td>
<td>1220 ± 50</td>
<td>215 ± 8</td>
<td>3360 ± 120</td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>3110 ± 70</td>
<td>1020 ± 20</td>
<td>187 ± 11</td>
<td>3100 ± 100</td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>3020 ± 130</td>
<td>930 ± 60</td>
<td>194 ± 22</td>
<td>3340 ± 40</td>
<td></td>
</tr>
<tr>
<td>40 min</td>
<td>2950 ± 50</td>
<td>740 ± 10</td>
<td>135 ± 3</td>
<td>3080 ± 100</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>3170 ± 100</td>
<td>780 ± 100</td>
<td>166 ± 11</td>
<td>3040 ± 110</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>2880 ± 80</td>
<td>705 ± 30</td>
<td>140 ± 5</td>
<td>3250 ± 120</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data are given as means ± SEM ($n = 6-8$).
Fig. 5. Correlation between the ATP/ADP × P ratio in the total liver cell and the concentration of long-chain acyl-CoA in the liver during starvation (▲) and after glucose refeeding (○). Data for each individual rat were plotted. Starvation, $r = -0.64$, $P < 0.01$; glucose refeeding, $r = -0.61$, $P < 0.0005$.

Fig. 6. Alteration in the cytoplasmic and mitochondrial redox states of the NAD system (as represented by the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios) during starvation and after glucose refeeding. Data are given as means ± SEM ($n = 6-8$).

The lactate/pyruvate ratio was correlated inversely to the ATP/ADP × P overall ratio throughout the whole starvation-refeeding cycle (Fig. 7). This result confirms the predicted moment-to-moment equilibrium of the glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase reaction, since it has been demonstrated in the isolated perfused rat liver by direct measurement that 75-80% of the...
total adenine nucleotides are located within the cytoplasmic compartment (42). Accordingly, it supports the validity of the calculation of the cytoplasmic ATP/ADP × P ratio from the measured concentration of glyceraldehyde phosphate, 3-phosphoglycerate, lactate, and pyruvate.

After glucose refeeding, the cytoplasmic NADPH/NADP ratio was also shifted toward the oxidized state within minutes (Fig. 8).

In contrast to the cytoplasmic redox state of the NAD and NADP systems, the mitochondrial NADH/NAD ratio, as represented by the β-hydroxybutyrate/acetocetate ratio, was decreased only slightly by glucose refeeding (Fig. 6).

Glucose refeeding resulted in significant alterations in the concentration of all intermediates of the citric acid cycle (Fig. 9). The essential findings can be summarized as follows: (i) The concentration of acetyl-CoA was reduced within 15 min. (ii) The citrate level of the total liver cell was elevated significantly, representing an increase of citrate concentration within the mitochondria. (iii) The alterations in the concentration of α-ketoglutarate were very similar to those of citrate with respect to both the overall and the mitochondrial levels. (iv) The concentration of succinyl-CoA was rapidly yet only slightly elevated. (v) The succinate level was elevated, similar to the level of mitochondrial citrate and α-ketoglutarate. (vi) The concentration of free CoA was increased. (vii) In contrast, the malate level in the total liver cell was continuously lowered, predominantly due to the rapid decrease in metabolite concentration in the mitochondria. (viii) Glucose refeeding increased the concentration of oxaloacetate in the total liver cell, representing the increased metabolite level in the cytoplasm, whereas oxaloacetate concentration in the mitochondria remained practically unchanged.

Within 1 h, glucose refeeding reduced nitrogen excretion in the urine by about 50% (equivalent to 54 ± 4 μmol of N/100 g body weight × h), indicating the rapid switching off of gluconeogenesis from amino acids.

Following glucose refeeding, characteristic changes in the concentrations of the intermediates of the Embden-Meyerhof pathway were observed (Fig. 10). The predominant finding was the excessive increase in the concentration of phosphoenolpyruvate and 3-phosphoglycerate. In contrast, the concentration of fructose 1,6-diphosphate remained unchanged, whereas that of hexose phosphates was only slightly elevated.
Fig. 9. Alteration in the hepatic concentration of the intermediates of the citric acid cycle after glucose refeeding. Measured values (●—●); calculated values for the cytoplasm (□——□); calculated values for the mitochondria (○——○). Measured values are given as means ± SEM (n = 6-8). Calculations were performed as described under Materials and Methods.
Alteration in the concentration of the intermediates of the Embden-Meyerhof pathway in the liver after glucose refeeding. Values of 100% represent the metabolite concentrations in the liver of starved controls. The respective measured values expressed as nanomoles per gram wet weight (means ± SEM, n = 6–8) were as follows: Lactate (LAC), 312 ± 25; pyruvate (PYR), 17 ± 2; phosphoenolpyruvate (PEP), 23 ± 3; 3-phosphoglycerate (3-PGA), 98 ± 13; glyceraldehyde phosphate (GAP), 1 ± 0.3; dihydroxyacetone phosphate (DAP), 10 ± 2; fructose 1,6-diphosphate (FDP), 7 ± 2; fructose 6-phosphate (F-6-P), 4 ± 1; glucose 6-phosphate (G-6-P), 34 ± 3. ●, 5 min; ▲, 20 min; ■, 60 min after glucose refeeding.

After glucose refeeding, the activity of the key regulatory enzymes of the Embden-Meyerhof pathway in the liver (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase, glucokinase, phosphofructokinase, and pyruvate kinase) was estimated at 5, 10, 30, and 240 min. Under these conditions, all activities remained unchanged with the exception of phosphoenolpyruvate carboxykinase, which was slightly yet significantly reduced (data not shown).

To perform the different calculation procedures, the hepatic concentrations of further metabolites were measured at 0, 5, 10, 20, 40, 60, and 120 min after glucose refeeding. The results in nanomoles per gram wet weight (means ± SEM, n = 6–8) were as follows: aspartate, 362 ± 30, 373 ± 29, 381 ± 21, 470 ± 39, 656 ± 54, 540 ± 35, 447 ± 14; glutamate, 1470 ± 110, 1490 ± 80, 1950 ± 130, 2300 ± 180, 2480 ± 120, 2670 ± 210, 2050 ± 200; alanine, 853 ± 51, 855 ± 66, 897 ± 11, 918 ± 30, 1083 ± 53, 1475 ± 118, 1682 ± 144; NH₄⁺, 693 ± 48, 671 ± 20, 641 ± 23, 607 ± 37, 575 ± 19, 500 ± 22, 546 ± 9.

DISCUSSION

Regulation of hepatic long-chain acyl-CoA concentration. One essential finding of the present study is the demonstration that the concentration of long-chain acyl-CoA in the liver is gradually elevated during starvation and reduced within minutes after glucose refeeding (cf. Fig. 3). This result could be explained by the previous observation that, in the isolated perfused rat liver, the tissue concentration of long-chain acyl-CoA can be coordinately increased by increasing the concentration of oleic acid in the perfusion medium (43). In fact, in our experiments, there was a direct correlation between both parameters, the concentration of long-chain acyl-CoA in the liver and that of free fatty acids in the blood (cf. Fig. 3), the latter depending on the ratio of circulating insulin and glucagon (cf. Fig. 1).

Regulation of the mitochondrial and cytoplasmic energy states in the liver. Recently it has been demonstrated in isolated mitochondria of rat and guinea pig liver that different long-chain acyl-CoA derivatives inhibited effectively the adenine
nucleotide translocation across the mitochondrial inner membrane, thus inducing an asymmetric distribution of ATP and ADP on both sides of the membrane (7, 44). Applying this result to the in vivo situation, one could expect that an increase in tissue long-chain acyl-CoA concentration should reduce the ATP/ADP ratio in the cytoplasm, whereas within the mitochondria this ratio should be elevated coordinately. Vice versa, the energy state in both compartments should be reversed as the long-chain acyl-CoA concentration drops. In fact, this prediction has been demonstrated by our experiments: During the whole starvation-refeeding cycle, the expected changes in the ATP/ADP × P ratio did occur in close correlation with the tissue level of hepatic long-chain acyl-CoA (cf. Fig. 5). A low phosphate potential in the cytoplasm and its simultaneous elevation in the mitochondria of the liver have been obtained recently by direct measurement in the isolated perfused liver of starved rats (42).

Regulation of pyruvate conversion to either oxaloacetate or acetyl-CoA. The physiological importance of the observed increase in the mitochondrial ATP/ADP ratio and its rapid decline after glucose refeeding could be deduced from the recent in vitro observation that, in isolated rat liver mitochondria, a decrease in the mitochondrial ATP/ADP ratio favors the conversion of pyruvate to acetyl-CoA effectively, whereas pyruvate conversion to oxaloacetate was diminished (9). This regulatory effect of the energy state can be explained by the following observations: (i) Purified rat liver pyruvate carboxylase is inhibited by lowering the ATP/ADP ratio in the assay (8). (ii) In isolated rat liver mitochondria, a decrease in the ATP/ADP ratio led to an increased interconversion of the inactive to the active form of pyruvate dehydrogenase (10, 48). Although the results cited suggest the intramitochondrial energy state to be of major importance in directing pyruvate to either oxaloacetate or acetyl-CoA, it should be noted that other regulatory factors are probably involved.

As for pyruvate carboxylase, regulation by acetyl-CoA, an established activator of the enzyme, is still being discussed. The physiological significance of this type of regulation has been questioned, since the $K_a$ of purified rat liver pyruvate carboxylase for acetyl-CoA ranges from 10 to 110 $\mu$M, depending on the condition of the assay with respect to the concentration of different cofactors (46). This $K_a$ is below the mitochondrial acetyl-CoA concentration which can be estimated from our data, 100–1400 $\mu$M, suggesting that the enzyme is almost saturated with its effector under in vivo conditions. However, for isolated rat liver mitochondria, an apparent $K_a$ of pyruvate carboxylase for acetyl-CoA of up to 230 $\mu$M has been published, the actual value being inversely related to the mitochondrial ATP/ADP ratio (47). Applying the latter in vitro finding to our results, it would appear that, in addition to the direct effect of the mitochondrial ATP/ADP ratio upon pyruvate carboxylase activity, the enzyme is activated effectively by acetyl-CoA during starvation and deactivated within minutes after glucose refeeding (cf. Fig. 9).

As for pyruvate dehydrogenase, the following factors have been demonstrated in different rat tissues to stimulate the conversion of PDH$_d$ to PDH$_a$, in addition to the decrease in the intramitochondrial ATP/ADP ratio: (i) a drop in long-chain acyl-CoA concentration (48), (ii) a decline in the mitochondrial NADH/NAD ratio (49, 50), and (iii) a decrease in the acetyl-CoA/CoA ratio (50). Although it is still uncertain which of the factors cited above is the predominant one in vivo, our results show that all of them were altered simultaneously in the direction expected for an accelerated conversion of pyruvate to either oxaloacetate or acetyl-CoA during starvation or after glucose refeeding, respectively.

Regulation of citric acid cycle activity. Following glucose refeeding, citrate synthesis from acetyl-CoA and oxaloacetate

\footnotetext[2]{The estimation is based upon the assumption that up to 95% of the total acetyl-CoA is located within the mitochondria (42).}
\footnotetext[3]{Abbreviation used: PDH, pyruvate dehydrogenase.
should be accelerated immediately as a consequence of the decline in the concentration of long-chain acyl-CoA, which has been previously demonstrated to inhibit citrate synthase effectively in both rat liver (51) and pig heart (52) independently of their detergent properties (52). It is unlikely that the deinhibition of citrate synthase by the decreasing concentration of long-chain acyl-CoA is counteracted by the simultaneous drop in the concentration of acetyl-CoA. Although glucose refeeding reduced the acetyl-CoA level from 1200 to 420 \mu M after 120 min, even the latter value is approximately 25 times above the \( K_m \) of rat liver citrate synthase for acetyl-CoA: 10–16 \mu M (53, 54).

Purified citrate synthase from rat liver and beef heart has been shown recently to be inhibited effectively by succinyl-CoA (54), suggesting a feedback control of the initial step of the citric acid cycle. It appears, however, that the physiological significance of succinyl-CoA as a regulator of citrate synthesis is questionable under the present experimental conditions, since no alteration in its concentration could be detected.

In addition to the increase in citrate synthesis, the conversion of isocitrate to \( \alpha \)-ketoglutarate should coordinately be stimulated. As has been demonstrated recently in isolated rat liver mitochondria, inhibition of isocitrate oxidation after the addition of palmitoyl-CoA was correlated closely with the increase in the intramitochondrial NADH/NAD ratio (55), probably due to the high sensitivity of NAD-dependent isocitrate dehydrogenase toward NADH (\( K_i = 0.02 \text{ mM} \)) (55, 56). Accordingly, after glucose refeeding, the activity of the enzyme should increase as a consequence of the observed decline in the mitochondrial NADH/NAD ratio (cf. Fig. 6). Moreover, isocitrate dehydrogenase activity should be elevated as a consequence of the decrease in the mitochondrial ATP/ADP ratio, as can be derived from the previous observation that the enzyme is inhibited effectively by ATP and activated by ADP (57).

Finally, it has been demonstrated in rat liver mitochondria that flux through \( \alpha \)-ketoglutarate dehydrogenase, as measured by \( \alpha \)-ketoglutarate uptake, varied inversely with the succinyl-CoA/CoA ratio at different intramitochondrial NADH concentrations (58). The proposed physiological significance of this finding is supported by our present data: After glucose refeeding, the previously elevated succinyl-CoA/CoA ratio rapidly declined from 15.1 to 5.6 after 120 min (cf. Fig. 9).

The decrease in the succinyl-CoA/CoA ratio is not unexpected, assuming an equilibrium of the succinate thiokinase reaction as defined by the equation

\[
\frac{[\text{GTP}]}{[\text{GDP} \times [P]]} = \frac{[\text{succinyl-CoA}]}{[\text{succinate}] 	imes [\text{CoA}] \times \frac{1}{K}}.
\]

Following glucose refeeding, in this equation, the ratio GTP/GDP \times P should decrease parallel to the decrease in the mitochondrial ATP/ADP \times P ratio, since both the ATP/ADP and the GTP/GDP couples are interlinked through the catalytic action of nucleoside diphosphate kinase (58). Consequently, the succinyl-CoA/succinate \times CoA ratio and, thereby, the succinyl-CoA/CoA ratio should diminish coordinately. In fact, both were actually observed (cf. Fig. 9).

**Regulation of the transport of reducing equivalents across the mitochondrial membrane.** During starvation, the increased rate of gluconeogenesis is associated with an increased transport of reducing equivalents from the mitochondria to the cytoplasm. Vice versa, hydrogen transport is channeled into the opposite direction by glucose refeeding. Recent results obtained with isolated rat liver mitochondria reveal that a major part of the hydrogen is translocated as malate in exchange for \( \alpha \)-ketoglutarate within the malate-aspartate shuttle. In addition, it has been demonstrated that the direction and the rate of transport of these anions depend on both the concentration of the anion on either side of the mitochondrial membrane and the apparent \( K_m \) of the specific translocator (for a review, see Ref. 11). The suggested physiological significance of these findings is supported by the results depicted in Fig. 11. After glucose

\(^{1}\) Cf. Footnote 2.
refeeding, when hydrogen is transported from the cytoplasm into the mitochondria, the molar ratio of cytoplasmic to mitochondrial malate declined from about 4 to about 1 within 20 min, while the molar ratio of \(\alpha\)-ketoglutarate increased simultaneously from about 1.5 to about 6. Moreover, the concentration of mitochondrial \(\alpha\)-ketoglutarate was observed to change within the range of the apparent \(K_m\) of the appropriate translocator system, 1.5 mM (59).

Another shuttle for the transfer of reducing equivalents from cytoplasm to mitochondria has been proposed under lipo-}

FIG. 11. Concentration of the reactants in the malate/\(\alpha\)-ketoglutarate translocator system and in the malate/citrate shuttle on both sides of the mitochondrial membrane after glucose refeeding. The values were calculated as described under Materials and Methods from the measured data depicted in Fig. 9. The content of water in rat liver cytoplasm and mitochondria was taken from Soboll et al. (42).
chain acyl-CoA, dropped within minutes; (ii) the level of intramitochondrial citrate was elevated rapidly from about the apparent $K_m$ of the citrate transporter, 220 $\mu$M (62), to about the saturation concentration (cf. Fig. 11); (iii) the molar ratio of cytoplasmic to mitochondrial malate declined coordinately from about 4 to about 1 (cf. Fig. 11).

Regulation of the cytoplasmic NAD system by the adenine nucleotide phosphate potential. As derived above, the adenine nucleotide phosphate potential in the cytoplasmic compartment was gradually reduced during starvation and rapidly reversed after glucose refeeding. One major implication of a change in the cytoplasmic ATP/ADP $\times$ P ratio has been pointed out by Krebs and Veech (36). These authors demonstrated that in rat liver any shift in the cytoplasmic phosphate potential induced either by feeding different diets for several days (35) or by treatment with ethionine or carboxyl cyanide $p$-trifluoromethoxyphenylhydrazone (63) was followed by an inverse shift in the cytoplasmic NAD system. The observed relationship between both parameters was interpreted as a consequence of the equilibrium of the combined glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase reaction.

Applying the hypothesis of Krebs to our experimental conditions, one could expect that, during the drastic switching over of hepatic glucose intermediary metabolism after glucose refeeding, a moment-to-moment correlation between the ATP/ADP $\times$ P ratio and the cytoplasmic NAD couple, lactate/pyruvate, should be observed. In fact, this has been verified by our data.

Stimulation of fatty acid biosynthesis. Switching over of hepatic intermediary metabolism induced by glucose refeeding involves the rapid onset of fatty acid biosynthesis from carbohydrate precursors (64). The overall process is believed to be stimulated by activation of acetyl-CoA carboxylase, by the increase in citrate supply, and by an accelerated generation of NADPH in the pentose phosphate shunt. All three types of regulation are consistent with the changes in the metabolite concentration observed under our experimental conditions. As mentioned above, after glucose refeeding the intrahepatic level of long-chain acyl-CoA, an established inhibitor of acetyl-CoA carboxylase (65), rapidly declined. In addition, the enzyme should be activated by citrate. At high insulin/glucagon ratios the enzyme probably exists predominantly in its activated a-form (66). Since the $K_c$ of the latter for citrate (0.2 mM) is about one-tenth below that of the b-form (67), it could be suggested that activation by citrate should occur in response to an increase in the insulin/glucagon ratio, although the concentration of citrate in the cytoplasmic compartment remained unaltered.

Increase of acetyl-CoA supply after glucose refeeding as a consequence of long-chain acyl-CoA-mediated stimulation of the malate–citrate shuttle has been pointed out already.

Finally, accelerated generation of NADPH in the pentose phosphate shunt can be concluded from the decrease in the cytoplasmic NADPH/NADP ratio from 350 to 40, since this in vivo change has been demonstrated in vitro to deinhibit both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and, thereby, to increase the catalytic capacity of these enzymes by a factor of 30 (68).

Regulation of the final gluconeogenetic pathway. Refeeding glucose to starved rats resulted in a rapid switching off of hepatic gluconeogenesis, as can be derived from the reduction of nitrogen excretion in the urine. Analogous results have been published recently: Glucose refeeding diminished the incorporation in vivo of $^{14}$Cpyruvate into blood glucose by about 80% within 1 h (69). The switching over of hepatic carbohydrate metabolism from glucose production to glucose utilization within minutes is also reflected by the drastic changes in the metabolite pattern of the Embden–Meyerhof pathway (cf. Fig. 10).

As for the mechanism of the rapid regulation of the Embden–Meyerhof pathway in the liver, it has been shown by Taunton et al. (70) that insulin in vivo produced an immediate increase in the activity of
rat liver phosphofructokinase and pyruvate kinase and a coordinate decrease in fructose 1,6-diphosphatase activity, whereas glucagon produced an equally rapid but reciprocal response of these enzymes. The physiological significance of these findings appears, however, to be questionable, since in our experiments the activities of the gluconeogenic or glycolytic key enzymes were unchanged 5, 10, 30, and 60 min after glucose refeeding, despite the drastic increase in the insulin/glucagon ratio.

Besides the unexplained activation/deactivation mechanism proposed by Taunton et al., acceleration of the hepatic metabolite flux through the Embden-Meyerhof pathway during starvation and its rapid reversal after glucose refeeding have been ascribed to the deactivation/inhibition of gluconeogenetic and a coordinate activation/deinhibition of glycolytic key regulatory enzymes, brought about by changes in the concentration of a multiplicity of intermediates acting as effectors. However, the results summarized in Table II question the physiological significance of this concept: In the cytoplasmic compartment, the concentration of none of the relevant effector intermediates was altered in the direction expected, with the exception of fructose 6-phosphate.

Finally, regulation of the gluconeogenic pathway by cyclic AMP has to be considered. Stimulation of hepatic glucose production by cyclic AMP and hormones known to elevate the tissue level of the second messenger, e.g., glucagon and catecholamines, has been demonstrated in different isolated liver systems (for a review, see Ref. 72). Vice versa, the stimulating action of glucagon is counteracted by insulin, the latter hormone reducing both the hepatic cyclic AMP concentration and the glucagon- or cyclic AMP-induced stimulation of gluconeogenesis. It would therefore be reasonable to assume that under in vivo conditions the gradual decline of the insulin/glucagon ratio during starvation and its drastic increase after glucose refeeding are followed by coordinate inverse changes in hepatic cyclic AMP concentrations. In fact, this has been confirmed by our present results, suggesting the physiological significance of the cyclic AMP-mediated control of the Embden-Meyerhof pathway in the liver.

Synopsis. Considered as a whole, our present data offer a concept of the moment-to-moment control of hepatic intermediary glucose metabolism: Any change in the glucose availability brings about a response of the endocrine pancreas, immediately changing the insulin/glucagon ratio.

### Table II

<table>
<thead>
<tr>
<th>Effector metabolite</th>
<th>Regulatory function</th>
<th>Change in effector concentration after glucose refeeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Inhibitor of phosphofructokinase</td>
<td>↓</td>
</tr>
<tr>
<td>AMP</td>
<td>Inhibitor of fructose 1,6-diphosphatase</td>
<td>↑</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>Activator of phosphofructokinase</td>
<td>↑</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>Activator of pyruvate kinase</td>
<td>↑</td>
</tr>
<tr>
<td>Citrate</td>
<td>Inhibitor of phosphofructokinase</td>
<td>↓</td>
</tr>
<tr>
<td>Alanine</td>
<td>Inhibitor of pyruvate kinase</td>
<td>↓</td>
</tr>
</tbody>
</table>

* Values were considered only when the effector action was established for rat liver and when the hepatic effector concentration was within the range of the respective $K_i$ or $K$.  

* For a review, see Ref. 71.
ratio in the blood. In the liver, the latter controls the level of long-chain acyl-CoA—secondary to the alteration in free fatty acid release from adipose tissue—as well as the concentration of cyclic AMP, both of which regulate the major pathways of hepatic intermediary glucose metabolism in an interlinked fashion as described above.

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REFERENCES