# The disposition of carbohydrate between glycogenesis, lipogenesis and oxidation in liver during the starved-to-fed transition

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A comparison was made between the time courses of restoration of pyruvate dehydrogenase activities, fructose 2,6-bisphosphate concentrations and lipogenic rates, together with net hepatic glucose flux and glycogen synthesis/deposition in livers of 48 h-starved rats provided with laboratory chow *ad libitum* for up to 24 h. Increased glycogenesis, lipogenesis and net glucose uptake were observed after 1 h of re-feeding, preceding re-activation of pyruvate dehydrogenase, which occurred after 3–4 h. Increased concentrations of fructose 2,6-bisphosphate were only observed after 5–6 h. The implication of the temporal relationship between these parameters is discussed.

## INTRODUCTION

Long-term adaptations of the liver to starvation include decreases in the maximal activities of glucokinase, acetyl-CoA carboxylase and fatty acid synthase, whereas the activities of overt carnitine palmitoyltransferase (CPT 1) and phosphoenolpyruvate carboxykinase are increased. Consequently the predominant direction of carbon flux is towards gluconeogenesis rather than glycolysis, and to fat oxidation rather than synthesis. This pattern continues to be observed immediately after re-feeding, except that the gluconeogenic product (glucose 6-phosphate) is directed towards glycogen synthesis, not glucose output [1–5]. The maintenance of the metabolic profile is facilitated by a failure to restore fructose 2,6-bisphosphate (Fru-2,6-$P_2$) and malonyl-CoA concentrations [6,7], continued inactivation of hepatic pyruvate dehydrogenase (PDH) [8], increased CPT 1 activity [3] and decreased sensitivity of CPT 1 to inhibition by malonyl-CoA [3].

Marchington *et al.* [9] demonstrated partial re-activation of hepatic PDH in response to insulin after 2 h of hepatocyte culture. *In vivo*, in an investigation of the effects of experimental hyperthyroidism on the hepatic response to re-feeding, we observed re-activation of PDH in the euthyroid controls by 4 h after chow administration [4]. It might be expected that re-activation of hepatic PDH after re-feeding would be coupled to increased glycolysis, and would result in increased lipogenesis. However, it has been suggested [7,10] that the switch from gluconeogenesis to glycolysis is observed only when glycogen repletion is essentially complete and Fru 2,6-$P_2$ concentrations exceed a critical value. This occurs at approx. 5 h after the provision of chow [7]. Furthermore, malonyl-CoA concentrations recover only slowly after re-feeding [6], suggesting a restriction on lipogenesis. Consequently PDH re-activation may occur independently of changes in glycolytic rate, and factors other than PDH activity may limit lipogenesis. Measurement of these parameters has previously been performed in separate studies and by independent groups. Consequently the temporal sequence of events remains equivocal, and firm conclusions cannot be made. We have therefore compared the time courses of restoration of hepatic PDH activities with Fru-2,6-$P_2$ concentrations and rates of lipogenesis, glycogen synthesis and glycogen deposition in the same experiment. The results indicate that increases in glycogenesis and lipogenesis occur concomitantly and precede re-activation of PDH, whereas repletion of Fru-2,6-$P_2$ occurs only as a relatively late event.

## MATERIALS AND METHODS

### Materials

Sources of materials were as in [1]. Standard laboratory chow was purchased from E. Dixon and Sons (Ware) Ltd., Ware, Herts., U.K. and contained 52% digestible carbohydrate.

### Methods

**Treatment of rats.** Female albino Wistar rats (180–220 g) were starved for 48 h (in grid-bottomed cages) before use. Experiments were started between 08:30 and 09:30 h, when chow was provided *ad libitum*. Rats were sampled at intervals from 1 h to 24 h after the provision of chow, after anaesthesia with sodium pentobarbital (5 min; 6 mg/100 g body wt.). Chow intakes are shown in Table 1. $^3H_2O$ was administered by intraperitoneal injection at 1 h before sampling. In some experiments, (−)-hydroxycitrate (0.85 μmol/100 g body wt. in 0.15 M-NaCl, pH 7.4) was given at the time of administration of $^3H_2O$ (see [11] for details).

**Lipogenesis and glycogen synthesis.** Rates of lipogenesis and glycogen synthesis were estimated as $^3H$ incorporation from $^3H_2O$ into tissue saponifiable fatty acid or glycogen respectively [12,13].

The incorporation of $^3H$ from $^3H_2O$ into glycogen

Abbreviations used: CPT 1, overt carnitine palmitoyltransferase; Fru-2,6-$P_2$, fructose 2,6-bisphosphate; PDH, pyruvate dehydrogenase.

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**Table 1. Chow intakes after the provision of chow *ad libitum* to 48 h-starved rats**

For experimental details see the text. Results are means ± S.E.M. for five to seven rats.

| Time after chow administration (h) | Chow intake (g/100 g body wt.) |
|---|---|
| 1 | 2.31 ± 0.65 |
| 2 | 2.91 ± 0.49 |
| 3 | 3.03 ± 0.49 |
| 4 | 4.05 ± 0.34 |
| 5 | 4.31 ± 0.44 |
| 6 | 5.49 ± 0.58 |
| 7 | 5.72 ± 0.46 |
| 8 | 6.04 ± 0.36 |
| 24 | 14.24 ± 1.13 |

occurs at the hexose phosphate level. There is no intracellular compartmentation of $^3H_2O$, and the method has been validated at high glycogen concentrations [13]. $^3H_2O$ structurally bound to glycogen was removed by extraction of freeze-clamped liver (< 200 mg) in 0.3 ml of 30% (w/v) KOH (5 min, 100 °C), precipitation of glycogen (overnight, 2 °C) with 1 ml of ethanol containing a drop of satd. $Na_2SO_4$, and washing of the precipitate with ethanol (twice) and acetone (once). After drying at 90 °C, the glycogen was digested with amyloglucosidase in 20 mM-sodium acetate buffer, pH 4.8, and freeze-dried.

**Enzyme assays.** PDH complex (active form, $PDH_a$) and citrate synthase activities were measured in freeze-clamped liver extracts (see [14]). $PDH_a$ activities are expressed relative to citrate synthase, to correct for possible differences in mitochondrial extraction [4,8,14]. A unit of enzyme activity converts 1 μmol of substrate into product/min at 30 °C.

### Metabolite assays

Glucose, lactate and pyruvate were determined in KOH-neutralized $HClO_4$ extracts of blood sampled from the hepatic portal and the hepatic veins. Concentrations of non-esterified fatty acids were measured in arterial plasma. Glycogen was determined in extracts of freeze-clamped liver (see [15] for details of methods). Fru-2,6-$P_2$ was measured by activation of 6-phosphofructo-1-kinase as described by Richards & Uyeda [16], after its extraction from freeze-clamped liver as described by Hue *et al.* [17].

### Statistics

Statistical significance of differences was assessed by Student's unpaired *t* test. Results are means ± S.E.M. for the numbers of rats (*n*) indicated.

## RESULTS

### Blood metabolite concentrations after chow re-feeding

Results are shown in Fig. 1. Increases in portal glucose concentrations after chow re-feeding were associated with a decline in concentrations of non-esterified fatty acids, indicating inhibition of lipolysis. Both concentration changes occurred within 1 h of the provision of

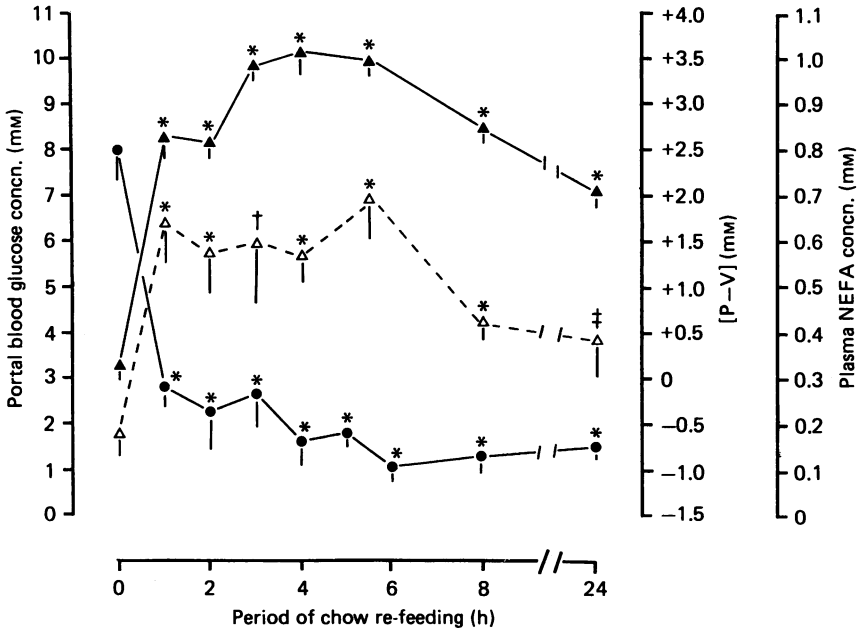


**Fig. 1. Blood glucose and fatty acid concentrations after chow re-feeding**

Chow was provided *ad libitum* to 48 h-starved rats, which were sampled at the times indicated. Glucose (▲) was assayed in portal blood. Concentrations of non-esterified fatty acids (NEFA; ●) were measured in arterial blood. Portal minus hepatovenous glucose concentration differences ([P−V]) are also shown (△), a positive value of [P−V] indicating net hepatic uptake. Results are shown as means ± S.E.M. for a minimum of six rats. Statistically significant effects of re-feeding are indicated by: †$P < 0.05$; ‡$P < 0.01$; *$P < 0.001$.

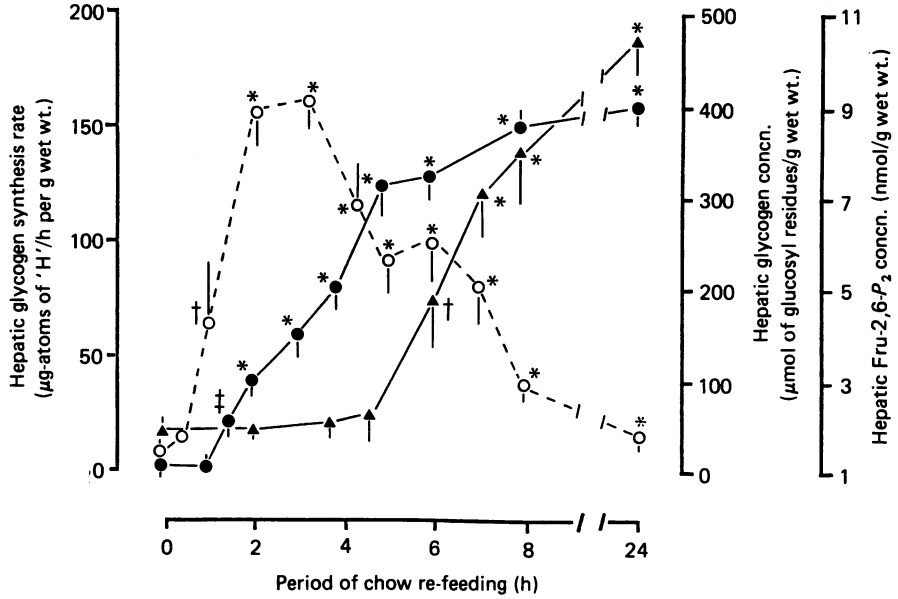


**Fig. 2. Hepatic glycogen synthesis and deposition and Fru-2,6-$P_2$ concentrations after chow re-feeding**

Glycogen synthesis rates (○) and glycogen (●) and Fru-2,6-$P_2$ (▲) concentrations were measured as described in the Materials and methods section. Results are shown as means ± S.E.M. for a minimum of three rats. Statistically significant effects of re-feeding are indicated by: †$P < 0.05$; ‡$P < 0.01$; *$P < 0.001$.

chow. Elevated portal glucose concentrations were maintained for at least 6 h. Measurement of portal minus hepatovenous concentration differences ([P−V]) indicated a reversal of hepatic glucose flux, with a switch from net output towards net uptake. Positive values of [P−V] were observed from 1 h after the provision of chow and thereafter. The decline in [P−V] observed after 6 h paralleled decreased portal glycaemia, hepatovenous concentrations remaining constant (results not shown). This contrasts with the variation in the value of [P−V] observed after the administration of a glucose load, which is occasioned by an increased hepatovenous concentration [18] and which can be suppressed by inhibition of gluconeogenesis [5].

### Hepatic glycogen synthesis and Fru-2,6-$P_2$ concentrations after chow re-feeding

Results are shown in Fig. 2. Glycogen synthesis rates, estimated by the incorporation of $^3H$ from $^3H_2O$ into glycogen, rapidly increased after 1 h, were maximal between 2 h and 3 h, and subsequently decreased. A similar pattern of $^3H$ incorporation into glycogen has been observed previously in response to meal-feeding in the absence of prolonged starvation [13]. In this latter study, the combination of a low rate of $^3H$ incorporation into glycogen with a high glycogen concentration was interpreted as a low rate of glycogen turnover. In the present work, $^3H$ incorporation was approximately paralleled by glycogen deposition only during the first 4 h after re-feeding. During this period up to 80% of hepatic glycogen is synthesized from $C_3$ derivatives of glucose via the gluconeogenic pathway [12]. However, the remainder of the glycogen would be expected to be formed from glucose directly (for review see [19]). This may account, at least in part, for the net hepatic glucose uptake observed during this initial period. The additional possibility of glucose utilization via glycolysis in the perivenous zone of the liver has been discussed elsewhere [19,20].

Kuwajima *et al.* [7] originally observed that increases in hepatic Fru-2,6-$P_2$ concentrations after chow re-feeding occurred only after glycogen stores were largely repleted. Essentially similar results were obtained in the present study, where a longer period of starvation (48 h) before re-feeding was utilized. Increases in Fru-2,6-$P_2$ were not evident for at least 4 h after re-feeding. A 3-fold increase was observed by 6 h, with further increases between 6 h and 24 h.

Recently the importance of a low Fru-2,6-$P_2$ concentration for the maintenance of gluconeogenic flux *in vivo* has been questioned [21]. A high Fru-2,6-$P_2$ concentration (12 nmol/g) was observed within 1 h of the administration of sucrose, yet glycogen synthesis occurred via the gluconeogenic pathway (for review, see [22]). We observed significant increases in concentrations of both Fru-2,6-$P_2$ and glycogen between 4 h and 8 h after chow re-feeding (Fig. 2). However, in liver, glycolytic flux from glucose is negligible at a Fru-2,6-$P_2$ concentration of $< 5$ nmol/g [10]. This concentration was reached only after approx. 80% of the maximum glycogen concentration had been achieved (Fig. 2).

Although we have no direct measurements of glycolysis in our system, it is expected, on the basis of Fru-2,6-$P_2$ concentrations, that significant glycolytic flux would be observed from 6 h. Interestingly, this period coincides with a marked decline in $^3H$ incorporation from $^3H_2O$ into glycogen. $^3H$ incorporation from $^3H_2O$ into glycogen at a given rate of glycogen deposition decreases if glucose rather than pyruvate is used as precursor [21]. In view of the finding that the culture of hepatocytes from starved rats with glucose for 5 h increases the direct utilization of glucose as glycogenic precursor [23], it would seem reasonable to suggest that there is increased direct utilization of glucose for glycogen synthesis after 6 h of

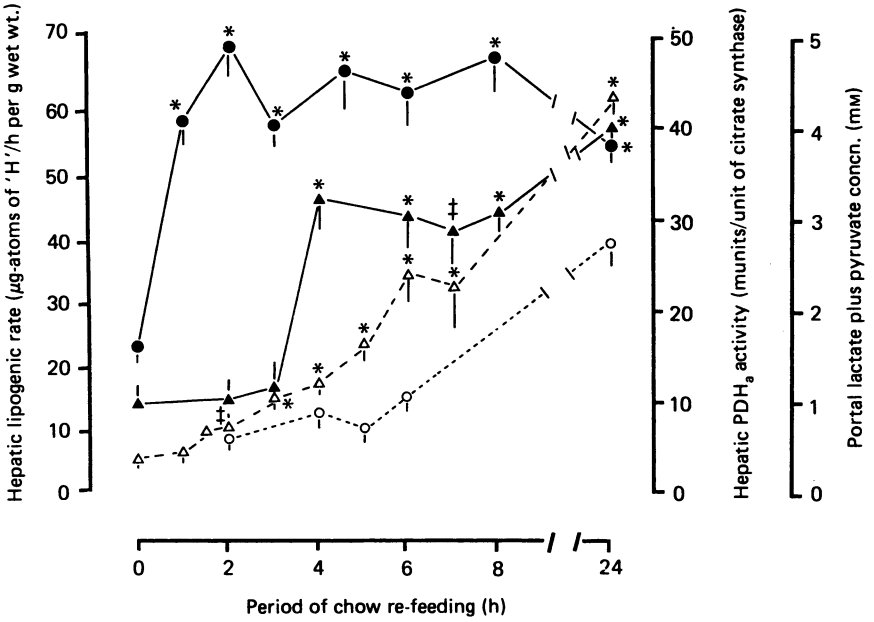


**Fig. 3. Hepatic PDH activities and lipogenic rates after chow re-feeding**

Hepatic PDH$_a$ (▲) and lipogenic rates were measured as described in the Materials and methods section. Lipogenic rates were measured in the absence (△) and the presence (○) of (−)-hydroxycitrate, which was administered at the same time as $^3H_2O$ (i.e. for the 1 h preceding sampling). Portal blood concentrations of lactate plus pyruvate are also shown (●). Results are shown as means ± S.E.M. for a minimum of six rats. Statistically significant effects of re-feeding are indicated by: †$P < 0.05$; ‡$P < 0.01$; *$P < 0.001$. Significant effects of (−)-hydroxycitrate were not observed in rats sampled at 2 h and 4 h, but significance ($P < 0.01$) was achieved at later time points.

re-feeding. This may be permitted by a restoration of glucokinase activities [24]. Notably, despite decreased rates of glycogen deposition, [P − V] for glucose remained positive after 6 h, suggesting that there may be diversion of glucose 6-phosphate towards glycolysis.

### Hepatic PDH activities after chow re-feeding

The culture of hepatocytes from starved rats for 21 h in the presence of a low concentration of glucose (5.5 mM) is not in itself sufficient to elicit PDH re-activation, but the further addition of insulin (1 munit/ml) effects partial re-activation within 1–2 h [9]. Complete reversal of the effects of starvation requires exposure of hepatocytes to insulin at a concentration of 0.1 munit/ml for 4 h [9]. A steady increase in insulin concentration occurs during the first 2 h after chow re-feeding, reaching approx. 0.06 munit/ml at 2 h, a value which is subsequently maintained [21]. In view of the striking resemblance between the time courses of PDH re-activation *in vitro* [9] and *in vivo* in response to chow re-feeding (the present study, and [4]), it is suggested that re-activation of hepatic PDH *in vivo* is mediated primarily via increased plasma insulin.

The increase in PDH activity clearly preceded the rise in Fru-2,6-$P_2$ concentrations (see Figs. 2 and 3), and, although an increased L-pyruvate kinase activity [4,20,25] may permit significant flux from triose phosphate to pyruvate (particularly after 4 h, when phosphoenolpyruvate carboxykinase activity is decreased [26]), glycolysis from glucose 6-phosphate and flux through PDH may nevertheless be uncoupled. Consequently lactate and pyruvate derived from glycolysis in extrahepatic tissues rather than in the liver (but see [20]) may be used as oxidative substrate. This may provide a mechanism for clearance of lactate in the face of diminished glycogenesis from lactate. In the present experiments, portal lactate plus pyruvate concentrations rose rapidly after the provision of chow (Fig. 3; see also [21]), and were approximately constant from 1 to 8 h, during which period positive portal-minus-hepatovenous concentration differences were observed, indicating net uptake (results not shown).

### Hepatic lipogenesis after chow re-feeding

Results are shown in Fig. 3. The provision of a limited amount of carbohydrate fails to elicit increases in lipogenesis within 2 h in previously starved rats [27]. In contrast, small yet significant increases in rates of lipogenesis were observed at 2 h and at 4 h after the provision of chow *ad libitum*. Further increases were observed from 4 to 24 h after re-feeding. Changes in lipogenic rates are paralleled by increases in the concentration of malonyl-CoA (see [6]). This can be taken to indicate greater stimulation of acetyl-CoA carboxylase than of fatty acid synthase in response to re-feeding.

### Lipogenic substrates after chow re-feeding

Hepatic lipogenesis in starved rats is insensitive to inhibition of ATP citrate lyase by (−)-hydroxycitrate [11,28,29]. The increases in lipogenic rates observed between 2 h and 4 h after chow administration to 48 h-starved rats (Fig. 3) were also not susceptible to inhibition by (−)-hydroxycitrate. Thus citrate is not the precursor of the cytoplasmic acetyl-CoA used for lipogenesis during this period. Increased rates of lipogenesis can also be observed within 2 h of the provision of carbohydrate if oxaloacetate utilization for gluconeogenesis is inhibited

[1]. Again, such increases precede re-activation of PDH [30], and citrate is not the lipogenic precursor [31].

The increases in lipogenesis from 4 to 24 h after re-feeding were preceded by PDHa re-activation and were inhibited by (−)-hydroxycitrate. Consequently, it can be concluded that there is a change in the location of the key regulatory step for lipogenesis from downstream to upstream of ATP citrate lyase at approx. 4 h after re-feeding. It is implied that after this time the supply of substrate via ATP citrate lyase assumes regulatory significance.

### Oxidative substrates after re-feeding

This study indicates that the metabolic response to re-feeding falls into two phases. Initially (0–3 h) the pattern of carbon flux resembles that of starvation, and is characterized by high rates of gluconeogenesis, low rates of flux from glucose to triose phosphate, limited pyruvate oxidation and low rates of lipogenesis. During this period the major distinction between the starved and the re-fed state is that glucose 6-phosphate is directed towards glycogen synthesis and glucose output is suppressed. Since Fru-2,6-$P_2$ concentrations (and presumably rates of glycolysis) remain low and PDH is maintained in the inactive (phosphorylated) state, we suggest that the energetic requirements of the liver continue to be met by the utilization of non-carbohydrate substrates. This could be achieved in part through continued mitochondrial oxidation of long-chain acyl-CoA: even though malonyl-CoA concentrations are increased, the maximal activity of CPT-1 remains high and the enzyme remains relatively insensitive to inhibition by malonyl-CoA [3].

Throughout the second phase of re-feeding (from 4 to 8 h after the provision of chow), hepatic carbon flux more closely resembles that found in the fed state, i.e. increased glycolysis and pyruvate oxidation, with a switch in emphasis from high rates of glycogen deposition towards high rates of lipogenesis. Malonyl-CoA concentrations exceed the fed value [6], and some sensitivity of CPT-1 to inhibition by malonyl-CoA is regained [3]. It is envisaged that during this period there is a switch to the utilization of carbohydrate as oxidative substrate.

### Regulation of carbon flux after chow re-feeding

Of the parameters selected for study, only three (suppression of glucose output, incorporation of $^3H$ from $^3H_2O$ into glycogen and lipogenesis) exhibited an immediate response to re-feeding, and one (an increase in Fru-2,6-$P_2$) required at least 5 h to be established. Since starvation leads to protein phosphorylation, the finding of discrete, non-identical, periods of hysteresis for re-activation of these various parameters implies a differential sensitivity or a selective response in terms of enzyme dephosphorylation.

For both glycogenesis and PDH, mechanisms have already been identified which may modulate the response to re-feeding. The binding of glucose to phosphorylase facilitates its dephosphorylation by phosphorylase phosphatase, whereas multi-site phosphorylation retards the dephosphorylation of PDH phosphate by PDH phosphatase. In the case of PDH a further mechanism, a chronic adaptive increase in PDH kinase [9], may retard net dephosphorylation by changing the set point of the kinase/phosphatase cycle. A notable finding from the present study is that a significant increase in lipogenesis can be observed in the absence of changes in Fru-2,6-$P_2$, even though, in view of their cytoplasmic location, both acetyl-CoA carboxylase and 6-phosphofructo-2-kinase may be dephosphorylated by the same ubiquitous phosphatase (2A). Future research will reveal whether this distinction resides in changes in enzyme concentration or susceptibility to dephosphorylation by modulation of phosphoprotein substrate (e.g. by ligand binding, multi-site phosphorylation) or whether additional regulatory factors are involved.

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