Glutamine is a good substrate for glycogen synthesis in isolated hepatocytes from 72 h-starved rats, but not from 24 h- or 48 h-starved rats

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In isolated hepatocytes from 24 h-starved rats, no glycogen synthesis was observed in the presence of glutamine. By contrast, glutamine was the best gluconeogenic substrate to induce glycogen synthesis in isolated hepatocytes from 72 h-starved rats. The effect of glutamine on glycogen synthesis was not accompanied by parallel changes in glucose or lactate production. Glutamine activated glycogen synthase independently of the starvation period; however, the extent of synthase activation was 2-fold higher in isolated hepatocytes from 72 h-starved rats than in hepatocytes from 24 h-starved rats. This increase in synthase activation was associated with increased cell swelling. The rate of glutamine transport was not significantly different in hepatocytes from 24 h- and 72 h-starved rats. By contrast, the intracellular glutamate concentration was 1.5-fold higher after 3 days of starvation in hepatocytes incubated with 5 mM-glutamine. We propose that glutamine may play a key role in the glycogen synthesis observed *in vivo* after 3 days of starvation.

INTRODUCTION

It has been well established 16 years ago that, after the depletion of glycogen during the first 2 days of starvation, glycogen synthesis occurred on day 3 of starvation and a persistent small glycogen content appeared in the liver [1]; this has been confirmed recently [2]. Despite the physiological importance of this phenomenon, little is known about the gluconeogenic capacity and the glycogen synthesis in the liver after 3 days of starvation. In the present work, we have compared the effects of various gluconeogenic precursors on glycogen synthesis in isolated hepatocytes from 24 h-, 48 h- and 72 h-starved rats. We show that glutamine is the best substrate for glycogen synthesis in isolated hepatocytes from 72 h-starved rats, but not in those from 24 h- and 48 h-starved rats. The specificity and the mechanism of glutamine action on glycogen synthesis are also discussed.

MATERIALS AND METHODS

Radiochemicals (Amersham International) and other biochemical reagents (Sigma or Boehringer Mannheim) were purchased as indicated. Mercaptopicolinate was a gift from Smith Kline & French Research Ltd. (U.K.).

Hepatocytes were prepared as described previously [3] from 24 h-, 48 h- and 72 h-starved male Wistar rats (200–220 g). The cells (usually 50–70 mg wet wt./ml) were shaken (120 strokes/min) in stoppered scintillation vials at 37 °C for the indicated times. In all experiments using cells from 72 h-starved rats, the cells were preincubated for 60 min without added substrates in order to deplete the intracellular glycogen content (no preincubation, $4.17 \pm 0.49 \mu\text{mol/g}$; 60 min preincubation, $0.45 \pm 0.09 \mu\text{mol/g}$; $n = 5$); so hepatocytes from 72 h-starved rats, like those from 24 h- or 48 h-starved rats, were used with a low initial glycogen content. In preliminary experiments, we found that preincubation did not affect either the cell ATP content or their metabolic capacities. The standard incubation medium was a Krebs–Henseleit bicarbonate buffer at pH 7.4 [4]. All media were in equilibrium with a gas phase of O_2/CO_2 (19:1). For the measurement of metabolites, the incubation was

stopped with 0.5 M- HClO_4 . Cell volume was measured as described by Baquet *et al.* [5].

Transport of glutamine was determined as described by Hayes & McGivan [6], by using silicone-layer centrifugation to terminate the transport process [7]. To inhibit exchange of ^{14}C label between glutamine and oxoglutarate, the cells were preincubated for 15 min at 37 °C in the presence of 1 mM-amino-oxyacetate, which inhibits aspartate aminotransferase, before starting transport measurement as indicated in [6]. Intracellular concentrations of metabolites were measured as previously described [8]. In experiments using $[\text{U-}^{14}\text{C}]$ glutamine, the specific radioactivity was 0.196 MBq/ μmol . Glutamate [9], glutamine [10], glucose [11], lactate [12], ammonia [13] and urea [14] were measured enzymically in neutralized extracts. Glycogen, glycogen synthase *a* and *a+b*, and glycogen phosphorylase *a* and *a+b* were measured as indicated in [15].

The results are expressed as means $\pm$ S.E.M. for observations on the indicated number (*n*) of different cell or homogenate preparations. Statistical significance of differences was calculated by Student's *t* test for paired or unpaired data.

RESULTS AND DISCUSSION

Comparison of effects of various gluconeogenic substrates on glucose and lactate production and on glycogen synthesis in isolated hepatocytes from 24 h-, 48 h- and 72 h-starved rats

In isolated hepatocytes from 48 h- and 72 h-starved rats, the order of efficiency for gluconeogenesis was similar to that reported in isolated hepatocytes from 24 h-starved rats, except that glutamine was a better gluconeogenic substrate than alanine in hepatocytes from both 48 h- and 72 h-starved rats (Table 1). The lactate production in the presence of the different substrates was also similar to the values found for isolated hepatocytes from 24 h-starved rats, except for alanine, which produced 3-fold more lactate after 48 h and 72 h starvation than after 24 h.

In isolated hepatocytes from 24 h-starved rats, there was no net glycogen synthesis in the presence of the different gluconeogenic substrates except for dihydroxyacetone. By contrast, a net glycogen synthesis was observed not only with dihydroxy-

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Table 1. Glucose and lactate production and glycogen content in isolated hepatocytes from 24 h-, 48 h- and 72 h-starved rats

Hepatocytes were incubated for 60 min without substrate (control), with 10 mM gluconeogenic substrates or with 20 mM-glucose. The values are means $\pm$ S.E.M. for six cell preparations. *Significantly different ($P < 0.05$) from the control values. †Significantly different from the corresponding values obtained with hepatocytes from 24 h-starved rats. ‡Significantly different from the corresponding values obtained with alanine.

Addition	Starvation ...	Glucose production ($\mu\text{mol}/\text{min per g}$)			Lactate ($\mu\text{mol}/\text{min per g}$)			Glycogen content ($\mu\text{mol}/\text{g}$)		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
—		0.044 $\pm$ 0.005	0.061 $\pm$ 0.011	0.066 $\pm$ 0.006	0.002 $\pm$ 0.002	0.003 $\pm$ 0.001	0.004 $\pm$ 0.002	0.48 $\pm$ 0.29	0.40 $\pm$ 0.04	0.62 $\pm$ 0.07
Alanine		0.271 $\pm$ 0.035*	0.238 $\pm$ 0.017*	0.187 $\pm$ 0.022*†	0.056 $\pm$ 0.015*	0.178 $\pm$ 0.018*†	0.184 $\pm$ 0.020*†	0.67 $\pm$ 0.26	0.69 $\pm$ 0.10	1.09 $\pm$ 0.18*†
Dihydroxyacetone		0.929 $\pm$ 0.058*	0.876 $\pm$ 0.051*	0.844 $\pm$ 0.059*	0.163 $\pm$ 0.020*	0.128 $\pm$ 0.024*	0.154 $\pm$ 0.018*	1.40 $\pm$ 0.40*	1.02 $\pm$ 0.17*	2.10 $\pm$ 0.36*†
Glutamine		0.221 $\pm$ 0.037*	0.267 $\pm$ 0.017*†	0.287 $\pm$ 0.042*†	0.030 $\pm$ 0.009*	0.018 $\pm$ 0.005*	0.021 $\pm$ 0.005*	0.68 $\pm$ 0.30	1.47 $\pm$ 0.38*†	3.27 $\pm$ 0.47*†
Lactate		0.480 $\pm$ 0.048*	0.482 $\pm$ 0.031*	0.557 $\pm$ 0.027*†	—	—	—	1.25 $\pm$ 0.050	0.74 $\pm$ 0.10	1.39 $\pm$ 0.27*
Proline		0.195 $\pm$ 0.024*	0.201 $\pm$ 0.015*	0.124 $\pm$ 0.017*†	0.006 $\pm$ 0.002	0.006 $\pm$ 0.002	0.004 $\pm$ 0.002	0.85 $\pm$ 0.26	0.98 $\pm$ 0.21	1.16 $\pm$ 0.21*†
Glucose		—	—	—	—	—	—	4.94 $\pm$ 1.39*	2.63 $\pm$ 0.70*†	2.70 $\pm$ 0.27*†

acetone as substrate but also with glutamine in hepatocytes from both 48 h- and 72 h-starved rats. Moreover, all the other gluconeogenic substrates induced a small but significant glycogen synthesis in isolated hepatocytes from 72 h-starved rats. In these cells, the best precursors of glycogen were, in decreasing order of efficiency, glutamine, dihydroxyacetone, lactate, proline and alanine. Therefore the substrate efficiency for glycogen synthesis was dependent on the duration of starvation. The efficiency of glucose as a precursor of glycogen synthesis decreased as the duration of starvation was prolonged. After 72 h starvation, 10 mM-glutamine was also better than 20 mM-glucose (Table 1). The results showed that glutamine was the best substrate to induce glycogen synthesis in hepatocytes from 72 h-starved rats, but not in hepatocytes from 24 h- and 48 h-starved rats. Moreover, the effect of glutamine on glycogen synthesis after 3 days of starvation was not accompanied by parallel changes in glucose or lactate production.

Glutamine as carbon source for glycogen synthesis in isolated hepatocytes from 72 h-starved rats

With various concentrations of [^{14}C]glutamine, carbon atoms of glutamine were incorporated into glycogen with a low rate in isolated hepatocytes from both 24 h- and 48 h-starved rats, and with a high rate in hepatocytes from 72 h-starved rats (Fig. 1). The high rate of glutamine incorporation into glycogen in isolated hepatocytes from 72 h starved rats cannot be due to the preincubation used in our experiments (see the Materials and methods section), since, firstly, 5 mM-[^{14}C]glutamine was incorporated at the same rate into glycogen in hepatocytes from 72 h-starved rats with or without preincubation (results not shown) and, secondly, preincubation of hepatocytes isolated from 24 h-starved rats does not significantly modify the incorporation rate of [^{14}C]glutamine into glycogen (results not shown). In all these experiments, the incorporation of [^{14}C]glutamine into glycogen increased in parallel with the glycogen synthesis measured with unlabelled glutamine. At 5 mM-glutamine, the incorporation of [^{14}C]glutamine into glycogen and glycogen synthesis measured with unlabelled glutamine were both 8-fold higher in isolated hepatocytes from 72 h-starved rats than in those from 24 h-starved rats, and 4-fold higher than in those from 48 h-starved rats. In hepatocytes from 72 h-starved rats, half-maximal incorporation rate was obtained with about 3 mM-glutamine. As expected, mercaptopicolinate, a known inhibitor of phosphoenolpyruvate carboxykinase, totally inhibited the glycogen synthesis from 10 mM-glutamine (control, $2.33 \pm 0.60 \mu\text{mol}/\text{h per g}$; + 1 mM-mercaptopicolinate, $-0.10 \pm 0.10 \mu\text{mol}/\text{h per g}$; $n = 5$; 72 h-starved rats).

All the results demonstrated that glutamine by itself was a good substrate for glycogen synthesis only in isolated hepatocytes from 72 h-starved rats.

Glutamine alone activated glycogen synthase in isolated hepatocytes from 24 h- and 72 h-starved rats

As shown in Figs. 2(a) and 2(b), glutamine activated glycogen synthase whatever the duration of starvation without any changes in total synthase activity. However, the activation was 2-fold larger in hepatocytes from 72 h-starved rats (Fig. 2b) than in those from 24 h-starved rats at both 5 mM- and 10 mM-glutamine (Fig. 2a). Moreover, synthase α activity significantly increased with 2 mM-glutamine in hepatocytes from 72 h-starved rats, but not in those from 24 h-starved rats.

Glutamine did not significantly affect total phosphorylase activity (Figs. 2c and 2d). However, by contrast with the results observed for synthase activation, glutamine was more effective to activate phosphorylase in hepatocytes from 24 h-starved rats

Fig. 1. Incorporation of $[U-^{14}C]$ glutamine into glycogen and comparison with glycogen synthesis in isolated hepatocytes from (a) 24 h-, (b) 48 h- and (c) 72 h-starved rats

Hepatocytes were incubated for 60 min with various concentrations of $[U-^{14}C]$ glutamine (○) or unlabelled glutamine (●). The values are means $\pm$ S.E.M. for five cell preparations. *Significantly different ($P < 0.05$) from the values obtained without glutamine. †Significantly different ($P < 0.05$) from the values obtained with hepatocytes from both 24 h- and 48 h-starved rats.

Fig. 2. Influence of various glutamine concentrations on glycogen synthase and phosphorylase activities in isolated hepatocytes from 24 h- and 72 h-starved rats

Hepatocytes were incubated for 45 min. The values are means $\pm$ S.E.M. for five cell preparations. (a) and (c) 24 h-starved rats; (b) and (d), 72 h-starved rats. ●, *a* activities; ○, *a* + *b* activities. *Significantly different ($P < 0.05$) from the values obtained without glutamine.

(Fig. 2c) than in hepatocytes from 72 h-starved rats (Fig. 2d). Moreover, 5 mM-glutamine activated phosphorylase in hepatocytes from 24 h-starved rats, but not in hepatocytes from 72 h-starved rats. These two phenomena, strong activation of glycogen synthase and absence or small activation of glycogen phosphorylase in hepatocytes from 72 h-starved rats, may contribute to the high rate of glutamine incorporation into glycogen in such hepatocytes. Moreover, it has been proposed that

Fig. 3. Time course of glycogen synthase activation in isolated hepatocytes from 72 h-starved rats

Hepatocytes were incubated without added substrate (□) or with 10 mM-dihydroxyacetone (○) or 10 mM-glutamine (●) for the indicated periods of time. The values are means $\pm$ S.E.M. for five cell preparations. *Significantly different ($P < 0.05$) from the corresponding control values.

glycogen depletion triggered a potential substrate cycling at the level of glycogen/glucose 1-phosphate in the liver of 24 h-starved rats [16,17]. The observed activation of phosphorylase by 5 mM-glutamine in hepatocytes from 24 h-starved rats, but not in those from 72 h-starved rats, may favour such cycling, and thus contribute to the low rate of glutamine incorporation into glycogen in hepatocytes from 24 h-starved rats.

Mechanism by which glutamine activated glycogen synthase

As shown in Fig. 3, a lag period was apparent for the activation of glycogen synthase in the presence of glutamine in hepatocytes from 72 h-starved rats, as described in hepatocytes from 24 h-starved rats [15]. Moreover, like the results obtained in hepatocytes from 24 h-starved rats, dihydroxyacetone activated glycogen synthase in the first minutes of the incubation period. Furthermore, the effects of glucose on both glycogen synthase and glycogen phosphorylase activities were similar in isolated hepatocytes from 24 h- and 72 h-starved rats (Fig. 4). Finally, all

Fig. 4. Influence of various glucose concentrations on glycogen synthase *a* and phosphorylase *a* activities in isolated hepatocytes from (a) 24 h- and (b) 72 h-starved rats

Hepatocytes were incubated for 45 min. The values are means for two cell preparations. ○, Synthase *a*; ●, phosphorylase *a*.

the results suggested a similar regulation of both glycogen synthase and glycogen phosphorylase in the presence of various substrates in hepatocytes from both 24 h- and 72 h-starved rats. Thus the fact that glutamine was better able to activate glycogen synthase in hepatocytes from 72 h-starved rats than in those from 24 h-starved rats seemed not to be related to a different regulation of glycogen metabolism after 3 days of starvation.

It has been recently proposed that cell swelling may contribute to the activation of glycogen synthase by glutamine in hepatocytes from 16 h-starved rats [5,18]. Therefore we have compared the effect of glutamine on cell swelling in isolated hepatocytes from 72 h- and 24 h-starved rats. As shown in Fig. 5, glutamine was better able to induce cell swelling in isolated hepatocytes from 72 h-starved rats than in those from 24 h-starved rats. In the presence of various concentrations of glutamine, a similar relationship between the increase in cell volume and the increase in glycogen synthase activity was observed with hepatocytes from both 24- and 72 h-starved rats (Fig. 6). Moreover, the synthase activation induced by 5 mM-glutamine persisted in the presence of 1 mM-mercaptopycolinate in hepatocytes from both 24 h-starved (glutamine, +25.5±6.6%; glutamine+mercaptopycolinate, +19.6±8.0%; *n*=3) and 72 h-starved (glutamine, +97.6±11.2%; glutamine+mercaptopycolinate, +83.7±7.1%; *n*=3) rats. Therefore, a similar mechanism seemed to be involved in the activation of glycogen synthase by glutamine in hepatocytes from both 24 h- and 72 h-starved rats. Such results also suggested that the greater extent of cell swelling after 3 days of starvation may contribute to the greater extent of synthase activation. Thus we tried to elicit the mechanism by which glutamine was more effective to induce cell swelling in hepatocytes from 72 h-starved rats than in those from 24 h-starved rats.

In the presence of glutamine, cell swelling reflects both the transport of this amino acid through the plasma membrane [19–21] and the accumulation of catabolites inside the cell (mainly glutamate). As shown in Table 2, there was no significant difference in the rate of glutamine transport in hepatocytes from 24 h- and 72 h-starved rats. The intracellular concentration of glutamine was also not significantly different in hepatocytes from 24 h- and 72 h-starved rats incubated in the presence of 5 mM-glutamine (24 h, 3.82±0.42 μmol/g; 72 h, 3.03±0.31 μmol/g; *n*=6). By contrast, in the presence of 5 mM-glutamine, the intracellular concentration of glutamate was 1.5-fold greater in hepatocytes from 72 h-starved rats than in those from 24 h-starved rats (24 h, 3.91±0.38 μmol/g; 72 h, 6.00±0.99 μmol/g; *P*<0.05; *n*=6). Moreover, in the presence of 5 mM-glutamine, production of ammonia plus urea was also greater in hepatocytes

Fig. 5. Influence of various glutamine concentrations on cell volume in isolated hepatocytes from 24 h- and 72 h-starved rats

Hepatocytes were incubated for 45 min. The values are means ± S.E.M. for eight cell preparations, except for 2 mM-glutamine (*n*=4): ●, 24 h-starved rats; ○, 72 h-starved rats. *Significantly different from the corresponding control values. †Significantly different from the corresponding values obtained with hepatocytes from 24 h-starved rats.

Fig. 6. Relation between increase in cell volume and increase in synthase activity

Hepatocytes were incubated for 45 min in the presence of 2 mM- or 5 mM-glutamine; isolated hepatocytes from 24 h- (●) and 72 h- (○) starved rats were used. The values are means ± S.E.M. for eight cell preparations.

Table 2. Effect of the duration of starvation on the overall initial rates of transport of glutamine

The initial rate of uptake at 37 °C was measured after 2 min. The values are means ± S.E.M. for four cell preparations. Each rate determination was performed in duplicate.

Glutamine concn. (mM)	Glutamine transport (nmol/min per mg)	
	24 h	72 h
1	0.67 ± 0.08	0.73 ± 0.06
2	0.96 ± 0.10	1.11 ± 0.10
5	1.45 ± 0.14	1.48 ± 0.12

from 72 h-starved rats than in those from 24 h-starved rats (24 h, $34.35 \pm 3.70 \mu\text{mol/h per g}$; 72 h, $55.59 \pm 3.22 \mu\text{mol/h per g}$; $P < 0.05$; $n = 5$). These results (no change in glutamine transport, increased intracellular concentration of glutamate, increased ammonia + urea production) suggested that the increased cell swelling induced by glutamine after 3 days of starvation reflected an increased intracellular metabolism rather than an increased transport of glutamine. Recently, Meijer *et al.* [22] reported that (i) cell swelling acts on glycogen metabolism through a decrease in intracellular Cl^- concentration (Cl^- ions are inhibitors of glycogen synthase phosphatase) and (ii) glutamate stimulates glycogen synthase phosphatase *in vitro*. Thus the increased intracellular glutamate concentration and the increased cell swelling observed after 3 days of starvation may both contribute to the greater extent of synthase activation.

Conclusion

Our results clearly demonstrate that glutamine is a good substrate for glycogen synthesis in hepatocytes from 72 h-starved rats, but not in those from 24 h-starved rats. This phenomenon seems to be related to an increased intracellular metabolism of glutamine after 3 days of starvation, which induces increased cell swelling and, in turn, an increase in the glycogen synthase activation. This increased synthase activation may explain, at least in part, why glutamine is a good substrate for glycogen synthesis in hepatocytes from 72 h-starved rats, without parallel changes in glucose or lactate production. Thus we propose that glutamine may play a key role for the glycogen synthesis observed *in vivo* after 3 days of starvation: firstly, as activator of glycogen synthase; secondly, as a potential substrate for glycogen synthesis.

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