Biochem. J. (1989) 263, 889–895 (Printed in Great Britain)

Pathways of glycogen synthesis from glucose during the glycogenic response to insulin in cultured foetal hepatocytes

Hervé BISMUT and Christiane PLAS
Laboratoire Interactions Cellulaires, U.E.R. Odontologie, Université Paris 7, 2 Place Jussieu, 75005, Paris, France

The pathways of glycogen synthesis from glucose were studied using double-isotope procedures in 18-day cultured foetal-rat hepatocytes in which glycogenesis is strongly stimulated by insulin. When the medium containing 4 mM-glucose was supplemented with [2-^{3}H,U-^{14}C]glucose or [3-^{3}H,U-^{14}C]glucose, the ratios of ^{3}H/^{14}C in glycogen relative to that in glucose were 0.23 ± 0.04 ($n = 6$) and 0.63 ± 0.09 ($n = 8$) respectively after 2 h. This indicates that more than 75% of glucose was first metabolized to fructose 6-phosphate, whereas 40% reached the step of the triose phosphates prior to incorporation into glycogen. The stimulatory effect of 10 nM-insulin on glycogenesis (4-fold) was accompanied by a significant increase in the (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio with ^{3}H in the C-2 position (0.29 ± 0.05, $n = 6$, $P < 0.001$) or in the C-3 position (0.68 ± 0.09, $n = 8$, $P < 0.01$) of glucose, whereas the effect of a 12 mM-glucose load (3.5-fold) did not alter these ratios. Fructose (4 mM) displaced [U-^{14}C]glucose during labelling of glycogen in the presence and absence of insulin by 50 and 20% respectively, and produced under both conditions a similar increase (45%) in the (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio when ^{3}H was in the C-2 position. 3-Mercaptopicolinate (1 mM), an inhibitor of gluconeogenesis from lactate/pyruvate, further decreased the already poor labelling of glycogen from [U-^{14}C]alanine, whereas it increased both glycogen content and incorporation of label from [U-^{14}C]serine and [U-^{14}C]glucose with no effect on the relative ^{3}H/^{14}C ratios in glycogen and glucose with ^{3}H in the C-3 position of glucose. These results indicate that an alternative pathway in addition to direct glucose incorporation is involved in glycogen synthesis in cultured foetal hepatocytes, but that insulin preferentially favours the classical direct route. The alternative foetal pathway does not require gluconeogenesis from pyruvate-derived metabolites, contrary to the situation in the adult liver.

INTRODUCTION

It has been established that glucose diverts gluconeogenic precursors to glycogen formation rather than being directly used itself for glycogen synthesis (Hems *et al.*, 1972; Seglen, 1974; Shikama & Ui, 1978; Boyd *et al.*, 1981). Beyond the importance of gluconeogenesis for glycogen synthesis, studies *in vivo* have shown that the classical direct pathway from glucose, following the reaction sequence: glucose → glucose 6-phosphate → glucose 1-phosphate → UDP-glucose → glycogen (for review, see Hers & Hue, 1983), is not the major pathway in the fasted-to-fed transition (Newgard *et al.*, 1983, 1984; Scofield *et al.*, 1985). Under these conditions, it has been shown that glucose follows an indirect pathway by which it is first metabolized into C_3 compounds prior to incorporation into glycogen. These findings, called the 'glucose paradox', suggest that the formation of pyruvate-derived metabolites is necessary for glycogen synthesis from glucose via the indirect pathway. However, the site of lactate formation, i.e. extrahepatic or hepatic according to the zonation model of liver metabolism, remains to be established (for reviews, see Pilkis *et al.*, 1985; Jungermann, 1987; McGarry *et al.*, 1987).

Foetal liver stores large amounts of glycogen towards the end of gestation. Since lactate/pyruvate is the obligatory intermediate for glycogenesis in the adult liver, the pathways implicated in glycogen synthesis in foetal-rat liver, in which phosphoenolpyruvate carboxykinase is not active when the mother is normally fed (Hanson *et al.*, 1975; Girard, 1986), remain to be elucidated. To account for foetal hepatocyte glycogenesis, one can postulate (1) that there is no 'glucose paradox' in these cells and that they synthesize glycogen by the classical direct pathway or (2) that gluconeogenesis from lactate/pyruvate is not totally inactive, allowing the use of the adult indirect pathway for glycogenesis. Another possibility is that glycogenesis is dependent on gluconeogenesis from precursors which bypass the phosphoenolpyruvate carboxykinase step. In this respect, it has been shown that in cultured foetal-rat hepatocytes, which accumulate glycogen *de novo*, glucose accounts for only 50–60% of glycogen synthesis, which is thus partially achieved from gluconeogenic precursors (Plas *et al.*, 1973, 1979). The purpose of the work described in this paper was to study the pathways involved in the incorporation of glucose into glycogen in such a cultured liver cell system by using double-isotope procedures. Moreover, the responsiveness of these cells to insulin, which produces a large stimulation of glycogen synthesis (Plas & Nunez, 1976; Plas *et al.*, 1979; DeSante *et al.*, 1984), permitted further study of the regulation of the pathways from glucose to glycogen. Our results show that in 18-day foetal hepatocytes, a major part of the glucose used for glycogen synthesis must first be converted to fructose 6-phosphate, while supplementation with insulin or fructose favours the contribution of the direct pathway. Moreover, it appears that one-third of glucose reaches the triose phosphate level before incorporation into glycogen. Finally, data obtained with 3-mercaptopicolinate in-

dicate that an alternative pathway exists in foetal hepatocytes despite the low activity of gluconeogenesis from pyruvate-derived metabolites.

MATERIALS AND METHODS

Materials

Insulin was purchased from Novo Laboratories. D-Glucose, D-fructose, glycerol, L-alanine and L-serine were obtained from Sigma. [U-^{14}C]Glucose, [U-^{14}C]serine and [U-^{14}C]alanine were from New England Nuclear and [2-^{3}H]glucose and [3-^{3}H]glucose were from Amersham, and were used without further purification. The sources of other materials have been specified previously (Plas & Nunez, 1976). 3-Mercaptopicolinate was a gift from Smith Kline & French Laboratories (Philadelphia, PA, U.S.A.).

Culture procedure

Primary cultures of hepatocytes were obtained from 18-day rat foetuses (Sprague–Dawley rats) as described previously (Plas *et al.*, 1973). After mild trypsin treatment, the isolated cells were plated on a collagen substratum to which only the hepatocytes adhered, and after 6 h the non-adhering haematopoietic cells were removed. At this point, the culture medium (1 ml/well) was replaced. This culture medium consisted of NCTC 109 medium (Evans *et al.*, 1964) containing a glucose concentration of 5.5 mM and supplemented with 10 μM-cortisol and 10% (w/v) foetal calf serum. All experiments were performed after 2 days of culture in the presence of cortisol, by which time the glycogenic effect of insulin is fully expressed (Plas & Nunez, 1976). At this time, the glucose concentration in the medium was close to 4 mM, which corresponds to the glycaemia of the rat foetus at the end of gestation.

Glycogen studies

Glycogen content and glycogen labelling were measured as previously described (Plas *et al.*, 1973, 1979). On day 2 of culture, labelled substrates were added together with 10 nM-insulin or its solvent (HCl, 2.5 μM final concentration in the medium). The radioactivity present in glycogen was determined after 2 h of incubation. In the double-labelling experiments, cells from the same dish were exposed to [U-^{14}C]glucose (1 μCi/mg) and to either [2-^{3}H]glucose (6 μCi/mg) or [3-^{3}H]glucose (2 μCi/mg). Incorporation of ^{3}H and ^{14}C into glycogen was measured simultaneously using a Beckman LS 7500 liquid scintillation counter (dual label d.p.m.) with appropriate channel discriminators. The relative ^{3}H/^{14}C ratio was defined as the ratio of the specific radioactivities of ^{3}H and ^{14}C in glycogen divided by the similar ratio for glucose in the medium.

Expression of results

Each protocol involved at least three independent experiments performed on different cell preparations. Data are presented as means ± S.D. for n experiments. For statistical analyses, Student's t test for paired samples was used, with triplicate treated cultures and the corresponding controls for each independent experiment. Each symbol in the Figure corresponds to a different culture. The results are expressed per mg of cell protein. The cell population of a culture well was of the order of 0.6×10^{6} hepatocytes, which corresponds to 230 μg of protein and to 1.60 mg of wet liver. In order to express the response to glycogenic agents, a 'stimulation index' (s.i.) was used, defined as the following ratio: nmol of glucosyl units in glycogen per mg of cell protein in treated cultures divided by nmol of glucosyl units in glycogen per mg of cell protein in control cultures.

RESULTS

Basal and insulin-stimulated glycogen synthesis from [2-^{3}H,U-^{14}C]glucose and from [3-^{3}H,U-^{14}C]glucose

Glucose doubly labelled with U-^{14}C and with ^{3}H in the C-2 or the C-3 position was used in order to estimate the contribution of glycolysis prior to incorporation into glycogen, which is indicated by the relative ratio of ^{3}H/^{14}C in glycogen to that in glucose (Newgard *et al.*, 1983). Indeed, the loss of ^{3}H from [2-^{3}H,U-^{14}C]glucose occurs during the isomerization of glucose 6-phosphate

Table 1. Incorporation of [2-^{3}H,U-^{14}C]glucose and [3-^{3}H,U-^{14}C]glucose into glycogen in the presence or absence of insulin

After 2 days of culture, the medium containing 4 mM-glucose was supplemented with [U-^{14}C]glucose (1 μCi/mg) and [2-^{3}H]glucose (6 μCi/mg) or [3-^{3}H]glucose (2 μCi/mg). At the same time, 10 nM-insulin or its solvent alone (control) was added to the medium. Glycogen content and radioactivity incorporated into glycogen were determined after 2 h of labelling. Results are presented as means ± S.D. for five (glycogen content), six (incorporation from [2-^{3}H,U-^{14}C]glucose) or eight (incorporation from [3-^{3}H,U-^{14}C]glucose) experiments performed with different cell preparations. *$P < 0.01$, **$P < 0.001$ compared with corresponding control in the absence of insulin.

Addition	Glycogen content (nmol of glucose/ mg of protein)	[^{14}C]Glucose incorporation into glycogen (nmol of glucose/ 2 h per mg of protein)	Insulin stimulation index	^{3}H/^{14}C in glycogen / ^{3}H/^{14}C in glucose
[2-^{3}H,U-^{14}C]Glucose				
Solvent	540 ± 275	26.1 ± 12.3		0.23 ± 0.04
Insulin	665 ± 270*	89.4 ± 24.4**	4.05 ± 1.43	0.29 ± 0.05**
[3-^{3}H,U-^{14}C]Glucose				
Solvent		31.7 ± 18.8		0.63 ± 0.09
Insulin		101.1 ± 33.0**	3.87 ± 1.62	0.68 ± 0.09*

Table 2. Effect of a glucose load on the incorporation of [2-^{3}H,U-^{14}C]glucose and [3-^{3}H,U-^{14}C]glucose into glycogen in the presence or absence of insulin

After 2 days of culture, the medium containing 4 mM-glucose was supplemented or not with 12 mM-glucose so that the concentration in the medium was 16 mM. [U-^{14}C]Glucose (1 μCi/mg) and [2-^{3}H]glucose (6 μCi/mg) or [3-^{3}H]glucose (2 μCi/mg) were also added, together with 10 nM-insulin or its solvent. Glycogen content and radioactivity incorporated into glycogen were determined after 2 h of labelling. Results are presented as means ± S.D. for three (glycogen content) or four (^{14}C incorporation) experiments performed with different cell preparations. $*P < 0.02$, $**P < 0.01$ compared with corresponding control in the absence of glucose.

Addition	Glycogen content (nmol of glucose/ mg of protein)	[^{14}C]Glucose incorporation into glycogen (nmol of glucose/ 2 h per mg of protein)	Insulin stimulation index	$\frac{^3H/^{14}C \text{ in glycogen}}{^3H/^{14}C \text{ in glucose}}$
[2-^{3}H,U-^{14}C]Glucose				
Solvent	715 ± 230	32.3 ± 13.1		0.23 ± 0.04
Insulin	810 ± 250	99.9 ± 27.7	3.15 ± 0.43	0.28 ± 0.06
Glucose	825 ± 270*	90.0 ± 21.3**		0.26 ± 0.05
Glucose + insulin	1055 ± 395	186.9 ± 27.7**	2.16 ± 0.20	0.33 ± 0.04
[3-^{3}H,U-^{14}C]Glucose				
Solvent		35.0 ± 17.7		0.62 ± 0.12
Insulin		111.1 ± 38.3	3.47 ± 0.61	0.68 ± 0.12
Glucose		93.7 ± 23.2**		0.63 ± 0.11
Glucose + insulin		211.3 ± 29.4**	2.34 ± 0.39	0.67 ± 0.11

into fructose 6-phosphate, whereas the detritiation from [3-^{3}H,U-^{14}C]glucose occurs at the level of triose phosphate formation (Katz & Rognstad, 1976). After 2 days of culture, when the 2 h-labelling experiments were performed, the hepatocytes already contained glycogen synthesized *de novo* (Table 1). When [2-^{3}H,U-^{14}C]glucose was tested at a concentration of 4 mM, the ratio of $^3H/^{14}C$ in glycogen to $^3H/^{14}C$ in glucose was 0.23. This result suggests that about 75% of the glucose 6-phosphate used for glycogen synthesis was not directly metabolized into glucose 1-phosphate. When [3-^{3}H,U-^{14}C]glucose was used at the same concentration, the ratio of $^3H/^{14}C$ in glycogen to that in glucose was 0.63, indicating that more than 35% of the glucose incorporated into glycogen could have first been metabolized at least up to the level of triose phosphates. In the presence of 10 nM-insulin during the 2 h labelling period, the glycogen content was significantly increased, and the incorporation of ^{14}C from [U-^{14}C]glucose was stimulated 4-fold. At the same time, there was a small but significant increase in the relative $^3H/^{14}C$ ratios in insulin-treated cultures as compared with controls.

Effects of a glucose load on basal and insulin-stimulated glycogen synthesis from double-labelled glucose

A glucose load was added to the medium together with double-labelled glucose so that the concentration of glucose varied from 4 to 16 mM. Under these conditions, a significant increase in the net glycogen content was obtained after 2 h, while incorporation of ^{14}C into glycogen from [U-^{14}C]glucose was stimulated 3.5-fold (Table 2). However, the ($^3H/^{14}C$ in glycogen)/($^3H/^{14}C$ in glucose) ratios were not significantly modified either with [2-^{3}H,U-^{14}C]glucose or with [3-^{3}H,U-^{14}C]glucose, and remained close to 0.25 and 0.63 respectively. When a glucose load and 10 nM-insulin were both added, they produced apparent synergistic effects on glycogenesis. The relative ratios of $^3H/^{14}C$ obtained with ^{3}H either in the C-2 position or the C-3 position were very close to those obtained with insulin alone. Thus it appears that the route of incorporation of glucose into glycogen was not affected by the glycogenic stimulus produced by the glucose load.

Effects of fructose and glycerol on basal and insulin-stimulated glycogen synthesis from double-labelled glucose

Fructose and glycerol, which are known to contribute to glycogen formation in this cell system (Plas *et al.*, 1973; Menuelle *et al.*, 1988), were tested as unlabelled substrates for their capacity to interfere with the pathways from glucose to glycogen. The presence of 4 mM-fructose, which is expected to enter gluconeogenesis at the level of fructose 6-phosphate in foetal hepatocytes (Walker, 1966), decreased the incorporation of [^{14}C]-glucose into glycogen by about 20% during the 2 h of labelling, but did not significantly affect the glycogen content (Table 3). Moreover, the addition of fructose increased the ($^3H/^{14}C$ in glycogen)/($^3H/^{14}C$ in glucose) ratios using [2-^{3}H,U-^{14}C]glucose by more than 45%, reaching a value close to 0.32. When insulin was simultaneously present, the incorporation of glucose into glycogen was decreased by 50%, so that the insulin stimulation index was reduced (2.6 versus 4.4 with or without fructose respectively), showing that fructose appeared to be a better competitor in the presence of insulin. As in the absence of insulin, fructose produced a 45% increase in the relative ratio of $^3H/^{14}C$ in glycogen and glucose when [2-^{3}H,U-^{14}C]glucose was used (0.38 versus 0.26), so that the two agents produced additive effects. By contrast, there was no modification in the relative ratio of $^3H/^{14}C$ by fructose when [3-^{3}H,U-^{14}C]glucose was used, whether insulin was present or not (Table 3). As glycerol enters gluconeogenesis at the level of dihydroxyacetone phosphate (Lin, 1977), one might have expected the presence of glycerol to interfere with

Table 3. Effect of fructose on the incorporation of [2-^{3}H,U-^{14}C]glucose and [3-^{3}H,U-^{14}C]glucose into glycogen in the presence or absence of insulin

The experiment protocol was as described in Table 2, except that the medium was supplemented with 4 mM-fructose instead of 12 mM-glucose. Results are presented as means ± S.D. for four experiments performed with different cell preparations. *$P < 0.02$, **$P < 0.01$ and ***$P < 0.001$ compared with corresponding control in the absence of fructose.

Addition	Glycogen content (nmol of glucose/ mg of protein)	[^{14}C]Glucose incorporation into glycogen (nmol of glucose/ 2 h per mg of protein)	Insulin stimulation index	^{3}H/^{14}C in glycogen / ^{3}H/^{14}C in glucose
[2-^{3}H,U-^{14}C]Glucose				
Solvent	595±285	22.5±12.4		0.22±0.03
Insulin	700±295	82.8±24.8	4.43±1.61	0.26±0.02
Fructose	610±240	19.0±10.1**		0.32±0.08*
Fructose+insulin	850±370	42.5±16.3***	2.50±0.54	0.38±0.05***
[3-^{3}H,U-^{14}C]Glucose				
Solvent		24.2±11.4		0.58±0.11
Insulin		89.4±21.5	4.44±2.01	0.65±0.11
Fructose		20.5±11.0**		0.57±0.12
Fructose+insulin		45.8±12.8***	2.67±0.92	0.63±0.10

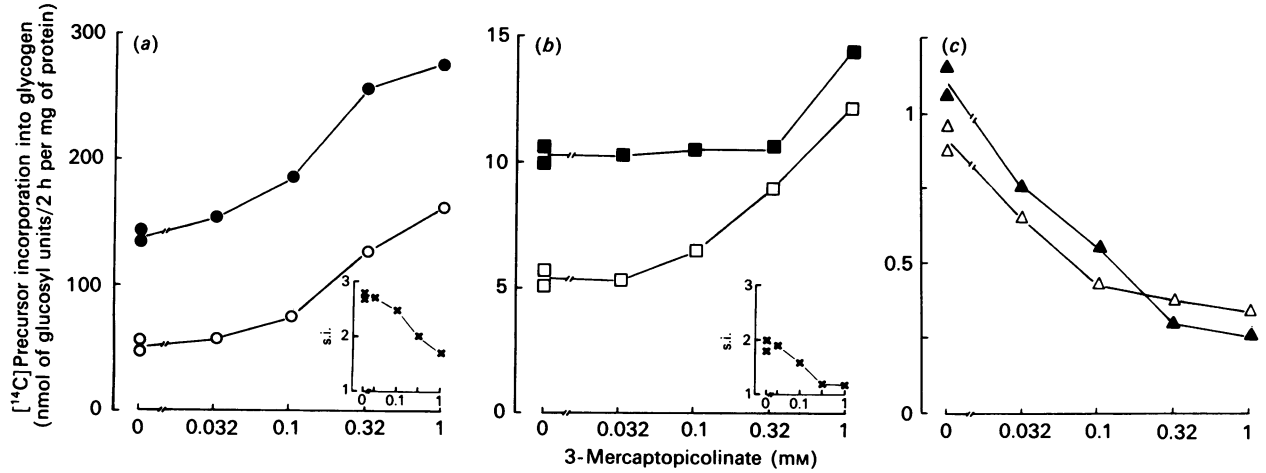

Fig. 1. Effect of 3-mercaptopicolinate on glycogen synthesis from alanine, serine and glucose in the presence or absence of insulin

After 2 days of culture, the medium containing 4 mM-glucose was supplemented with (*a*) [U-^{14}C]glucose (1 μCi/mg), (*b*) 1 mM-[U-^{14}C]serine (10 μCi/mg) or (*c*) 1 mM-[U-^{14}C]alanine (11 μCi/mg) together with 10 nM-insulin or its solvent. At the same time, 3-mercaptopicolinate was added at the concentrations indicated. The radioactivity incorporated into glycogen was determined 2 h later. The corresponding insulin stimulation index (s.i, ×), as defined in the Materials and methods section, is shown in the inset. Cultures supplemented with [U-^{14}C]glucose (○, ●), [U-^{14}C]serine (□, ■) or [U-^{14}C]alanine (△, ▲) in the presence of insulin (●, ■, ▲) or its solvent (○, □, △) are represented.

the detritiation of [3-^{3}H,U-^{14}C]glucose. The significant stimulation (about 20%) of the incorporation of ^{14}C into glycogen induced by 4 mM-glycerol did not alter the (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio when ^{3}H was in the C-3 position. The insulin stimulation index for glucose incorporation was reduced and the relative ratio of ^{3}H/^{14}C remained close to that obtained in the presence of the hormone alone (results not shown). Thus fructose and glycerol appear unable to modify the relative ratio of ^{3}H/^{14}C in glycogen and glucose with ^{3}H in the C3 position of glucose, despite their ability to either compete with or stimulate glycogenesis from glucose.

Effects of 3-mercaptopicolinate on basal and insulin-stimulated glycogen synthesis from [3-^{3}H,U-^{14}C]glucose, [U-^{14}C]serine and [U-^{14}C]alanine

The incorporation of ^{14}C into glycogen from [U-^{14}C]serine and [U-^{14}C]alanine was compared with incorporation from [U-^{14}C]glucose in the presence of increasing doses of 3-mercaptopicolinate, an agent that is known to inhibit gluconeogenesis from lactate/pyruvate (DiTullio *et al.*, 1974). In the absence of the inhibitor, the basal ^{14}C incorporation from 1 mM-[U-^{14}C]serine (5.4 nmol of glucosyl units/mg of protein) corresponded

to about 10% of the incorporation of 4 mM-[U-^{14}C]-glucose (50.7 nmol of glucosyl units/mg of protein), whereas the basal ^{14}C incorporation from 1 mM-[U-^{14}C]-alanine (0.9 nmol of glucosyl units/mg of protein) was less than 2% of glucose incorporation (Fig. 1). The insulin stimulation index was 2.7 and 1.9 with glucose and serine as glycogenic substrates respectively, whereas it was only 1.2 with alanine, indicating that glyconeogenesis from pyruvate remained poorly active in the presence of insulin. The already low incorporation of ^{14}C from [U-^{14}C]alanine was further decreased by the addition of 3-mercaptopicolinate to the medium, with a maximal inhibition at 0.32 nM of 60%, whether insulin was present or not. At the same time, 0.32 mM-3-mercaptopicolinate produced a significant increase in glycogen content, both in the presence of insulin (1380 ± 425 versus 1035 ± 335 nmol of glucose units/2 h per mg of protein, $n = 3$, $P < 0.01$) and in the absence of the hormone (1295 ± 260 versus 920 ± 280 nmol of glucose units/2 h per mg of protein, $n = 3$, $P < 0.001$). Labelling of glycogen from [U-^{14}C]glucose and [U-^{14}C]-serine was clearly increased in the presence of the inhibitor (Fig. 1). In the absence of insulin, this stimulation was already present with 0.1 mM-3-mercaptopicolinate and progressively increased with the dose of inhibitor (the stimulation index was 3.0 and 2.3 at 1 mM-mercaptopicolinate with [U-^{14}C]glucose or [U-^{14}C]serine respectively). In the presence of 1 mM-inhibitor, the stimulatory effect of insulin on glycogen synthesis from [U-^{14}C]glucose was depressed (stimulation index: 1.7 versus 2.7) and stimulation of glycogenesis from [U-^{14}C]-serine was virtually abolished (stimulation index: 1.2 versus 1.9) (Figs. 1*a* and 1*b* insets). On the other hand, when [3-^{3}H,U-^{14}C]glucose was used, the presence of 3-mercaptopicolinate did not significantly modify the (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio (0.69 ± 0.01 and 0.74 ± 0.05, $n = 4$, $P > 0.05$ in the absence of insulin, and 0.71 ± 0.04 and 0.76 ± 0.07, $n = 4$, $P > 0.2$ in the presence of insulin, in the absence or presence respectively of 0.32 mM-3-mercaptopicolinate). This observation was confirmed for all inhibitor concentrations tested from 0.032 to 1 mM (results not shown).

DISCUSSION

During the course of double-labelling experiments carried out in cultured foetal hepatocytes, the incorporation of radioactivity from glucose into glycogen was accompanied by a net uptake of glucose, as these cells do not release glucose into the medium (H. M'Zali & C. Plas, unpublished work). Moreover, the specific radioactivity of glucose in the medium remained constant, so that it can be considered that incorporation of glucose corresponded to a net carbon transfer into glycogen (Plas *et al.*, 1979). When [2-^{3}H,U-^{14}C]glucose was tested as a glycogenic substrate at 4 mM, the ratio of ^{3}H/^{14}C in glycogen to ^{3}H/^{14}C in glucose after 2 h was 0.23, showing that in cultured foetal hepatocytes only 20% of the glucose 6-phosphate used for glycogen synthesis was directly converted into glucose 1-phosphate. A similar ratio, close to 0.30, has been found in cultured adult hepatocytes from fed rats incubated with 8 mM-[2-^{3}H,U-^{14}C]glucose. This was ascribed to cycling through phosphoglucoisomerase to fructose 6-phosphate (Parniak & Kalant, 1985). When [3-^{3}H,U-^{14}C]glucose was used in cultured foetal hepatocytes, a (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio of close to 0.63 was found, which indicates that between 35 and 40% of glycogen synthesis could be accomplished via an alternative pathway in which glucose was first degraded at least up to the triose phosphate level. It is possible that the contribution of the alternative pathway could have been overestimated, since detritriation of [3-^{3}H]glucose also occurs in the pentose phosphate pathway (Katz & Rognstad, 1976). In fact, this is not the case, because a comparison of glycogen labelling from [1-^{14}C]glucose and [U-^{14}C]glucose gave similar values (P. Menuelle & C. Plas, unpublished work) indicating that flux through the pentose phosphate pathway is negligible (Williams *et al.*, 1987). The contribution of the alternative pathway via triose phosphate formation in the present study was similar to that of the indirect pathway in the adult determined in cultured hepatocytes from 24 h-fasted rats (Spence & Koudelka, 1985), and also in studies *in vivo* on 20 h-fasted rats refed on a solid diet (Newgard *et al.*, 1983).

The presence of 16 mM-glucose during the 2 h labelling period produced a 3.5-fold increase in glycogenesis from glucose but did not alter the relative contributions of the pathways of incorporation. This result suggests that the glycogenic effect of the glucose load was not related to a diversion of glucose 6-phosphate to the direct or to the alternative pathway. Thus it appears that the presence of a glucose load stimulates glycogen synthesis via both pathways. Indeed, a glucose load has been found to increase the contribution of glucose to glycogenesis (Menuelle *et al.*, 1987) and to increase the intracellular pool of glucose 6-phosphate (P. Menuelle & C. Plas, unpublished work). However, such an increase does not alter the relative channelling of glucose 6-phosphate into either pathway of glucose incorporation into glycogen. The inability of the glucose load to modify the route of glucose incorporation is in agreement with what has been shown in cultured adult hepatocytes from fed and from fasted rats (Parniak & Kalant, 1985; Spence & Koudelka, 1985), where glucose exerted a glycogenic effect by the activation of glycogen synthase via inhibition of phosphorylase *a* (Stalmans, 1976).

Displacement by fructose of [^{14}C]glucose during glycogen labelling was related to an increase in the (^{3}H/^{14}C in glycogen)/(^{3}H/^{14}C in glucose) ratio to 0.32 when ^{3}H was in the C-2 position. Because of the absence of hepatic fructokinase before birth, fructose would be expected to enter gluconeogenesis only at the level of fructose 6-phosphate in foetal hepatocytes (Walker, 1963, 1966). Thus it appears that fructose diluted the pool of labelled fructose 6-phosphate derived from glucose 6-phosphate, which enhanced the relative ratio of ^{3}H/^{14}C in glycogen. Similarly, the apparent increase in the direct pathway in the presence of fructose would be most likely to be due to competition with glucose 6-phosphate channelled into glycolysis, showing the necessity for the cells to divert to the glycolytic steps a major part of the pool of glucose 6-phosphate ultimately used to synthesize glycogen. In contrast, in adult hepatocytes from starved rats, a synergism of glucose and fructose for glycogen synthesis has been observed (Parniak & Kalant, 1988), and fructose caused a marked increase in the ratio of ^{3}H/^{14}C in glycogen compared with that in glucose when using [2-^{3}H,U-^{14}C]glucose (Katz & Rognstad, 1976).

The route followed by serine and alanine as intermediates for glycogen synthesis in adult hepatocytes may

be distinguished, since the former enters gluconeogenesis at the level both of pyruvate (serine dehydratase-mediated pathway) and of 2-phosphoglycerate (serine aminotransferase-mediated pathway), whereas the latter only enters at the level of pyruvate (Snell, 1984). The low rate of glycogenesis from alanine in cultured foetal hepatocytes was further decreased in the presence of 3-mercaptopicolinate at a concentration known to inhibit gluconeogenesis at the level of phosphoenolpyruvate carboxykinase (DiTullio *et al.*, 1974; Goodman, 1975). Such a situation can be attributed to a weak gluconeogenic activity between pyruvate and 2-phosphoglycerate. Thus it appears that a pathway of glycogenesis from glucose involving the intermediary metabolism of glucose into C_3 compounds occurred despite the inability to utilize pyruvate-derived metabolites. However, possible effects of 3-mercaptopicolinate on enzymes other than phosphoenolpyruvate carboxykinase could complicate such an interpretation. The efficient incorporation of ^{14}C from [^{14}C]serine suggests that a pathway of serine utilization in glycogen formation was operative. Low activity of serine aminotransferase has been reported in the foetal-rat liver at the 18-day stage, while serine dehydratase was totally absent (Snell & Walker, 1974; Snell, 1980). The presence of 3-mercaptopicolinate produced an unexpected enhancement of glycogen labelling from both serine and glucose, and this was accompanied by a net increase in glycogen formation which might rule out possible effects on dilution of the specific activities of labelled precursors. As the relative contribution of the alternative pathway was not significantly modified, an effect of the inhibitor at a level proximal to the final step of glycogen formation from these precursors is most probably involved. This would be in agreement with studies on isolated hepatocytes from fasted rats, which led to the conclusion that the inhibition of phosphoenolpyruvate carboxykinase by 3-mercaptopicolinate had produced the accumulation of an unknown activator of glycogen synthase (Okajima & Katz, 1979). In contrast to the situation in cultured foetal hepatocytes, the administration of 3-mercaptopicolinate to fasted adult rats produced a 85% decrease in glycogen synthesis, and the less active direct pathway became primary (Newgard *et al.*, 1984).

Though glucose destined for glycogen synthesis in cultured foetal hepatocytes cannot be metabolized as far as the stage of pyruvate/lactate formation prior to incorporation, the participation of non-phosphorylated C_3 intermediates cannot be excluded. Some 3-phosphoglycerate could be channelled into the reversible pathway of serine biosynthesis and, assuming that the serine aminotransferase pathway is operative in our cell system, the amino acid could subsequently enter gluconeogenesis at the level of 2-phosphoglycerate. Thus one can postulate that serine-derived metabolites are possible intermediates of the alternative pathway of foetal glucose incorporation which would be: glucose → glucose 6-phosphate → serine-derived metabolites → glucose 6-phosphate → glycogen. This hypothesis needs further investigation of the pathway flux rates from glucose and serine.

The significant enhancement by insulin of the relative ratio of $^3H/^{14}C$ in glycogen, compared with that in glucose, more marked with [2-^{3}H,U-^{14}C]glucose, shows that the hormone preferentially diverted glucose 6-phosphate into the direct pathway, in accordance with the known insulin-stimulated contribution of glucose to glycogen synthesis (Plas *et al.*, 1979). However, the striking (4-fold) stimulation of glycogenesis by the hormone suggests a less pronounced but concomitant stimulatory effect on the alternative pathway. The fact that 3-mercaptopicolinate reduced the glycogenic effect of insulin is in agreement with an action of the hormone at a final metabolic step in the glycogen synthetic pathway. In any case, the preferential effect of the hormone with glucose as a glycogenic precursor (Plas *et al.*, 1982; Menuelle *et al.*, 1988; this paper) remains to be characterized. In conclusion, this study shows that in cultured foetal hepatocytes, glucose can be partially incorporated into glycogen via an alternative pathway in which the sugar is first metabolized into triose phosphates. The low activity of gluconeogenesis from pyruvate in this system indicates that in foetal liver, the alternative pathway of glycogenesis, whose contribution is depressed in the presence of insulin, does not involve the intermediary formation of pyruvate-derived metabolites.

This investigation was supported by Institut National de la Santé et de la Recherche Médicale grants C.R.E. 847010 and 897008 and by a financial support from the Aide aux Jeunes Diabétiques, from the Société Française de Recherche en Pédiatrie and from the Association de l'Ecole Odontologique. We thank Dr. H. Clauser for advice and helpful discussions. The skilful secretarial services of Mr. E. Marie-Rose are also acknowledged.

REFERENCES

Boyd, M. E., Albright, E. B., Foster, D. W. & McGarry, J. D. (1981) J. Clin. Invest. **68**, 142–152
DeSante, D. C., Little, L., Peavy, D. E. & Vinicor, F. (1984) Biochem. J. **223**, 39–46
DiTullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) Biochem. J. **138**, 387–394
Evans, V. J., Bryant, J. C., Kerr, H. A. & Schilling, E. L. (1964) Exp. Cell Res. **36**, 439–474
Girard, J. (1986) Biol. Neonate **50**, 237–258
Goodman, M. N. (1975) Biochem. J. **150**, 137–139
Hanson, R. W., Reshef, L. & Ballard, J. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. **34**, 166–171
Hems, D. A., Whitton, P. D. & Taylor, E. A. (1972) Biochem. J. **129**, 529–538
Hers, H. G. & Hue, L. (1983) Annu. Rev. Biochem. **52**, 617–653
Jungermann, K. (1987) Diabetes Metab. Rev. **3**, 269–293
Katz, J. & Rognstad, R. (1976) Curr Top. Cell. Regul. **10**, 237–289
Lin, E. C. C. (1977) Annu. Rev. Biochem. **46**, 765–795
McGarry, J. D., Kuwajima, M., Newgard, C. B., Foster, D. W. & Katz, J. (1987) Annu. Rev. Nutr. **7**, 51–73
Menuelle, P., Buc, H. A. & Plas, C. (1987) Biochim. Biophys. Acta **928**, 332–340
Menuelle, P., M'Zali, H., Forest, N. & Plas, C. (1988) Int. J. Biochem. **20**, 777–782
Newgard, C. B., Hirsh, L. J., Foster, D. W. & McGarry, J. D. (1983) J. Biol. Chem. **258**, 8046–8052
Newgard, C. B., Moore, S. V., Foster, D. W. & McGarry, J. D. (1984) J. Biol. Chem. **259**, 6958–6963
Okajima, F. & Katz, J. (1979) Biochem. Biophys. Res. Commun. **87**, 155–162
Parniak, M. & Kalant, N. (1985) Can. J. Biochem. Cell Biol. **63**, 333–340
Parniak, M. A. & Kalant, N. (1988) Biochem. J. **251**, 795–802
Pilkis, S. J., Regen, D. M., Claus, T. H. & Cherrington, A. D. (1985) BioEssays **2**, 273–276
Plas, C. & Nunez, J. (1976) J. Biol. Chem. **251**, 1431–1437

Plas, C., Chapeville, F. & Jacquot, R. (1973) Dev. Biol. **32**, 82–91
Plas, C., Menuelle, P., Moncany, M. L. J. & Fulchignoni-Lataud, M. C. (1979) Diabetes **28**, 705–712
Plas, C., Forest, N., Pringault, E. & Menuelle, P. (1982) J. Cell. Physiol. **113**, 475–480
Scofield, R. F., Kosugi, K., Schumann, W. C., Kumaran, K. & Landau, B. R. (1985) J. Biol. Chem. **260**, 8777–8782
Seglen, P. O. (1974) Biochim. Biophys. Acta **338**, 317–336
Shikama, H. & Ui, M. (1978) Am. J. Physiol. **235**, E354–E360
Snell, K. (1980) Biochem. J. **190**, 451–455
Snell, K. (1984) Adv. Enzyme Regul. **22**, 325–400
Snell, K. & Walker, D. G. (1974) Biochem. J. **144**, 519–531
Spence, J. T. & Koudelka, A. P. (1985) J. Biol. Chem. **260**, 1521–1526
Stalmans, W. (1976) Curr. Top. Cell. Regul. **11**, 51–97
Walker, D. G. (1963) Biochem. J. **87**, 576–581
Walker, D. G. (1966) Essays Biochem. **2**, 33–67
Williams, J. F., Arora, K. K. & Longenecker, J. P. (1987) Int. J. Biochem. **19**, 749–817

Received 6 February 1989/12 June 1989; accepted 23 June 1989