Turnover of Liver Glycogen in the Rat Foetus

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Incorporation and release of the radioactivity in the liver glycogen of 18.5- and 19.5-day-old rat foetuses were studied after intravenous injection of [$1\text{-}^{14}\text{C}$]glycerol. Incorporation occurred during 1 h after injection of the radioactive tracer to the foetus; then, the incorporated radioactivity decreased. Glycogen content in the liver, and glycogen phosphorylase and glycogen synthase were not modified during the experiment. It is therefore postulated that a physiological turnover of glycogen exists in the liver of the rat foetus.

The question of a turnover of glycogen in the foetal liver remains controversial. Goldwater & Stetten (1947), by using $^2\text{H}_2\text{O}$, estimated the normal rate of turnover of glycogen in the liver of the rat foetus at term to be as high as 5 mg/g of liver per day.

It is usually assumed that towards the end of pregnancy the foetus is able to mobilize its glycogen stores in several situations. Experimentally, glycogenolysis was induced in the liver of the rat foetus *in vivo* by anoxia (Shelley, 1961) or glucagon (Girard & Bal, 1970) or *in vitro* in organ culture by adrenaline, glucagon or cyclic AMP (Eisen *et al.*, 1973; Schwartz & Rall, 1973), and in the perfused foetal monkey liver by hypoglycaemia, glucagon or cyclic AMP (Glinsmann *et al.*, 1975). However, this does not prove that hepatic glycogen turnover actually occurs in the foetus under normal circumstances. Devos & Hers (1974) studied the fate of radioactivity incorporated into hepatic glycogen of the rat foetus after administration of [$6\text{-}^3\text{H}$]glucose to the mother. They observed no turnover and concluded that, before birth, only accumulation and no degradation of glycogen occurs. Watts & Gain (1976) criticized these results, suggesting that in experiments by Devos & Hers (1974) labelled glycogen could have been rapidly buried under subsequently added non-radioactive glucose molecules. They also pointed out that the existence of a turnover of the foetal hepatic glycogen would explain the fact that there is always a significant amount of phosphorylase activity in the foetal liver, even when net glycogen synthesis occurs. From their experimental enzymic determinations, they calculated that the rat foetus would produce at least 7 times more glycogen than it effectively accumulates from day 19 to day 20, if no degradation simultaneously occurred (Watts & Gain, 1976).

The present study is an attempt to reinvestigate this problem by labelling the glycogen of rat foetuses through an injection of a radioactive tracer directly into the foetal bloodstream. [$1\text{-}^{14}\text{C}$]Glycerol was pre-

ferred to labelled glucose as a precursor for glycogen, since it has been shown that the radioactivity of labelled glycerol is rapidly incorporated into the liver glycogen (Gilbert, 1977). Moreover, glycerol crosses the placenta slowly from foetus to mother, and conversion into glucose in the mother is negligible (Gilbert, 1977). The fate of labelled glycogen was studied in a 6 h period in 19.5-day-old rat foetuses. Glycogen synthase and phosphorylase activities were assayed over the period of turnover study. To follow the extent of labelling during the longest possible period before parturition (72 h), the study was also carried out in 18.5-day-old foetuses, the age when glycogen synthesis begins.

The results are in agreement with the existence of a glycogen turnover in the liver of the rat foetus, which means that deposition and utilization of hepatic glycogen occur simultaneously.

Experimental

Materials

Animals. The study was made on female rats of the Wistar strain weighing approx. 250–300 g and fed *ad libitum*. Gestation was verified 14 days after mating by abdominal palpation and the foetal age determined by the method of Jost & Picon (1970).

Chemicals. [$1\text{-}^{14}\text{C}$]Glycerol (sp. radioactivity 57 mCi/mmol) was purchased from CEA, Saclay, France; UDP-[$U\text{-}^{14}\text{C}$]glucose (sp. radioactivity 312 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; oyster glycogen, UDP-glucose, glucose 6-phosphate and glucose 1-phosphate were obtained from Calbiochem (San Diego, CA, U.S.A.); $\alpha(1\rightarrow4)$ glucosidase (amylglucosidase, EC 3.2.1.3) and glucose oxidase were provided by Boehringer (Mannheim, Germany). Other chemicals were obtained from Prolabo (Paris, France).

Surgical procedure. Pregnant rats were anaesthetized by intraperitoneal administration of 30 mg of

pentobarbital/kg body wt. and laparotomized. Injections to the foetuses were made with a Hamilton syringe (Bonaduz, Switzerland) into a vitellin vein through the uterine wall, by the method of Petter & Bourbon (1970); glycerol ($1\mu\text{Ci}/\text{foetus}$) was given in a saline solution (0.9% NaCl, 0.02ml). Foetuses injected with radioactive glycerol were not suitable for the determination of glycogen synthase activity, since the enzyme assay is based on the incorporation of radioactivity into glycogen. For the enzymic determinations, foetuses of other animals were injected with saline alone (0.02ml).

After injections were made, the abdominal wall was closed and the rats kept anaesthetized until removal of the foetuses. Cooling of the rat under anaesthesia was prevented by controlling the environmental temperature.

At the end of selected time intervals (0.25, 0.5, 1, 2, 4 or 6 h) uterine horns were rapidly exposed and foetuses taken one after the other. Liver of injected foetuses (3 or 4 per litter) was dissected out and immediately frozen in liquid N_2 .

In long-term studies on 18.5-day-old foetuses, the rats were anaesthetized with ether. After the injections, the mothers were allowed to awake in their cages and re-anaesthetized when the foetuses were taken out, 6 to 72 h later. Livers were kept frozen at -25°C until assays were carried out (no more than a week).

Glycogen concentration and radioactivity determination

Glycogen concentration of the liver and radioactivity incorporated into glycogen were simultaneously determined by the method of Chan & Exton (1976). Frozen livers were rapidly weighed and homogenized (2.5%) in double-distilled water with a glass/Teflon homogenizer. Homogenate (0.1 ml) was spotted on a piece ($2\text{cm} \times 3\text{cm}$) of Whatman 3MM chromatography paper and the papers were washed ($3 \times 10\text{min}$) with 66% ethanol. They were then rinsed with acetone, dried and placed in plastic tubes containing 0.4 ml of 0.05M-acetate buffer, pH 4.5, 1.6 ml of water and 0.02 ml of $\text{exo}(\alpha 1 \rightarrow 4)\text{glucosidase}$ suspension (3 units). The tubes were heated at 55°C for 30 min to allow hydrolysis of glycogen. Glucose was determined in 0.01 ml of hydrolysate by the glucose oxidase method. Hydrolysed oyster glycogen standard was used as reference. Hydrolysate (1 ml) was placed in a vial containing 10 ml of PCS scintillation fluid (Amersham/Searle, Arlington Heights, IL, U.S.A.) and the activity was determined with a liquid scintillation system (Mark I; Nuclear-Chicago, Des Plaines, IL, U.S.A.).

Enzyme assays

Phosphorylase α (EC 2.4.1.1) was assayed by measuring P_i released during incorporation of

glucose 1-phosphate into glycogen (Hue *et al.*, 1975). Fragments of liver were homogenized in 10 vol. of ice-cold medium containing 100mM-NaF, 20mM-EDTA, 0.5% (w/v) glycogen and 50mM-glycylglycine, pH 7.4. Homogenate (0.1 ml) was incubated for 30 min at 20°C with an equal volume of a mixture containing 100mM-glucose 1-phosphate, 2% (w/v) glycogen, 0.3M-NaF and 1mM-caffeine, pH 6.1. The reaction was stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid. The mixture was centrifuged ($16000g$ for 2 min) and P_i assayed in the supernatant as described by Fiske & SubbaRow (1925). The activity was expressed in μmol of P_i liberated from glucose 1-phosphate/min per mg wet wt. of liver.

Glycogen synthase (EC 2.4.1.11) activity was measured by incorporation of $[\text{U}-^{14}\text{C}]\text{glucose}$ from $\text{UDP}-[\text{U}-^{14}\text{C}]\text{glucose}$ into glycogen, by the method of De Wulf & Hers (1968). The liver was homogenized with 3 vol. of ice-cold solution containing 150mM-sucrose and 100mM-glycylglycine buffer, pH 7.4. A 0.02 ml portion of homogenate was incubated for 10 min at 20°C in a final volume of 0.2 ml containing $0.2\mu\text{mol}$ of $\text{UDP-glucose} + \text{UDP}-[\text{U}-^{14}\text{C}]\text{glucose}$ (100 d.p.m./nmol), 1 mg of glycogen, $6\mu\text{mol}$ of glycylglycine buffer, pH 7.4, $1\mu\text{mol}$ of Na_2SO_4 and $0.5\mu\text{mol}$ of EDTA. The labelled glycogen was measured after adsorption on filter paper (Thomas *et al.*, 1968). A portion (0.1 ml) of the mixture was placed on a piece ($2\text{cm} \times 2\text{cm}$) of Whatman 31 ET chromatography paper and the reaction stopped by placing the paper in cold ethanol. The papers were washed ($3 \times 10\text{min}$) with 66% ethanol. Papers rinsed with acetone and dried were placed in vials containing 10 ml of scintillation liquid (PCS; Amersham/Searle) for determination of the radioactivity incorporated. The activity was expressed in nmol of glucose incorporated from UDP-glucose into glycogen/min per mg wet wt. of liver, i.e. units/mg wet wt. of liver.

Results

Short-term studies (19.5-day-old foetuses)

During the first hour after the injection of $[\text{U}-^{14}\text{C}]\text{glycerol}$ into the foetus, the incorporation of radioactivity was roughly proportional to time (Fig. 1); it reached a maximum after 1 h. Thereafter the radioactivity of liver glycogen decreased sharply between 1 and 2 h and slowly between 2 and 6 h. More than two-thirds of the radioactivity incorporated during the first hour was lost between 1 and 2 h and five-sixths between 1 and 6 h after the injection. Neither liver glycogen content (Fig. 1) nor phosphorylase and glycogen synthase activities (Table 1) varied significantly during the period of study.

Table 1. *Activities of glycogen phosphorylase a and glycogen synthase a in the liver of 19.5-day-old rat foetuses at different times after the injection of 0.02 ml of 0.9% NaCl*
 The values are the means $\pm$ s.e.m. Each mean value includes foetuses from litters. The number of foetuses is indicated in parentheses in each column heading.

Time (h) ...	Enzyme activity (unit/g wet wt. of liver)					
	Zero (9)	0.5 (14)	1 (13)	2 (13)	4 (16)	6 (12)
Glycogen phosphorylase (a)	2.30 $\pm$ 0.20	2.75 $\pm$ 0.11	2.46 $\pm$ 0.10	2.42 $\pm$ 0.13	2.06 $\pm$ 0.16	3.02 $\pm$ 0.27
Glycogen synthase a	0.098 $\pm$ 0.019	0.064 $\pm$ 0.012	0.050 $\pm$ 0.009	0.047 $\pm$ 0.07	0.123 $\pm$ 0.018	0.070 $\pm$ 0.07

Table 2. *Incorporation of radioactivity in the liver of 19.5-day-old rat foetuses*
 A time zero all the foetuses received [$1\text{-}^{14}\text{C}$]glycerol (1 μCi). At time 1.5 h a first group (single injection) was not reinjected; a second group (double injection) received either saline injection or [$1\text{-}^{14}\text{C}$]glycerol (1 μCi). Livers were removed at 2 h.

Time zero ... Time 1.5 h ... Glycogen (mg/g liver) Radioactivity of glycogen (d.p.m./mg)	Double injection			
	Single injection		Double injection	
	No injection (n = 21)	Glycerol	Saline (n = 15)	Glycerol
	19.3 $\pm$ 1.6		26.4 $\pm$ 3.3	Glycerol (n = 15)
	2032 $\pm$ 631		1490 $\pm$ 467	14.4 $\pm$ 1.2
				7037 $\pm$ 993

Table 3. *Time studies of labelling of the liver glycogen of rat foetuses between 18.5 and 21.5 days of gestation*
 On day 18.5 the foetuses were injected with 1 μCi of [$1\text{-}^{14}\text{C}$]glycerol by the vitellin vein. The values are means $\pm$ s.e.m. Each of the mean values includes foetuses from 2 or 4 litters. The number of foetuses is indicated in parentheses in each column heading.

Time after injection (h) ... Glycogen (μg /mg wet wt. of liver) Radioactivity in glycogen (d.p.m. in total)	Glycogen content and radioactivity					
	0 (12)	1 (12)	2 (8)	6 (6)	24 (8)	72 (12)
	2.4 $\pm$ 0.2	2.5 $\pm$ 0.7	1.8 $\pm$ 0.4	1.8 $\pm$ 0.4	34.9 $\pm$ 3.9	102.2 $\pm$ 7.7
		7977 $\pm$ 2939	5538 $\pm$ 1643	3135 $\pm$ 505	2531 $\pm$ 163	3010 $\pm$ 402
					80.5 $\pm$ 9.6	3022 $\pm$ 511

Fig. 1. Liver glycogen content (■) and incorporation of radioactivity into glycogen (●) after the injection of [$1\text{-}^{14}\text{C}$]glycerol into the rat foetus (19.5 days of gestation). The numbers in the body of the Figure indicate the number of foetuses for each point.

The ability of the foetal liver to simultaneously reincorporate newly injected labelled glycerol during the sharp loss of radioactivity (between 1 and 2h) was investigated. Foetuses were reinjected 90 min after the first injection either with $1\mu\text{Ci}$ of [$1\text{-}^{14}\text{C}$]glycerol or with the same volume of 0.9% NaCl (Table 2); 30 min later (2h after the first injection) labelling was 5-fold higher in the glycogen from the [$1\text{-}^{14}\text{C}$]glycerol-injected group than in the saline-injected group. In the latter group the radioactivity incorporated into the glycogen was similar to that observed in the group that had received only a single injection of [$1\text{-}^{14}\text{C}$]glycerol (Table 2).

Long-term studies (18.5-day-old foetuses)

Although 18.5-day-old foetuses still had little glycogen in their liver, radioactive glycerol was actively incorporated into glycogen. The radioactivity in the glycogen was high after 1h and decreased between 1 and 6h. Thereafter it remained constant for 66h (Table 3).

Discussion

The loss of the radioactivity incorporated in the foetal liver glycogen is obviously the result of glycogen degradation. But whether this loss results from a physiological turnover or only from glycogenolysis induced by experimental conditions is questionable. Several experimental results support a physiological turnover: (a) the glycogen concentration in the liver of the 19.5-day-old foetuses did not decrease significantly over the 6h experimental period. Even after 2h, when the loss of radioactivity

was very great, the liver glycogen content was not statistically different from that observed after 0 or 15 min; (b) the activity of the enzymes that control liver glycogen metabolism were not modified by the experiment. It is unlikely that glycogenolysis could be induced by the experimental conditions without any change in the activity of both phosphorylase α and glycogen synthase α ; (c) in foetuses injected twice, despite possible stress due to a second manipulation, the second incorporation of labelled glycerol was approximately equal to that incorporated after the first injection. This would mean that degradation and synthesis can occur simultaneously. It can be assumed that the decrease of radioactivity incorporated into liver glycogen actually corresponds to a physiological turnover.

Between 1 and 2h after the injection of labelled glycerol, the release of labelled molecules from liver glycogen is more important than after the second hour. A variation of the turnover rate during this period is not likely. The most recently incorporated glucose molecules are probably located in the outer chains of glycogen and easily submitted to degradation. Afterwards, newly incorporated non-radioactive molecules cover the labelled molecules and the probability of a radioactive molecule being removed from glycogen decreases. This would also explain why, in the 72h studies, no loss of radioactivity was observed after 6h.

The present results are in agreement with the observations of Watts & Gain (1976) with regard to the existence of a turnover of glycogen in the liver of the rat foetus.

These conclusions raise the question why Devos & Hers (1974) failed to observe a turnover of liver glycogen in the rat foetus. They determined foetal glycogen radioactivity for the first time at 4–5h after the injection of tracer, which according to our results is too late. Moreover, they injected labelled glucose, not directly into the foetuses, but into their mother; it is likely that not insignificant amounts of labelled molecules (glucose or metabolites) are found in the maternal blood for some time, with recycling of labelled glucose. These molecules could probably reach the foetal circulation and be incorporated into foetal glycogen for a prolonged time after initial injection. The use of glycerol directly injected into the foetal blood avoids these perturbing effects.

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