

Glycogen Synthesis in Rat Liver from a Pool of Free Glucose

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Glycogen synthesis in isolated perfused livers or livers of anesthetized rats (*in situ*), was studied using radioactively labelled fructose, lactate, and inositol as substrates. The specific radioactivity of glucose and glycogen was measured at various times and compared with that of some intermediates.

The results suggest that liver glycogen is formed from the pool of free glucose which in turn is fed by the so-called “direct and indirect pathway” of glycogen synthesis. This points to an important role of glucose-6-phosphatase, an enzyme complex subject to regulation by glucocorticoids, well known promoters of hepatic glycogen synthesis.

Introduction

At least since 1976 many papers have been published discussing whether liver glycogen is synthesized *via* the so-called “direct” (free glucose from the blood) or the “indirect” (*via* gluconeogenesis) pathway (see [1–8]), review [9].

There are arguments for both pathways, depending from the experimental conditions that were considerably different in the various reports. However, it seems doubtful to differentiate and separate the supply of glucose for glycogen synthesis in such a way. Physiologically both “pathways” should contribute to glycogen synthesis.

The data presented here suggest that in rat liver glycogen is synthesized from the pool of free glucose which can be supplied by both, the so-called “direct” and the “indirect” pathway.

Materials and Methods

Male Wistar rats (170–270 g), strain FW 49, obtained from Thomae, Biberach, were kept in steel cages under a 12 h light/dark cycle (7 a.m. to 7 p.m.) and were fed a standard diet (Sniff, Soest) and water *ad libitum*.

Abbreviations: DAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-bisphosphate; G6Pase, glucose-6-phosphatase (EC 3.1.3.9); α -GP, glycerol-3-phosphate; PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); 3-PG, 3-phosphoglycerate; SRA, specific radioactivity in cpm/ μ mol; UDP-glc, uridine diphosphoglucose; UDP-glucUA, uridine diphosphoglucuronic acid.

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Biochemicals were from Boehringer, Mannheim, ^{14}C -labelled substrates from Amersham-Buchler, Braunschweig, Heparin (Liquemin®) from Hoffmann-La Roche, Grenzach, Evipan® from Bayer, Leverkusen, chinolinic acid from Fluka, Buchs (Switzerland), and fibrinogen and ion exchange resins from Serva, Heidelberg. Prednisolone (soludecortin H®) as well as all other chemicals (analytical grade) were from Merck, Darmstadt.

All experiments were performed between 9 and 11 a.m. to avoid differences by circadian rhythm. Starved rats (16–18 h) (except the experiments A in Table I) were used and pretreated with prednisolone (3 mg per 100 g rat i.p.) 2 h before the experiments. For PEPCK inhibition chinolinic acid (400 μ mol per 100 g rat, pH 6.5 in 0.9% NaCl) [10] was injected i.p. 10 min before substrate infusion.

Liver perfusion and collection of biological samples

Rats were anesthetized with a 10% solution of Evipan (initially 25–30 mg/100 g rat i.p. followed by smaller doses as required). The abdomen was opened and due to the great variation in “zero-glycogen” values after starvation plus prednisolone, “zero-glycogen” determination was done from a small tissue sample (about 50–100 mg) taken from the major liver lobe with a razor blade. The wound was covered with fibrinogen which stopped bleeding immediately.

A syringe was inserted into the *V. cava* and labelled substrates were infused at a rate of 0.6 ml/h. Infusion of substrates through the portal vein did not change the results. Infusions were stopped a



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few minutes before cutting off a piece of liver (about 4 g) which was frozen immediately with a Wollenberger clamp precooled in liquid nitrogen. Blood was collected from the *V. cava* or, during the experiments, from the tail veins. Animals were killed by decapitation. Radioactivity was measured ("β-scint" BF 8000, Berthold, with "Ready Solv EP", Beckmann) in blood and urine as well as in samples of tissue from muscle, heart, kidney, and liver previously digested in 2 N KOH. Recovery of infused radioactivity in these probes was between 75–85%. The contribution of expired $^{14}\text{CO}_2$ (occasionally trapped by insertion of a small tracheal tube) was lower than 10% of the radioactivity infused, irrespective of the substrate or the experimental conditions used. Substrates and metabolites were determined enzymatically according to standard procedures [11]. Protein was determined with the Biuret reagent and glycogen as described in [12], or for smaller samples as in [13]. Liver perfusion *in vitro* was performed as described earlier [15, 16].

Determination of blood metabolites

The sugars of neutralized perchloric acid extracts (1:3) were separated by ion exchange chromatography (anion exchange resin Dowex 1 × 8 – Serva cat. No. 41111, column 1 × 2 cm per ml extract). The aqueous fraction was dried and redissolved in a small amount of water. The individual sugars were separated by ion exchange chromatography after enzymatical conversion of glucose into gluconate (column 1 × 5 cm, Dowex 1 × 8, borate). Fructose was eluted with 60 mM borate, gluconate with 1 N formic acid.

Liver

Separation and determination of sugars was done in the same way as described for blood. Separation of phosphorylated metabolites is explained in Fig. 1 and followed essentially the method of [14]. Hexose phosphates were overlapping in the different fractions.

Specific radioactivity

The content of metabolites is quite low. In order to get precise estimates of their specific radioactivity (SRA), they were converted enzymatically into derivatives prior to separation *e.g.* G6P into

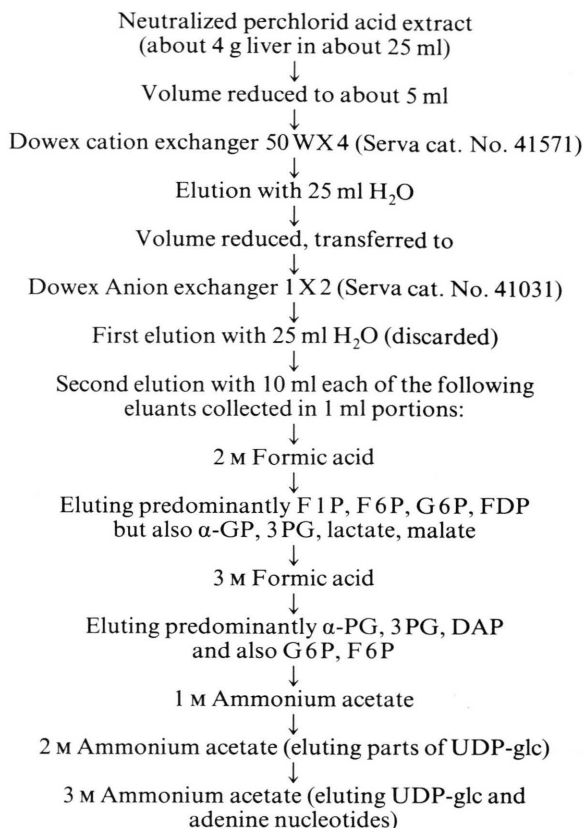


Fig. 1. Experimental protocol for the separation of phosphorylated sugar metabolites of rat liver [14].

6-phosphogluconate. In a few experiments the SRA of G1P was determined. In this case the whole available liver tissue (about 4 g) had to be used, and 1 μmol of cold G1P was added as a tracer. F1P was calculated from the fructose fraction by subtraction, determining first fructose and F6P.

Results

One major argument against glycogen synthesis *via* the "direct" pathway is the need of an unphysiologically high glucose concentration (20–30 mM) in order to obtain glycogen synthesis *in vitro*. On the other hand reasonable rates of glycogen synthesis can be observed with fructose as a precursor which points to a preference of the "indirect" pathway. However in these experiments fructose is only effective in livers (or liver cells) from fed rats where the glycogen had been depleted previously by gly-

cogenolytic treatment [17]. In livers from starved rats nearly no glycogen synthesis from fructose does occur. Since starvation is accompanied by a decrease in the level of insulin and glucocorticoids, the livers from starved rats, as usually taken for *in vitro* experiments, are highly unsuitable for glycogen synthesis emphasizing the dominant role of glucocorticoids in the regulation of glycogen synthesis [18–20].

As indicated in Table I, glycogen is synthesized *in vitro* from fructose under appropriate conditions in a time-dependent manner. Also in agreement with earlier observations is the fact that pretreatment with prednisolone leads to a marked glycogen synthesis from glucose (Table I/C).

Further information is obtained from Table I by comparing the distribution of radioactivity of free glucose and glycogen, respectively. Thus, in the experiments with fructose, the greater portion of ^{14}C

radioactivity of utilized fructose appears in the perfusion as ^{14}C glucose and not as glycogen *i.e.* in the free glucose pool. In experiment A about 120 μmol of glycogen were synthesized within the first 15 min. During the same time about 150 μmol of ^{14}C glucose (from the infused fructose) were available for synthesis *via* the gluconeogenic pathway. Although this glucose could be used directly for glycogen synthesis, nevertheless it is found almost completely in the medium and the SRA of glycogen at that time was only $\frac{1}{10}$ of the SRA of glucose in the medium.

The validity of these results obtained by liver perfusion *in vitro* was confirmed by experiments on anesthetized rats *in vivo*. Again, rats were starved and pretreated with prednisolone. Labelled precursors were infused into the *V. cava in situ*, and the SRA of glycogen, glucose and some intermediates was measured as described in Mate-

Table I. Glycogen synthesis from $[\text{U-}^{14}\text{C}]$ labelled fructose and glucose in isolated perfused rat livers and the distribution of radioactivity between glycogen and free glucose.

A) The liver from a fed rat was perfused 30 min with glucagon thereby lowering the glycogen level from 285 to 90 $\mu\text{mol/g}$ liver. The medium was replaced by 100 ml of a new medium containing 5 mM glucose to which at “zero” $[\text{U-}^{14}\text{C}]$ labelled fructose (600 $\mu\text{mol/h}$, SRA 10,500 cpm/ μmol) was infused. For further experimental details see text. It should be noted that without preceding glycogen breakdown there is nearly no glycogen synthesis [17].

B) Perfusion of the liver from a starved rat, pretreated with prednisolone (see Methods). At “zero” to 100 ml of the medium containing 10 mM glucose $[\text{U-}^{14}\text{C}]$ labelled fructose (500 $\mu\text{mol/h}$, SRA 18,700 cpm/ μmol) was infused. Notably, without pretreatment with prednisolone the rate of glycogen synthesis in starved rats is very small even with fructose as a substrate (data not shown).

C) Perfusion of the liver from a starved rat, pretreated with prednisolone as in B). At “zero” $[\text{U-}^{14}\text{C}]$ labelled glucose (SRA 11,000 cpm/ μmol) was added as a single dose to 100 ml medium resulting in a final glucose concentration of about 15 mM. This concentration is only slightly higher than the glucose level normally adjusted by the perfused rat liver in the medium and no glycogen synthesis would take place without prednisolone pretreatment.

For the experimental conditions in A, B and C see text. Recovery of radioactivity from glycogen and glucose was about 90%.

Experimental conditions		Glycogen content (whole liver) [μmol]	Specific radioactivity [cpm/ μmol]		Total cpm	
			Glycogen	Free glucose (Medium)	Glycogen	Free glucose (Medium)
A	“zero”	756	—	—	—	—
Liver of a fed rat preperfused with glucagon	15 min	891	230	2000	—	—
Substrate: $[\text{U-}^{14}\text{C}]$ fructose	55 min	1295	1300	3500	—	—
	95 min	1400	1800	4700	2.5×10^6	5.6×10^6
B	“zero”	40	—	—	—	—
Liver of a starved rat pretreated with prednisolone	45 min	240	9600	6500	—	—
Substrate: $[\text{U-}^{14}\text{C}]$ fructose	90 min	400	13300	8100	5.3×10^6	8.1×10^6
C	“zero”	21	—	—	—	—
Conditions as in B	60 min	145	11100	10800	—	—
Substrate: $[\text{U-}^{14}\text{C}]$ glucose	120 min	185	12200	10700	2.6×10^6	12.8×10^6

Table II. Comparison of the specific radioactivity of newly synthesized glycogen and blood glucose after infusion of radioactively labelled fructose. The amount and specific radioactivity of infused fructose as well as the time scale of the experiment varied. Glycogen synthesis without substrate infusion ranged between 5 and 15 $\mu\text{mol g}^{-1}\text{h}^{-1}$. The glycogen level at time zero was determined in each animal prior to substrate infusion. For further experimental conditions see Methods.

Substrate infused [μmol]	Glycogen synthesized [$\mu\text{mol g}^{-1}\text{h}^{-1}$]	Specific radioactivity [cpm/ μmol] of Substrate infused	Synthesized glycogen	Blood glucose
[U- ^{14}C]Fructose				
200 (20 min)	120	70000	5800	9200
400 (40 min)	70	56000	9500	12300
600 (60 min)	60	59000	8700	9800
400 (40 min)	50	80000	17470	26000
1200 (40 min)	150	10500	2700	4700
380 (10 min)	110	29000	1600	4200
970 (20 min)	130	28000	8800	10600
1800 (40 min)	85	18800	16600	16600

rials and Methods. In the experiments listed in Table II varying amounts of [U- ^{14}C]fructose were infused over varying periods of time. If glucose units from the "indirect" pathway were transferred directly to glycogen, under the experimental conditions (low level of glycogen, prednisolone pretreatment and high substrate supply) one might expect the SRA of synthesized glycogen to be higher than that of free glucose, especially since the synthe-

sized glycogen is not metabolized during the time course of the experiment [21] and glucose undergoes dilution in the entire glucose space. The SRA of both products, however, is almost equal or even higher in the glucose fraction (Table II).

Again the question arises whether glycogen is synthesized from the pool of free glucose. This should be detectable by short-term experiments. The data in Tables II and III show that the SRA of

Table III. Comparison of specific radioactivities of newly synthesized glycogen, blood glucose and some phosphorylated sugar intermediates after infusion of radioactively labelled substrates. Experimental conditions are the same as described for the experiments.

Substrates infused [μmol] (minutes)	Substrate infused	Specific radioactivity (cmp/ μmol) of				
		Glycogen synthesized	Free glucose	G 6P	UDP-glc	α -GP
[U- ^{14}C]Fructose						
180 4 min	32100	940	3100	6400	8800	10000
315 7 min	33000	4000	6000	16200	14000	16000
450 10 min	60000	7500	13000	17900	37000	17000
1000 30 min	31100	16700	13100	24700	28300	9400
1200 40 min	28500	18000	15300	15000	22500	11900
Chinolinic acid pretreatment						
500 40 min	59200	23200	22900	45200	56700	17800
[U- ^{14}C]Lactate						
400 40 min	29400	6800	6500	8600	14200	4600
[U- ^{14}C]Inositol						
670 40 min	39200	900	4300	2600	1400	n.d.
Fructose, without prednisolone pretreatment						
1800 40 min	14000	no synthesis	8400	12700	13000	6900

free glucose is definitely higher than the SRA of the simultaneously synthesized glycogen, if determined during the first 15 min. Taking the data from the 4 min experiment (Table III), fructose was infused with a SRA of 32,000 cpm/ μ mol. Irrespective of the dilution with unlabelled internal intermediates, the SRA of newly synthesized glycogen was only 940 cpm/ μ mol as compared to 3100 cpm/ μ mol of free glucose in the blood neglecting the dilution of glucose in the glucose space. Other short-term experiments showed similar results. Comparison of the radioactivity incorporated after 4 min of infusion of labelled fructose into glucose and glycogen, respectively, with the amount of synthesized glycogen, more strongly elucidates the differences. Total glycogen contains 50,000 cpm (in about 8 g of liver) whereas free glucose contains 150,000 cpm (calculated from the circulating blood, *i.e.* about 8 ml). This means that during the first 4 min most of the glycogen is synthesized from unlabelled glucose despite the ample rates of substrate supply and its conversion to glucose (180 μ mol) which was in excess of the amount of glycogen synthesized (about 80 μ mol).

Coming back to the perfusion (Table I/A) which demonstrates a closed, simplified and clearly arranged system. As mentioned above, within the first 15 min about 150 μ mol of [14 C]fructose were metabolized to [14 C]glucose and about 130 μ mol of glycogen were synthesized. If the "indirect" pathway for glycogen synthesis is the predominant one, the SRA of glycogen should be about 1600 cpm/ μ mol instead of 230 cpm/ μ mol as was measured. Supposing [14 C]glucose (from labelled fructose) was transferred entirely to the medium, the SRA of glucose theoretically calculated should be about 2300 cpm/ μ mol. The measured value was 2000 cpm/ μ mol. Therefore one may conclude that metabolized fructose is delivered nearly completely into the medium, and glycogen is synthesized nearly exclusively from unlabelled glucose although the liver cell is filled up with labelled glucose molecules.

The results draw attention to an exchange mechanism transferring at least one glucose molecule from the inside to the outside in exchange with one molecule glucose from the outside. As long as the free glucose in the glucose pool – source for the glycogen synthesis – contains only a few labelled glucose molecules (within the first minutes of the

experiment) the SRA of the glycogen synthesized during that time must be low. Consequently, the SRA of glycogen *in vivo* (Table III), after 4 min on infusion of labelled fructose amounts to only 14% of the SRA of G6P, a central intermediate, to 24% after 7 min and, to 40% after 10 min with the clear tendency to reach equilibrium parallel to the continuous increase of labelled glucose in the glucose pool (Table III). The exchange of glucose molecules exceeds the rate of glycogen synthesis. Pretreatment of the animals with chinolinic acid, an inhibitor of the PEPCK, did not change these relationships. The SRA of UDP-glucose (UDP-glc) is exceptionally high which is discussed below.

Table III also shows data from experiments with two other substrates, lactate (a regular source of gluconeogenesis), and inositol, a hexose which is only scarcely metabolized in the rat to either glucose or glycogen. The results with lactate as a substrate are rather similar to those with fructose but completely different to those obtained by using inositol.

Discussion

The results indicate that rat liver glycogen is synthesized from the pool of free glucose involving necessarily the complex of the G6Pase and an exchange mechanism. It seems unusual to connect glycogen synthesis with an active hydrolase but such considerations are not even completely new [20].

The question then arises of how free glucose is channelled towards glycogen synthesis. At present only a few indications from the literature may be mentioned. In experiments with isolated microsomes an exchange mechanism between G6P and free glucose was described involving glucose transphosphorylation and a G6P-specific translocase as part of the G6Pase complex [23–28]. The translocase activity of the G6Pase is inducible by glucocorticoid hormones and this should provide an increased supply of glucose for the transfer to glycogen. Although these and other findings are helpful in explaining the well known stimulatory action of glucocorticoid hormones on hepatic glycogen synthesis, a full understanding of the interrelationships must await the complete isolation of the G6Pase complex, which may include the exchange mechanism too. A partial purification has been described in [29]. The postulated special link between

glycogen synthesis and gluconeogenesis *e.g.* from C 3-units [30], may be seen under similar considerations.

Last not least, morphological studies have shown that the initially appearing glycogen in rat liver closely follows cell regions rich in smooth endoplasmic reticulum, and more specifically, at sites where G 6Pase activity stains [31–34]. Other studies with radioactive precursors showed that pretreatment with dexamethasone for 2 h led to a pronounced increase of labelling of these areas as measured by autoradiography [35, 36].

The proposal of glucokinase as a critical enzyme step for glycogen synthesis [37, 38] has become less likely [39]. It has been reported that glucokinase activity is detectable in rat liver not until the 16th day *post partum*, whereas G 6Pase appears already by the 19th day of gestation and it is the G 6Pase activity which parallels the liver's capacity to synthesize glycogen [40–43].

The data presented cannot explain the high SRA of UDP-glc which is also described in [44]. However, their validity was confirmed as follows: The content of UDP-glc was measured enzymatically in the pooled fractions followed by acid hydrolysis and determination of the liberated free glucose *via* ion exchange chromatography (nearly 100% recovery). Subsequently glucose was converted enzymatically to gluconate which was separated by ion exchange chromatography, and again determined enzymatically (95–100% recovery). Even under these conditions the SRA of the glu-

conate remained high similar to that of UDP-glc in the pooled fractions.

It should be noted that the SRA of G 6P is more closely related to that of UDP-glc than to that of glycogen, especially in short-term experiments. In further support of the validity of the UDP-glc data, the SRA of UDP-glc is low with [¹⁴C]inositol as a substrate. The high SRA of UDP-glc may be explained either by a special pool of UDP-glc for the synthesis of glycogen [45] or by the requirement of UDP-glc for the phosphorylation of primer molecules only. UDP-glc is not only used for glycogen synthesis. A major portion is consumed for the glucuronidation of many metabolites and foreign compounds [46]. Therefore it might be useful to investigate glycogen synthesis and glucuronidation in parallel. Although such experiments exist, they were done under different aspects and are not directly comparable to the present problem [46–49]. However some of the results described in [45] can be explained by the concept of this paper and possibly will become even more distinct by short-term experiments.

The concept of the so-called "zonation" of the liver [50, 51] should not be ignored in this context. There are still open fundamental questions regarding this concept as critically discussed in [52]. The different description of the lobular localization of glycogen deposits [18] and the specific isotope distribution along the products from gluco- and gluconeogenic substrates [53] also do not correspond with the concept postulated in [50].

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