Glucosamine Metabolism in Regenerating Rat Liver

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(Received 26 April 1976)

1. Glycoprotein synthesis was investigated with [1-¹⁴C]glucosamine *in vivo*. [¹⁴C]Glucosamine was administered intravenously 24 h after hepatectomy to rats. 2. Incorporation into the acid-soluble fraction was maximum at 15 min after injection both in sham-operated and hepatectomized rats. 3. Enhancement of incorporation into UDP-*N*-acetylhexosamine in regenerating liver was observed. However, its specific activity was lower, because of a greater enhancement of synthesis *de novo* of the amino sugar. 4. In the liver acid-insoluble fraction, maximum incorporation of [¹⁴C]glucosamine was at 30 min in sham-operated rats and 2 h in hepatectomized rats respectively. 5. In sham-operated rats, incorporation into the plasma acid-insoluble fraction followed that of the liver acid-insoluble fraction, but hepatectomy resulted in a rapid enhancement of incorporation into plasma. 6. It is concluded that synthesis of liver glycoproteins is stimulated after partial hepatectomy and that glycoproteins synthesized are released rapidly into the plasma.

In a previous communication (Akamatsu & Maeda, 1971), evidence was presented that glutamine-fructose 6-phosphate aminotransferase [glucosamine phosphate isomerase (glutamine forming), EC 5.3.1.19], which participates at the initial step of glycoprotein synthesis, was induced after partial hepatectomy.

To study further the synthesis of glycoprotein *in vivo*, D-[1-¹⁴C]glucosamine was injected into hepatectomized rats and its incorporation into liver acid-soluble and acid-insoluble fractions was investigated.

Glucosamine enters the glycoprotein synthetic pathway below the glutamine-fructose 6-phosphate aminotransferase step and is involved in UDP-*N*-acetylhexosamine synthesis (McGarrahan & Maley, 1962). Thus the rate of incorporation of [¹⁴C]glucosamine into amino sugar nucleotides and glycoprotein in liver reflects the activities of enzymes below glucosamine synthesis.

Materials and Methods

Chemicals

D-Glucosamine hydrochloride and 2,5-diphenyloxazole were from Wako Pure Chemical Ind., Osaka, Japan. *N*-Acetyl-D-glucosamine was from Tokyo Kasei Kogyo Co., Tokyo, Japan. D-Glucosamine 6-phosphate and *N*-acetylneuraminic acid were from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-Acetyl-D-glucosamine 6-phosphate was obtained by the phosphorylation of *N*-acetyl-D-glucosamine with *N*-acetyl-D-glucosamine kinase (EC 2.7.1.59),

which was prepared from pig spleen ((NH₄)₂SO₄ fraction) by the method of Datta (1970). The reaction product was applied to small column (1 cm×5 cm) of Dowex-1 (formate form) and the column was washed with water. *N*-Acetyl-D-glucosamine 6-phosphate was eluted by 4 M-formic acid and evaporated to dryness. This was dissolved in small volume of water and determined by the method of Reissig *et al.* (1955). *N*-Acetyl-D-glucosamine 6-phosphate has the absorbance as is obtained with an equimolar solution of *N*-acetyl-D-glucosamine (Reissig *et al.*, 1955). UDP-*N*-acetyl-D-glucosamine was from Boehringer, Mannheim, Germany. D-[1-¹⁴C]Glucosamine was from New England Nuclear Corp., Boston, MA, U.S.A. Dowex resins were from Dow Chemical Co., Midland, MI, U.S.A. NCS solubilizer was from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. Other chemicals were of commercial origin.

Animals

Male Wistar rats (Nippon Rat Co., Urawa, Japan) weighing 100–120 g were fed *ad libitum* with laboratory chow in a room at a constant temperature (23.5°C) with 12 h each of light (06:30–18:30 h) and darkness.

Partial hepatectomy (Higgins & Anderson, 1931) was performed between 09:00 h and 10:00 h, two-thirds of the liver being removed while the animals were kept under light diethyl ether anaesthesia. As a sham-operation, the abdominal wall was simply opened and closed. D-[1-¹⁴C]Glucosamine (1 or 2 μCi/0.5 μmol) was dissolved in 0.85% NaCl and injected into the tail vein 24 h after operation. At

desired intervals after injection, the animals were killed. The livers were immediately removed, frozen in solid CO₂/acetone, and pooled at -20°C. Blood was collected into a heparinized syringe from the abdominal aorta. Plasma was separated by centrifugation.

Preparation of liver acid-soluble extracts

The pooled livers were homogenized in a Waring Blender with 10 vol. of ice-cold 10% (w/v) trichloroacetic acid. The homogenates were centrifuged at 1000g and the residues were re-extracted with 5 vol. of 10% (w/v) trichloroacetic acid and pooled for the preparation of the acid-insoluble fraction. The supernatants were combined, extracted with diethyl ether three times to remove trichloroacetic acid, neutralized with 0.5 M-NH₃, and concentrated at 30°C under reduced pressure. The extracts were adjusted to pH 8 with 0.5 M-NH₃ and applied to Dowex 1 (X8; formate form; 200-400 mesh) columns (1 cm × 15 cm). Each column was washed with 75 ml of water (fraction I). Radioactivity adsorbed by the column was eluted by a modification of the method of Lukie & Forstner (1972) as follows: D-glucosamine 6-phosphate, 75 ml of 0.01 M-formic acid (fraction II); N-acetylneuraminic acid, 50 ml of 1 M-formic acid (fraction III); N-acetyl-D-glucosamine 6-phosphate, 75 ml of 4 M-formic acid (fraction IV); UDP-N-acetyl-D-glucosamine, 50 ml of 4 M-formic acid/0.4 M-ammonium formate (fraction V). Fraction V was passed through a Dowex 50 (H⁺ form; 100-200 mesh) column (1.2 cm × 24 cm) to remove NH₄⁺. The residues after evaporation of the above fractions were dissolved in water. The samples were adsorbed on filter paper and radioactivity was determined in 0.6% 2,5-diphenyloxazole in toluene with a Beckman LS-100 liquid-scintillation counter.

Quantitative analysis of UDP-N-acetylhexosamine

The sample was hydrolysed in 0.01 M-HCl at 100°C for 15 min, neutralized with 0.01 M-NaOH and N-

acetylhexosamine was determined by the method of Reissig *et al.* (1955) with N-acetyl-D-glucosamine as a standard.

Preparation of liver and plasma acid-insoluble fractions

Protein in plasma was precipitated by the addition of an equal vol. of ice-cold 20% (w/v) trichloroacetic acid, followed by centrifugation at 1000g for 10 min. It was then washed with 10% (w/v) trichloroacetic acid.

Acid-insoluble fractions of liver and plasma were washed once with 15 vol. of methanol/diethyl ether/chloroform (1:1:2, by vol.), twice with 5 vol. of 95% (v/v) ethanol, and dried overnight at 37°C. To 10 mg of the samples, 0.15 ml of NCS solubilizer was added, and the mixture was kept overnight at 50°C. Then 10 ml of 0.6% 2,5-diphenyloxazole in toluene was added and radioactivity was determined.

Determination of protein

Protein was determined colorimetrically by the method of Lowry *et al.* (1951) with egg albumin as a standard.

Results

Time-course of glucosamine incorporation after partial hepatectomy

Results in Table 1 show the incorporation of glucosamine into the acid-soluble fraction of liver and acid-insoluble fractions of liver and plasma during 48 h after hepatectomy. Incorporation reached a maximum at 24 h. Therefore subsequent experiments were carried out 24 h after the operation.

Incorporation of glucosamine into the liver acid-soluble fraction

[1-¹⁴C]Glucosamine (1 μCi; 0.5 μmol) was injected into rats 24 h after the operation. The animals were

Table 1. Incorporation of [¹⁴C]glucosamine into liver acid-soluble and acid-insoluble fractions, and the plasma acid-insoluble fraction

Animals were killed 20 min after injection of [1-¹⁴C]glucosamine (2 μCi, 0.5 μmol). Each value represents pooled samples from three animals.

Time after partial hepatectomy (h)	[¹⁴ C]Glucosamine incorporation		
	Liver		Plasma
	Acid-soluble fraction (10 ⁻³ × d.p.m./g of liver)	Acid-insoluble fraction (d.p.m./mg of protein)	Acid-insoluble fraction (d.p.m./mg of protein)
6	133	351	242
12	129	304	152
24	142	370	293
48	93	352	222

Table 2. Incorporation of [^{14}C]glucosamine into the acid-soluble fraction of liver

[^{14}C]Glucosamine ($1\mu\text{Ci}$, $0.5\mu\text{mol}$) was injected 24h after operation. The results are values from the pooled liver of three animals.

Time after injection (min)	$10^{-4} \times$ Incorporation of [^{14}C]glucosamine (d.p.m./g wet wt. of liver)	
	Sham operation	Partial hepatectomy
15	6.42	7.99
30	5.29	6.78
60	1.79	3.64
180	0.44	0.64
480	0.22	0.32

killed after 15, 30, 60, 180 and 480min successively. Liver acid-soluble fractions were prepared and total radioactivity was determined. As shown in Table 2, the incorporation of [^{14}C]glucosamine both in sham-operated and hepatectomized rats increased rapidly and reached a maximum 15 min after injection, thereafter decreasing gradually. Incorporation in hepatectomized rats was higher than that of sham-operated rats.

UDP-N-acetylhexosamine in liver acid-soluble fractions

Incorporation of [^{14}C]glucosamine into the UDP-N-acetylhexosamine fraction was 70–80% of the total acid-soluble fraction. This indicates that injected glucosamine is rapidly converted into UDP-N-acetylhexosamine. The result in sham-operated rats was consistent with that of Molnar *et al.* (1964).

As is shown in Table 3(a), a significant difference both in sham-operated and hepatectomized rats was not observed 15 min after [^{14}C]glucosamine injection. However, the incorporation increased 1.5 and 2.4 times 30 and 60 min after the operation respectively. In addition, the delayed decrease in radioactivity of UDP-N-acetylhexosamine was observed in regenerating liver.

The UDP-N-acetylhexosamine content of the liver was 0.21 and $0.35\mu\text{mol/g}$ wet wt. in sham-operated and hepatectomized rats respectively. This supports our previous report (Akamatsu & Maeda, 1971) that the activity of glutamine-fructose 6-phosphate aminotransferase, the enzyme of the rate-limiting step of glycoprotein synthesis, was increased after partial hepatectomy.

As was shown in Table 3(b), the specific activity of UDP-N-acetylhexosamine was lower in hepatectomized rats 15 min after injection. This fact indicates that the larger UDP-N-acetylhexosamine pool and its fast

Table 3. Incorporation of [^{14}C]glucosamine into liver UDP-N-acetylhexosamine

[^{14}C]Glucosamine ($1\mu\text{Ci}$, $0.5\mu\text{mol}$) was injected 24h after operation. Animals were killed at the time indicated. Each value represents pooled samples from three animals.

Time after injection (min)	$10^{-4} \times$ Incorporation of [^{14}C]glucosamine (d.p.m./g wet wt. of liver)		
	15	30	60
(a)			
Sham operation	5.15	3.68	1.14
Partial hepatectomy	5.88	5.41	2.74
	$10^{-5} \times$ Incorporation of [^{14}C]glucosamine (d.p.m./ μmol of UDP-N-acetylhexosamine)		
(b)			
Sham operation	2.45	1.75	0.55
Partial hepatectomy	1.68	1.56	0.78

rate of synthesis are diluting the glucosamine label more than in the control. The reversed tendency 60 min after the injection reflects the delayed decrease of incorporation of [^{14}C]glucosamine into UDP-N-acetylhexosamine.

Incorporation of [^{14}C]glucosamine into the acid-insoluble fraction of liver

As shown in Fig. 1, incorporation of [^{14}C]glucosamine into the liver acid-insoluble fraction was maximal 30 min after injection in sham-operated rats and decreased thereafter. This agrees with the result in normal rat liver of Robinson *et al.* (1964). In regenerating rat liver, however, the incorporation rose rather slowly and reached maximum 2h after injection. The delay seen is most likely to correspond to a slower flux of label through larger intermediary pools.

Incorporation of [^{14}C]glucosamine in acid-insoluble fraction of plasma protein

In sham-operated rats, incorporation of [^{14}C]glucosamine into plasma proteins increased with a maximum at 2–3h and decreased thereafter. The results presented here together with the data from the liver acid-insoluble fraction is consistent with the label in the liver acid-insoluble fraction being a precursor of the plasma protein radioactivity (Robinson *et al.*, 1964).

Fig. 1. Time-course of [^{14}C]glucosamine incorporation into liver and plasma acid-insoluble fractions of rats 24h after operation

[1- ^{14}C]Glucosamine ($1\mu\text{Ci}$, $0.5\mu\text{mol}$) was injected into rats. Liver and plasma acid-insoluble fractions were prepared as described in the text. $\circ$, Livers from sham-operated animals; $\bullet$, livers from partially hepatectomized animals. Δ , Plasma of sham-operated animals; $\blacktriangle$, plasma of partially hepatectomized animals.

In hepatectomized rats, however, the incorporation of [^{14}C]glucosamine into plasma increased rapidly without delayed incorporation as in sham-operated rats. Moreover, enhancement of the incorporation of [^{14}C]glucosamine into plasma protein was observed in regenerating liver, which shows the increase of glycoprotein synthesis.

Discussion

During the process of liver regeneration, increases of liver-cell size and number are observed (Bresnick, 1971). Since the glycoproteins are essential constituents of tissue stroma and of plasma membranes, there should be an increase of glycoprotein synthesis in regenerating liver.

Liver is the tissue most actively implicated in the incorporation of glucosamine into plasma protein (Robinson *et al.*, 1964). After partial hepatectomy, the residual liver cells have to synthesize plasma proteins for the maintenance of their concentrations. As was reported by several authors (Braun *et al.*, 1962; Majumdar *et al.*, 1967; Chandler & Snider, 1970), synthesis of plasma albumin was increased during the course of liver regeneration. Plasma glycoproteins have been shown to be synthesized mainly in liver (Schultze & Heremans, 1966), and the present study in regenerating liver shows marked enhancement of plasma glycoprotein synthesis.

The biosynthesis of glycoprotein occurs in a step-wise manner. Sugar units are attached to the protein moieties during the growth of the polypeptide chain in the endoplasmic reticulum and Golgi apparatus (Clauser *et al.*, 1972; Molnar, 1975). In the early stage

of liver regeneration, residual cells in liver may first have to synthesize protein. This is consistent with the fact that liver cells 24h after partial hepatectomy contain little endoplasmic reticulum and numerous free ribosomes (Stenger & Confer, 1966) and that secretory proteins including glycoproteins are produced on endoplasmic reticulum, whereas protein for the use of the cell itself is formed on free ribosomes. This may explain the delayed increase in the synthesis of glycoprotein in liver during the early stage of regeneration. In sham-operated rats, the synthesis of plasma glycoproteins followed that of liver glycoprotein synthesis. Synthesis of liver glycoproteins was stimulated after partial hepatectomy and glycoproteins synthesized were released rapidly into the plasma.

Bauer *et al.* (1976) have reported that galactosyl transferase (UDP-galactose-glycoprotein galactosyl-transferase) was induced after partial hepatectomy. Since, however, they assayed the enzyme activity only in the presence of exogenous acceptor, it is not clear whether pool size and capacity of the endogenous acceptor are increased or activity of the transferase is enhanced.

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