Metabolism of Sialic Acid in Regenerating Rat liver

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In regenerating rat liver slices 24 h after partial hepatectomy, the incorporation of [^{14}C]glucosamine into 'free sialic acid' (*N*-acetylneuraminic acid + CMP-*N*-acetylneuraminic acid) decreased to below 50% of the control values and the incorporation into protein-bound sialic acid decreased to the same extent. The incorporation of [^{14}C]glucosamine into 'free sialic acid' decreased during the period from 6 to 47 h after hepatectomy, showing a minimum at 12 h, and recovered to the control value by 96 h. At 12 h, the activities of UDP-*N*-acetylglucosamine 2-epimerase (UDP-2-acetoamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14) and *N*-acyl-D-mannosamine kinase (ATP: 2-acylamino-2-deoxy-D-mannose 6-phosphotransferase, EC 2.7.1.60) in the liver were significantly decreased. The amount of protein-bound sialic acid in the liver was not changed after partial hepatectomy, but the amount in plasma was changed, with a similar pattern to that of the incorporation of [^{14}C]glucosamine into slice 'free sialic acid'. These results indicate that the synthesis of sialic acid in the liver much decreases in the early stage of the regeneration and that this may be correlated with the decreased synthesis of plasma sialoglycoproteins.

During early periods of liver regeneration, an accumulation of UDP-*N*-acetylhexosamine and a low incorporation of [^{14}C]glucosamine into glycoproteins were observed in rat-liver slices (Okamoto & Akamatsu, 1977). These changes were mainly attributed to the decreased activity of *N*-acetylglucosamine transfer from UDP-*N*-acetylglucosamine to endogenous acceptors. But it was also observed that the decrease of the activity was less significant than that of [^{14}C]glucosamine incorporation into glycoproteins within 12 h after partial hepatectomy (Okamoto *et al.*, 1978).

Since glucosamine is also utilized for synthesis of sialic acid via UDP-*N*-acetylglucosamine (Kornfeld *et al.*, 1964), we studied the activity of sialic acid synthesis in slices and crude extract of liver as well as its correlation with the amount of protein-bound sialic acid in both liver and plasma.

Materials and Methods

Chemicals

N-Acetyl-D-mannosamine, *N*-acetylneuraminic acid, phosphoenolpyruvate, and ATP were from Sigma. UDP-*N*-acetyl-D-glucosamine was from Boehringer-Mannheim. *N*-Acetyl-D-mannosamine

6-phosphate was prepared from *N*-acetyl-D-mannosamine with *N*-acyl-D-mannosamine kinase (EC 2.7.1.60) which was prepared from rat liver up to the protamine sulphate step of the method of Kundig & Roseman (1966). The reaction product was applied to a Dowex 1 (X8; formate form; 200–400 mesh) column (1.2 cm $\times$ 30 cm). The column was washed with water. *N*-Acetyl-D-mannosamine 6-phosphate was eluted by 4 M-formic acid and lyophilized. The product was dissolved in a small volume of water. The purity was checked by paper chromatography before and after treatment with calf intestine alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1; Miles-Seravac, Holyport Berks., U.K.]. *N*-Acetyl-D-mannosamine 6-phosphate was determined by the method of Reissig *et al.*, (1955) with *N*-acetyl-D-mannosamine as a standard. D-[1- ^{14}C]Glucosamine hydrochloride (58 Ci/mol) and *N*-acetyl-D-[U- ^{14}C]mannosamine (270 Ci/mol) were from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine (235 Ci/mol) was from New England Nuclear, Boston, MA, U.S.A. Other chemicals were of commercial origin.

Animals

Male Wistar rats (100–120 g, Nihon CLEA,

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Tokyo, Japan) were fed *ad libitum* with laboratory chow in a room at a constant temperature (23.5°C) with 12h each of light (06.30–18.30h) and darkness. Partial hepatectomy (Higgins & Anderson, 1931) was performed between 09.00h and 10.00h and sham-operated rats were used as controls.

Preparation of liver slices and incubation procedure

Liver slices were prepared and incubated with D-[1-¹⁴C]glucosamine as described (Okamoto & Akamatsu, 1977).

Preparation of acid-soluble and acid-insoluble fractions

The acid-soluble and acid-insoluble fractions from the slices and the acid-insoluble fraction from the incubation medium were prepared as reported (Okamoto & Akamatsu, 1977).

Acid-insoluble fractions of livers and plasma were prepared as follows. Approx. 0.4g of liver was homogenized with 10vol. of 0.154M-NaCl, and 0.5vol. of ice-cold 30% (w/v) trichloroacetic acid was added to the homogenate. Blood (1–2ml) taken by cardiac puncture was immediately transferred into a tube containing 15mg of sodium citrate and the plasma was obtained by centrifugation at 1000g for 10min. Ice-cold 10% (w/v) trichloroacetic acid (4ml) was added to 0.2ml of the plasma. Both samples were cooled in ice for 20min, centrifuged at 1000g for 10min and the precipitates were washed once with chloroform/methanol/diethyl ether (2:1:1, by vol.), twice with 95% (v/v) ethanol, and then were dried under reduced pressure.

Separation and determination of sialic acid from acid-soluble and acid-insoluble fractions

Trichloroacetic acid was extracted from the acid-soluble fraction with diethyl ether. The fraction was adjusted to pH8 with 0.5M-NH₄OH, applied to a Dowex 1 (X8; formate form; 200–400 mesh) column (0.6cm × 13cm) and eluted with 50ml of 0.01M-formic acid followed by 25ml of 1M-formic acid. The latter fractions contained 'free sialic acid' (free *N*-acetylneuraminic acid and *N*-acetylneuraminic acid derived from CMP-*N*-acetylneuraminic acid) (Okamoto & Akamatsu, 1977).

The acid-insoluble fraction was suspended in 5% (w/v) trichloroacetic acid at a protein concentration of 15mg/ml or less and was hydrolysed at 80°C for 1h (Molnar *et al.*, 1965). After centrifugation, trichloroacetic acid was extracted from the supernatant with diethyl ether. The supernatant was adjusted to pH8 and sialic acid (derived from protein-bound sialic acid) was separated by column chromatography, as described for the acid-soluble fraction.

The fractions that contained sialic acid were

evaporated at 40–50°C. The residues were dissolved in a small volume of water and sialic acid was determined by the method of Warren (1959) with *N*-acetylneuraminic acid as a standard. To the radioactive samples, (1ml), 10ml of 0.6% 2,5-diphenyloxazole in toluene/Triton X-100 (2:1, v/v) was added. The radioactivity was determined by using a Beckman LS-100 liquid-scintillation counter.

Determination of protein-bound hexosamine in plasma

Protein-bound hexosamine was determined as previously reported (Okamoto *et al.*, 1978) in the residues of the hydrolysate of the plasma acid-insoluble fraction.

Enzyme assays

Livers were homogenized with 9vol. of 0.154M-KCl containing 10mM-potassium phosphate buffer (pH 7.6), 5mM-EDTA and 2mM-dithiothreitol in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 10000g for 10min and the supernatant was centrifuged again at 105000g for 1h. The post-microsomal supernatant was used as the crude extract. The activity of UDP-*N*-acetylglucosamine 2-epimerase (UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14) was assayed essentially by the method of Spivak & Roseman (1966). The reaction mixture contained the following components in a final vol. of 0.4ml: 0.2M-Tris/HCl buffer (pH 7.5), 2mM-UDP-*N*-acetyl-D-glucosamine, 50mM-MgSO₄ and 0.1ml of the liver extract. After incubation at 37°C for 30min, the reaction was stopped by heating the mixture at 100°C for 2min. To the reaction tube was added 180–220mg of dried Dowex 1 (X8; formate form; 200–400 mesh) resin and 0.6ml of water. The tube was shaken and was allowed to stand for 5min. After centrifugation, *N*-acetyl-D-mannosamine in the supernatant was determined by the method of Reissig *et al.* (1955) with *N*-acetyl-D-mannosamine as a standard.

The *N*-acyl-D-mannosamine kinase (ATP: 2-acylamino-2-deoxy-D-mannose 6-phosphotransferase, EC 2.7.1.60) activity was assayed as described below. The reaction mixture contained the following components in a final vol. of 0.5ml: 0.1M-Tris/HCl buffer (pH 8.1), 8mM-*N*-acetyl-[U-¹⁴C]mannosamine (0.27Ci/mol), 12mM-ATP, 10mM-MgCl₂, 24mM-NaF and 0.05ml of the liver extract. After incubation at 37°C for 20min, the tube was heated at 100°C for 2min and was then centrifuged at 1000g for 10min. The supernatant was applied to a Dowex 1 (X8; formate form; 200–400 mesh) column (0.6cm × 3.6cm). The column was washed with 8ml of water. *N*-Acetylhexosamine 6-phosphate was eluted with 12ml of 4M-formic acid and

evaporated. The residue was dissolved in 1.5 ml of water and an aliquot (0.5 ml) was treated with 5–6 units of alkaline phosphatase in 1.0 ml of 0.1 M-Tris/HCl buffer (pH 8.5) containing 2 mM-MgCl₂ at 37°C for 18 h. (One unit of alkaline phosphatase is the amount of the enzyme catalysing the liberation of one μ mol of *p*-nitrophenol from *p*-nitrophenyl phosphate/min at pH 10.25 and 37°C.) Then the mixture was deionized by passing it successively through columns of Dowex 50 W (X8; H⁺ form; 200–400 mesh) and Dowex 1 (X8; formate form; 200–400 mesh). The eluate was evaporated to dryness and dissolved in a small volume of water. A portion was spotted on borate-treated paper (Cardini & Leloir, 1957; Toyo no. 51). *N*-Acetylmannosamine was separated from *N*-acetylglucosamine by descending paper chromatography with a solvent system of butan-1-ol/pyridine/water (6:4:3, by vol.). The area containing *N*-acetylmannosamine was cut out and the radioactivity was measured in 10 ml of 0.6% 2,5-diphenyloxazole in toluene. The activity of *N*-acetylneuraminase-9-phosphate synthase [*N*-acetylneuraminase-9-phosphate pyruvate-lyase (pyruvate-phosphorylating), EC 4.1.3.20] was assayed essentially by the method of Watson *et al.* (1966). The reaction mixture contained the following components in a final vol. of 0.5 ml: 0.1 M-Tris/HCl buffer (pH 7.8), 2 mM-*N*-acetyl-D-mannosamine 6-phosphate, 2 mM-phosphoenolpyruvate, 20 mM-MgCl₂, 4 mM-dithiothreitol and 0.1 ml of the liver extract. The mixture was incubated at 37°C for 30 min, then heated at 100°C for 2 min. After centrifugation, the supernatant was used for the assay of *N*-acetylneuraminic acid 9-phosphate by the method of Warren (1959) with *N*-acetylneuraminic acid as a standard.

Determination of protein

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

Results

Time course of [¹⁴C]glucosamine incorporation into 'free' and protein-bound sialic acid

In regenerating liver slices and controls at 24 h after the operation, the incorporation of [¹⁴C]-glucosamine into 'free sialic acid' increased at nearly constant rates up to 120 min (Fig. 1a). The incorporation into protein-bound sialic acid in the slices increased after a lag of 30 min and exceeded the incorporation into 'free sialic acid' at 120 min (Fig. 1b). Uptake into protein-bound sialic acid of the medium rapidly increased after 60 min (Fig. 1c). These patterns of uptake indicate that the radioactivity taken up first into 'free sialic acid' was transferred to glycoproteins and then released

Fig. 1. Time course of [¹⁴C]glucosamine incorporation into (a) 'free sialic acid' of slices, (b) protein-bound sialic acid of slices and (c) protein-bound sialic acid of medium. Liver slices (about 200 mg each) prepared from rats at 24 h after partial hepatectomy or sham operation were incubated with 0.5 μ Ci of [1-¹⁴C]glucosamine. ○, Controls; ●, regenerating liver. Each point represents the mean $\pm$ S.E.M. for five animals.

into the medium. In the regenerating liver, the assimilation of [^{14}C]glucosamine into 'free sialic acid' was lower than in the control, and the incorporation into protein-bound sialic acid in the slices and the medium was also decreased to the same extent. These results suggest that the rate of synthesis of 'free sialic acid' in the liver may restrict the amount of sialic acid transferred to glycoproteins at 24 h after partial hepatectomy.

Changes in [^{14}C]glucosamine incorporation into 'free sialic acid' during liver regeneration

The decreased incorporation of [^{14}C]glucosamine into 'free sialic acid' was already significant at 6 h after partial hepatectomy, reached the lowest value (20% of the control) at 12 h and continued up to 48 h. Then, the incorporation returned to the control value at 96 h (Fig. 2). These decreased values may be due to the decreased synthesis of sialic acid. Thus the enzyme activities that participate in the synthesis of sialic acid were assayed at 12 h after the operation.

Enzyme activities of sialic acid synthesis

UDP-*N*-acetylglucosamine 2-epimerase, which has been shown to play a regulatory role in sialic acid synthesis (Kornfeld *et al.*, 1964), catalyses the formation of *N*-acetyl-D-mannosamine. *N*-Acetyl-D-mannosamine is converted via *N*-acetyl-D-mannosamine 6-phosphate to *N*-acetylneuraminic acid 9-phosphate by *N*-acyl-D-mannosamine kinase and

N-acylneuraminate 9-phosphate synthase respectively. Accordingly, the activities of these three enzymes were assayed at 12 h. They were all localized in the 105 000g supernatant (data not shown). Under the assay conditions described above, the formation of the reaction products was linear with respect to time and amounts of enzyme proteins.

In the assay of UDP-*N*-acetylglucosamine 2-epimerase activity, the possibility of the formation of *N*-acetyl-D-glucosamine via *N*-acetyl-D-glucosamine 1-phosphate from UDP-*N*-acetyl-D-glucosamine by UDP-acetylglucosamine pyrophosphatase and phosphatase and/or from *N*-acetyl-D-mannosamine by *N*-acylglucosamine 2-epimerase (2-acetylamido-2-deoxy-D-glucose-2-epimerase, EC 5.1.3.8; Datta, 1970) was checked. The composition of the reaction mixture was the same as in the UDP-*N*-acetylglucosamine 2-epimerase assay except that 2 mM-UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine (0.125 Ci/mol) was used. The supernatant that was obtained after the treatment with Dowex 1 resin was evaporated and dissolved in a small volume of water. The separation of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine by paper chromatography and determination of the radioactivity were as described for the *N*-acylmannosamine kinase assay. *N*-Acetyl-D-mannosamine was only 3–4% of *N*-acetyl-D-mannosamine in both control and regenerating livers.

In the regenerating liver, the activities of UDP-

Fig. 2. Changes in [^{14}C]glucosamine incorporation into 'free sialic acid' during liver regeneration. Liver slices (about 200 mg each) were incubated with 0.5 μCi of [^{14}C]glucosamine for 1 h. The incorporation into regenerating livers is expressed as the differences from controls. The mean values of three to five each of hepatectomized and control rats were used for calculation at each time point. * The differences from controls are statistically significant ($P < 0.05$); **, the differences from controls are statistically significant ($P < 0.01$).

Table 1. Activities of UDP-N-acetylglucosamine 2-epimerase, N-acylmannosamine kinase and N-acylneuraminase 9-phosphate synthase

Cytosol (105 000g supernatant) fractions were prepared from rat livers 12 h after partial hepatectomy and sham operation and were used for the assay of the enzyme activities. One unit is defined as the amount of enzyme that catalysed the formation of 1 nmol of product/30 min. Each value represents the mean $\pm$ S.E.M. for four to seven animals. * $P < 0.05$ as compared with controls.

Animals	Specific activity (units/mg of protein) of		
	UDP-N-acetylglucosamine 2-epimerase	N-Acylmannosamine kinase	N-Acylneuraminase 9-phosphate synthase
Controls	46.6 $\pm$ 3.1	64.1 $\pm$ 4.6	21.2 $\pm$ 0.9
Regenerating livers	33.6 $\pm$ 3.5*	40.7 $\pm$ 6.3*	18.3 $\pm$ 1.6

Fig. 3. Concentration of protein-bound sialic acid in plasma at various times after operation

Acid-insoluble fractions of plasma were prepared and the concentration of protein-bound sialic acid was determined as described in the Materials and Methods section. O, Controls; ●, regenerating livers. Each point represents the mean $\pm$ S.E.M. for three to five animals.

N-acetylglucosamine 2-epimerase and N-acylmannosamine kinase were 72 and 64% respectively of the control values (Table 1). No significant difference was observed in the activity of N-acylneuraminase-9-phosphate synthase. These results indicate that the substantial decrease in sialic acid synthesis is mainly caused by the decreased formation of N-acetyl-D-mannosamine and N-acetyl-D-mannosamine 6-phosphate.

Amount of protein-bound sialic acid in liver and plasma

The correlation of the decrease in the activity of sialic acid synthesis with the amount of protein-

bound sialic acid *in vivo* was examined. Differences were not observed between the regenerating liver and the control. A similar result was reported by Harms *et al.* (1973). On the other hand, the amount in plasma was 45 and 60% respectively of the control at 12 and 48 h after hepatectomy, then increased and returned to the control level at 144 h (Fig. 3). The pattern of the changes was very similar to that of the incorporation of [14 C]glucosamine into liver-slice 'free sialic acid'. The decreased synthesis of sialic acid in liver may be correlated with the decreased incorporation of sialic acid into plasma glycoproteins at early periods of regeneration.

Larmo *et al.* (1962) reported that the total

Table 2. *Sialic acid and hexosamine contents of plasma proteins*

The plasma was taken from rats 12h after partial hepatectomy or sham operation. Each value represents the mean $\pm$ S.E.M. for five or six animals. Values in parentheses are percentage differences from controls. * $P < 0.05$ as compared with controls.

Animals	Contents (nmol/mg of protein) of	
	Sialic acid	Hexosamine
Controls	48.8 $\pm$ 2.7	89.6 $\pm$ 4.1
Regenerating livers	29.4 $\pm$ 5.0* (-40)	71.9 $\pm$ 6.4* (-20)

hexosamine concentration in serum declined soon after partial hepatectomy. In the present experiments, the hexosamine content in plasma proteins was also decreased at 12h. In addition, the sialic acid content of plasma proteins became significantly lower (Table 2).

Discussion

The synthesis of sialic acid was decreased in regenerating rat liver within 48h after partial hepatectomy, and this can be explained by the lowered activities of UDP-*N*-acetylglucosamine 2-epimerase and *N*-acetylmannosamine kinase at 12h after hepatectomy. Okubo *et al.* (1977) reported that the activity of UDP-*N*-acetylglucosamine 2-epimerase in regenerating rat liver was higher than in controls at 24h after partial hepatectomy, but decreased thereafter and became lower than in controls at 72 and 120h. They also showed that the concentration of CMP-*N*-acetylneuraminic acid decreased during the period from 24 to 120h after partial hepatectomy. These observations, together with our results, suggest that a decrease of sialic acid synthesis is a characteristic common to rat liver at early periods of regeneration, though the pattern of the enzyme response to partial hepatectomy may vary according to the differences in the age of the rats or other experimental conditions.

The activities of UDP-*N*-acetylglucosamine 2-epimerase and *N*-acetyl-D-mannosamine kinase are extremely low in a variety of rat hepatomas and in foetal liver as compared with adult rat liver (Kikuchi *et al.*, 1971), corresponding with our results of the changes of the enzyme activities at 12h after partial hepatectomy. Possibly, the lowered activities of these two enzymes are intimately correlated with liver growth.

Sialyltransferase activities towards exogenous acceptors have been shown to increase significantly after partial hepatectomy (Serafini-Cessi, 1977;

Ip, 1979). But the enzyme activities towards endogenous acceptors are not changed significantly from 7 to 48h after such treatment (Serafini-Cessi, 1977). Thus the decreased synthesis of CMP-*N*-acetylneuraminic acid may lower the addition of sialic acid to glycoproteins. During early periods of liver regeneration, the synthesis of plasma glycoproteins decreases (Okamoto & Akamatsu, 1977; Serafini-Cessi, 1976). The decreased synthesis of sialic acid in the liver was linked with the low level of protein-bound sialic acid in plasma. On the other hand, the level of protein-bound sialic acid in regenerating liver was not changed. Prolongation of the half-life of sialoglycoproteins in regenerating rat liver was reported by Tauber & Reutter (1978). This may contribute to the maintenance of the amount of protein-bound sialic acid in the liver.

The decline of sialic acid synthesis was most pronounced at 12h after partial hepatectomy. This is thought to be the time immediately before the initiation of DNA synthesis in regenerating rat liver (Bresnick, 1971). It is interesting to study how the changes in sialic acid metabolism are related to other pre-replicative changes in the liver. For example, a decrease of the synthesis of CMP-*N*-acetylneuraminic acid can contribute to the increase of the CTP pool in regenerating liver (Bucher & Swaffield, 1969).

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