INHIBITION OF PROTEIN SYNTHESIS BY HEXOSAMINE CONTAINING GLYCOGEN FORMED IN MOUSE LIVER AFTER TREATMENT WITH D-GALACTOSAMINE

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1. Introduction

Large doses of D-galactosamine induce acute hepatic injury in a variety of species [1,2]. One of the early effects of galactosamine is the inhibition of hepatic protein synthesis [3,4]. Uridylate deficiency has been considered as the primary biochemical damage caused by galactosamine [5], however uridine failed to revert the decrease of amino acid incorporation [6,7]. Moreover, direct damage to the protein synthesizing apparatus has been suggested by electron-microscopic findings of electron dense intracellular aggregates, tentatively identified as clusters of ribosomes and glycogen particles [6,8–10]. Anomaly of hepatic glycogen synthesis has been demonstrated by Maley et al. [11]; non-*N*-acetylated UDP-glucosamine, a major product of the hepatic metabolism of galactosamine is a false substrate for the glycogen synthetase enzyme [12].

Complexes of heavy microsomal membranes and anomalous glycogen distinguished by an unusually high density have been isolated previously, by ultracentrifugation in sucrose density gradient, from livers of mice treated with subtoxic doses of galactosamine and glucose [13]. In an effort to model the formation of intracellular aggregates, isolation and characterization of glucosamine containing glycogen was undertaken. The purified polysaccharides (named aminoglycogen-I and -II) precipitated mouse liver ribosomes and inhibited amino acid incorporation in the poly(U) directed ribosomal system as well as in the liver post-mitochondrial supernatant system.

2. Experimental

2.1. *Isolation of aminoglycogen*

Some properties of the basic polysaccharide (strong protein adsorption, relatively high solubility in aqueous ethanol) rendered the usual methods of glycogen isolation inapplicable, therefore the following method was devised.

White CLP mice, starved for 48 h to deplete liver glycogen, were injected intraperitoneally with D-glucose (2.5 g/kg body weight) and D-galactosamine·HCl (Calbiochem; 0.25 g/kg). Controls received D-glucose only. After 3 h the livers were removed and homogenized in 5 vol. ice-cold perchloric acid, 0.3 M. The pooled homogenate was centrifuged at 6000 × *g* for 20 min. The supernatant was neutralized to pH 7.0 with Na_2CO_3 crystals. Centrifugation at 10 000 × *g* for 20 min removed a small amount of (protein) precipitate, which was discarded. Ultracentrifugation of the supernatant at 40 000 × *g* for 90 min yielded a transparent polysaccharide gel at the bottom of the tube, which was dissolved in distilled water and precipitated by addition of 2 vol. ethanol, then dried. This procedure yielded aminoglycogen-I and normal glycogen, 4–7 mg/g of wet liver.

For the preparation of aminoglycogen-II mice were treated with D-galactosamine·HCl, in a dose of 1 g/kg, which is known to cause hepatic damage in mice. The isolation of the polysaccharide was performed as described above with the modification that because of the relatively high solubility of aminoglycogen-II in aqueous ethanol it was recovered from

the suspension in distilled water by ultracentrifugation. The yield was 0.4–0.6 mg/g wet liver.

2.2. *Properties and composition of aminoglycogen*

Solubility of aminoglycogen in water was similar to that of normal glycogen, however slow aggregation was observed in concentrated solutions (100 mg/ml). Since the polysaccharides were excluded from Sepharose 2B when subjected to gel filtration, it was assumed that their molecular weight was higher than 3×10^7 d.

Analytical data are summarized in table 1. Glucose was estimated by the primary cysteine–sulphuric acid reaction [14]. Protein was determined by the method of Lowry et al. [15] using bovine serum albumin as standard. Ninhydrin reaction was performed according to Lee and Takahashi [16] with L-glucosamine·HCl as standard. Dinitrophenylation of aminoglycogen-I was carried out as recommended by Fraenkel-Conrat et al. [17]; to remove adsorbed reagent the polysaccharides were dissolved in 1 M NaOH at 60°C and precipitated with two volumes of ethanol. The latter steps were repeated three times and resulted in the complete bleaching of normal glycogen processed simultaneously. *N*-2,4-Dinitrophenyl glucosamine was prepared as described by Annison et al. [18] and served as standard; the absorbance of the product in ethanolic solution was similar to that reported. Dissolved in 1 M NaOH, maximum absorbance was found at 362 nm, $E^{1\%}_{1\,cm} = 10\,500$. The absorbance spectrum of dinitrophenylated aminoglycogen in alkaline solution was similar, thus the amount of dinitrophenylated amino groups could be calculated on the basis of the estimation of absorbance. Correction for glycogen turbidity was made at each spectrophotometric assay.

The presence of glucosamine was demonstrated in the acid hydrolysate of aminoglycogen. Considering the stabilizing effect of the unsubstituted amino group on the glycosidic bond, and the destruction of glucosamine by acid [19], optimum conditions of hydrolysis were established in preliminary experiments. Maximum yield of glucosamine liberated from aminoglycogen was found when 1 mg/ml polysaccharide was treated with 4 M HCl at 105°C for 12 h. Losses (about 10%) were estimated by the addition of galactosamine as internal standard, which was shown to decompose at a rate similar to that of glucosamine treated with hot HCl in the presence of excess glucose. The hydrolysates were evaporated to dryness in vacuo, and applied to a JEOL amino acid analyser. Normal glycogen did not contain any hexosamine. Glucosamine but no galactosamine was found in aminoglycogen-I and -II; traces of amino acids were also detectable.

2.3. *Precipitation of liver ribosomes by aminoglycogen*

Mouse liver was homogenized in 5 vol. of TKM-glycerol buffer (50 mM Tris–HCl, pH 7.6, 80 mM KCl, 5 mM $MgCl_2$, 6 mM mercaptoethanol, and glycerol 2%, v/v), and centrifuged at 10 000 × *g* for 10 min, yielding the post-mitochondrial supernatant. Ribosomes were prepared by deoxycholate (0.2%) treatment of the pellet gained by the ultracentrifugation of the post-mitochondrial supernatant [20]. RNA content of the preparations was measured according to Logan et al. [21].

Table 1
Composition of aminoglycogen-I and -II

Component	Aminoglycogen I	Aminoglycogen II	Normal glycogen
Glucose residues	98.1	95.4	98.8
Protein	0.42	0.56	0.33
Glucosamine residues, estimated as free amino groups of the intact molecule by			
ninhydrin reaction	0.65	3.74	0
dinitrophenylation	1.34	–	0
Glucosamine residues, demonstrated in the hydrolysate (corrected values)	1.25	4.58	0

Results are expressed as g/100 g purified polysaccharide.

Solutions of ribosomes (0.5 mg RNA/ml) and of normal glycogen (10 mg/ml), or aminoglycogen-I or -II (10 mg/ml) in TKM-glycerol buffer were combined at ratios 1:10 to 10:1 (v/v) at room temperature. After thorough mixing, a flocculent precipitate was observed in each ribosome–aminoglycogen mixture; normal glycogen had no such effect. When equal volumes of solutions of ribosomes and aminoglycogen-II were mixed and centrifuged at 2000 × *g* for 10 min, the precipitate contained the whole amount of aminoglycogen and 69.7% of ribosomes.

The ionic strength of the suspending media was increased by adjusting the concentration of KCl to 1 M. No precipitation occurred in the mixtures in this case. However, ribosomes pelleted from the high-salt medium and resuspended in standard TKM-glycerol buffer precipitated as before when mixed with aminoglycogen in the same buffer. It was concluded that ionic interactions were of primary importance in the precipitation reaction.

2.4. *Effect of aminoglycogen on [^{14}C]phenylalanine incorporation in poly(U) directed ribosomal system*

In order to study the functional consequences of aminoglycogen–ribosome aggregation, a ribosomal system [20] with charged tRNA and excess synthetic mRNA was applied. Ribosomes were prepared as described above. The 110 000 × *g* supernatant of liver postmitochondrial fraction was treated with ammonium sulphate (530 g/litre); the precipitate was extensively dialysed against TKM-glycerol buffer and used as a source of soluble factors. Yeast tRNA was prepared [22] and fractionated according to Pearson et al. [23]. The synthesis of aminoacyl-tRNA from $tRNA^{Phe}$ fractions and L-[^{14}C]phenylalanine (270 Ci/mol; Radiochemical Centre, Amersham) was based on the method of Kaji [24].

In this system, polyphenylalanine synthesis was directly proportional to the amount of ribosomes. No incorporation occurred when poly(U) was omitted.

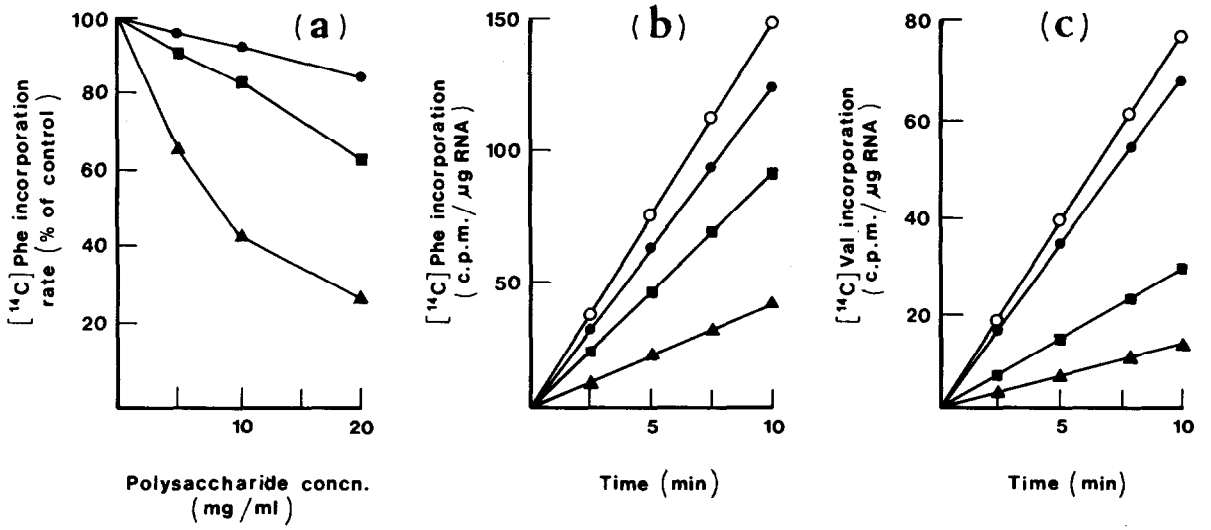

Fig.1. Effect of aminoglycogen-I and -II on amino acid incorporation in poly(U) directed ribosomal system (a and b) and in postmitochondrial supernatant system (c). Polysaccharides, (○) none, (●) normal glycogen, (■) aminoglycogen-I, (▲) aminoglycogen-II, were present either in different concentrations as indicated (experiment a) or at 20 mg/ml (experiments b and c). Assay mixtures contained 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase (Calbiochem), and TKM-glycerol buffer. The ribosomal systems (a and b) contained liver ribosomes (0.25 mg RNA/ml), soluble liver extract (7 mg protein per ml), 0.5 mg/ml poly(U), and 1.5 × 10^5 dpm/ml [^{14}C]phenylalanyl-tRNA; $MgCl_2$ concentration was adjusted to 15 mM. System (c) contained liver postmitochondrial supernatant (0.4 mg RNA/ml), 0.1 mg/ml tRNA, 3 µCi/ml [^{14}C]valine, and the 19 other amino acids, 10 µM each. Samples were pipetted into Whatman 3 MM filter paper discs, and the radioactivity incorporated into the hot trichloroacetic acid insoluble material was assayed by the method of Mans and Novelli [27]. Counting efficiency was about 50%.

Aminoglycogen caused some turbidity when mixed with the soluble liver extract, whereas it turned the complete, clear incubation mixture into a milky, viscous solution. Glycogen had no similar effects. Incorporation of [^{14}C]phenylalanine increased proportionally with time even when inhibited by aminoglycogen, thus allowing the calculation of incorporation rates (figs.1a and b).

2.5. *Effect of aminoglycogen on [^{14}C]valine incorporation in liver post-mitochondrial supernatant system*

In view of the predominant role of membrane-bound ribosomes in hepatic protein synthesis, the effect of aminoglycogen was also tested in a crude system described by Pain [25]. Mouse liver post-mitochondrial supernatant was prepared as described above, and it was used without gel filtration. Addition of tRNA increased the incorporation of L-[^{14}C]valine (125 Ci/mol; Isotope Institute, Prague).

Addition of aminoglycogen caused a marked increase in the turbidity of the reaction mixture and resulted in a strong inhibition of [^{14}C]valine incorporation into protein (precipitated with hot trichloroacetic acid). Radioactivity in charged tRNA (measured as the difference between the amounts of radioactivity precipitated with hot and cold acid) was unaltered in the presence of aminoglycogen.

3. Discussion

In order to investigate the possible role of modified glycogen in the hepatocellular damage induced by D-galactosamine, two types of glucosamine containing polysaccharides (aminoglycogen-I and -II) were isolated. The relatively high uridylate synthesizing capacity of mouse liver [5] may have acted in favour of polysaccharide synthesis even under conditions of high UTP demand. The presence of free amino groups was demonstrated by dinitrophenylation of the intact macromolecule, the result being in good agreement with that of hexosamine analysis of the hydrolysate (table 1). However, part of the amino groups did not react with ninhydrin as judged by the colour yield. Glucosamine was the only amino sugar found in our preparations, at variance with the report on simultaneous occurrence of glucosamine and galactosamine in liver glycogen of galactosamine treated rats [26]. Aminoglycogen-I and -II differed in their glucosamine content (1.24% and 4.58%, resp.), which values were considerably higher than that (0.1%) calculated from the data of Maley et al. [11].

Our experiments, modelling the interactions of aminoglycogen with rough endoplasmic membranes and ribosomes observed in liver cells after galactosamine treatment, show that aminoglycogen may attach to ribosomes as well as to heavy microsomes [13]. Ionic forces may have primary importance in the formation of complexes of negatively charged ribosomes and the polysaccharide bearing protonated amino groups. The protein synthesizing particles evidently suffer gross physicochemical changes when entering into complexes with aminoglycogen, and their deficient physiological activity is thus not unexpected; however, a strict parallelism between aggregation and inactivation is not necessary. The inhibition of amino acid incorporation in cell-free systems depended on the concentration and basic properties of the aminoglycogen added. It is reasonable to suppose a similar effect of aminoglycogen in vivo, too.

We wish to stress two unusual features of the phenomena described. (i) Large amounts of an ubiquitous sugar derivative (galactosamine) cause the intracellular transformation of a neutral storage polysaccharide into a toxic, basic heteropolysaccharide. (ii)A physiological interaction between particles of several million daltons was seen to occur.

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