Glucosamine is a normal component of liver glycogen

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There have been several reports of the incorporation of glucosamine into liver glycogen by an intraperitoneal injection of galactosamine, but it has not previously been considered that glucosamine is a normal component of liver glycogen. We now report that glucosamine occurs endogenously in rabbit- and pig-liver glycogens in the amount of about 1 nmol per 10 mg glycogen. Like the glucosamine incorporated by exogenous administration of galactosamine, the endogenous glucosamine takes the place of 1,4-linked α-glucose residues. It is found in both the outer and inner chains of the glycogen molecule.

Liver glycogen *Kidney glycogen* *Galactosamine* *Glucosamine* N-*Methylglucosamine*

1. INTRODUCTION

Intraperitoneal injection of D-galactosamine into rats has been studied from several viewpoints [1-5]. The process induces liver damage; it may be used as a model system for studies on human viral hepatitis [3]. One of the most overt biochemical manifestations is the result of D-galactosamine being metabolized by the same pathway as is galactose [1,2,4]. In this way [^{14}C]galactosamine injected intraperitoneally or administered by liver perfusion results in some of the glucose residues of glycogen being replaced by [^{14}C]glucosamine. The metabolic route is shown in fig.1 and from it one can understand the paradox that administration of glucosamine itself does not lead to the sugar being incorporated into glycogen. This is because the nucleoside diphosphate sugar formed from glucosamine is UDP-*N*-acetylglucosamine. This is not a substrate for glycogen synthase, where the non-esterified UDPglucosamine, formed from galactosamine, is a substrate [4]. We have already reported on this phenomenon [5], demonstrating

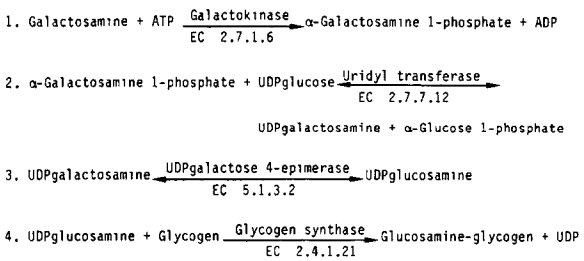

Fig.1. Route of incorporation of glucosamine into glycogen following the parenteral administration of glucosamine [1,2,4].

that as much as 20% of the injected galactosamine finds its way into glycogen and that as many as 10% of the glucose residues can be replaced by the amino sugar. Furthermore, it was observed that the glucosamine does not act as a metabolic block since it can be removed by phosphorylase with the formation of α-glucosamine 1-phosphate. We have been interested in the possible presence of a covalently bound protein in muscle and liver glycogens and, for example, reported the presence of such material in the liver glycogen fraction insoluble in cold 10% trichloroacetic acid [6]. We then turned our attention to the glycogen soluble in this extractant and found that we could apparently detect a very small amount of protein by reductive

We are pleased to join in tribute to Professor S.P. Datta, the first Managing Editor of FEBS Letters, by dedicating this paper to him on the occasion of his retirement

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methylation of supposed amino groups (N-terminal or ϵ-amino lysyl groups) with [^{14}C]formaldehyde in the presence of cyanoborohydride [7]. However, we found that the label introduced in this way was almost completely resistant to removal by pronase or treatment with 13.5 N sodium hydroxide at 120°C for 30 min, both of which procedures are destructive to protein. As a result, the ^{14}C label remained with the high-molecular-mass glycogen component, excluded on chromatography with Sephacryl S-200. Our research for what this pronase- and alkali-resistant label might be revealed that we had succeeded in *N*-methylating the amino groups of glucosamine present endogenously in the liver glycogen.

2. MATERIALS AND METHODS

2.1. *Materials*

DFP-treated crystalline pancreatic α-amylase was from Sigma (grade VII-A), crystalline *Rhizopus niveus* glucoamylase was from Miles, and crystalline sweet-potato β-amylase from Worthington. Pronase was a gift from Dr W.M. Awad. Sodium cyanoborohydride was from Aldrich, Sephadex and Sephacryl from Pharmacia and [^{14}C]formaldehyde from New England Nuclear. DEAE-cellulose was the Whatman DE-52 grade.

2.2. *Methods*

Rabbit-liver glycogen was obtained from a well-fed rabbit killed by cervical fracture. The liver was quickly excised and blended in an equal volume of ice-cold 10% trichloroacetic acid, centrifuged for 15 min at 3500 × *g* and the supernatant treated with 2 vols ethanol. The liver residue was then re-extracted with 2 vols of 5% trichloroacetic acid and similarly centrifuged followed by ethanol precipitation of the supernatant. The precipitated glycogen was dissolved in the minimum of water and centrifuged for 15 min at 3500 rpm to precipitate insoluble material. Next sodium sulfate was added to about 10 mM and the glycogen again precipitated with ethanol (2 vols). After centrifuging and redissolving in water, the solution was extracted with ⅓ vol. of chloroform:octanol (3:1), reprecipitated, re-extracted and precipitated, dissolved and lyophilized. The glycogen was then passed through a DEAE-cellulose column (5 × 17 cm) in 50 mM ammonium bicarbonate, pH 7.6. The glycogen, 99% of which did not bind, was then eluted through Sepharose CL-6B (5 × 17 cm) in the same medium, collecting the carbohydrate emerging in the excluded volume.

Reductive methylation [7] of the glycogen was carried out by treatment of 1 g glycogen in 10 ml of 0.1 M Hepes (pH 7.5) with [^{14}C]formaldehyde (0.4 μmol, 20 μCi) and 1 ml of 0.2 M sodium cyanoborohydride at room temperature overnight. Excess formaldehyde was removed by addition of 0.1 M sodium borohydride and the excess reducing agent destroyed by dropwise addition of acetic acid. The glycogen was recovered from Sepharose Cl-6B as above. A second and third *N*-methylation was found to increase further the specific radioactivity. A typical final specific radioactivity was 922 cpm/mg glycogen.

Glucosamine was released from glycogen (50 mg in 0.5 ml of 0.1 M NH_4Ac, pH 5.0) on incubation for 24 h at 37°C with glucoamylase (12 U) followed by addition of 1 M $CaCl_2$ (10 μl) and α-amylase (4 U) and incubation for a further 24 h. A sample (20 μl) was removed for determination of glucose and the remainder was loaded onto a Jeol amino acid analyzer for determination of glucosamine. When glycogen was digested separately with these 2 enzymes the conditions were similar, except that the pH of the α-amylase digest was 7.0. For digestion with β-amylase and for measurements of total carbohydrate concentration, reducing sugar and glucose, the methods in [5] were used. Pronase digestions were carried out using 5 mg/ml of enzyme in 0.1 M Tris-HCl buffer, pH 7.9, for 24 h at 45°C and another 24 h at 55°C. Electrophoresis was carried out on Whatman 3 mm paper in a Savant Instruments machine with 6% acetic acid/ 0.6% pyridine in water as the medium at 40 V/cm for 2 h. About 20000 cpm of each mixture to be separated was applied to the paper. To reveal radioactive spots the paper was exposed to X-ray film for 1 week at −70°C.

3. RESULTS

3.1. *Attempts to release the ^{14}C label from reductively methylated liver glycogen*

As noted above, the ^{14}C label incorporated from [^{14}C]formaldehyde into rabbit-liver glycogen in the presence of cyanoborohydride, a process which leads to the methylation of amino groups, was

almost completely resistant to the actions of pronase, or of strong alkali at 120°C. The ^{14}C label remained almost completely associated with the glycogen in the excluded volume of a Sephacryl S-200 column. Therefore, the majority of the incorporated label could not be present in the protein. When the glycogen was treated with α-amylase or glucoamylase, or a combination of the 2 enzymes, and the products were fractionated on Sephadex G-25, the results shown in fig.2 were obtained. When glucoamylase alone was used (fig. 2B), the glycogen was almost completely converted into glucose but the radiolabel was excluded, as if it were present in a glucoamylase-resistant macromolecule. By contrast, when the glycogen was digested with α-amylase (fig.2C), the profile of the radiolabel followed rather closely that of the carbohydrate. Therefore, the radiolabel was present within the glycogen molecule in such a way that the endo action of α-amylase could release the label in lower-M_r oligosaccharides. The resistance of the label to glucoamylase would be expected because, although unesterified 1,4-linked α-glucosamine is liberated by glucoamylase [5], the *N*-methylated sugar will act as a barrier to the exo action of this enzyme. When both glucoamylase and α-amylase were used to degrade the glycogen (fig.2D), the radiolabel was expectedly present in materials with M_r values in the oligosaccharide range, with the bulk of the glycogen converted into glucose.

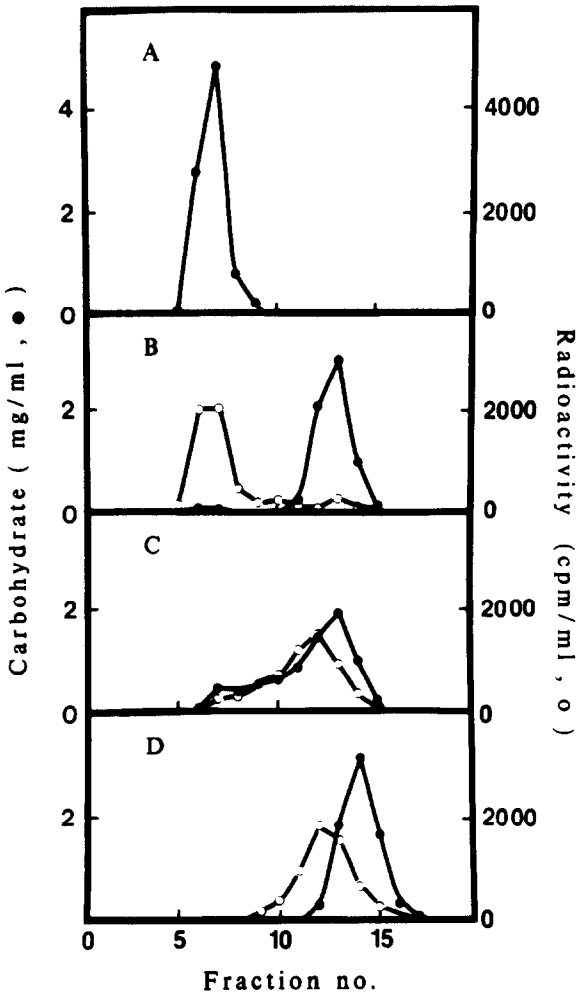

Fig.2. Behavior of the ^{14}C label when *N*-[^{14}C]methylated rabbit-liver glycogen (A) was treated with glucoamylase (B), α-amylase (C) and a mixture of the 2 enzymes (D). The products were fractionated on Sephadex G-25 (0.7×20 cm, 0.5 ml fractions).

When the same α-amylase/glucoamylase digest of the radiolabelled glycogen was passed through CM-Sephadex, almost all the radiolabel was retained, while most of the carbohydrate emerged from the column unhindered. The retained label could be displaced with 1% ammonium hydroxide (not shown).

3.2. *Identification of the ^{14}C-labelled component of liver glycogen as an amino sugar.*

Our investigations so far had revealed that radiolabelling of the glycogen by reductive methylation had detected a substance which was an integral part of the glycogen molecule and was released by α-amylase into material having the same M_r as the branched oligosaccharides, commonly referred to as the α-limit dextrins, that are among the products of α-amylase action. With the knowledge that the group that had been labelled was presumably an amino group, and our prior knowledge of the ability to incorporate exogenously administered galactosamine into glycogen as glucosamine, we reasoned that we may have detected the natural occurrence of glucosamine in glycogen. Our next efforts were therefore directed towards comparing the radiolabelled glycogen component with authentic *N*-methylglucosamine.

Accordingly, we carried out paper electrophoresis of the radiolabelled fragments released from glycogen by α-amylase/glucoamylase (fig. 2D) and concentrated by adsorption on CM-Sephadex. A radioautograph (fig.3) revealed a

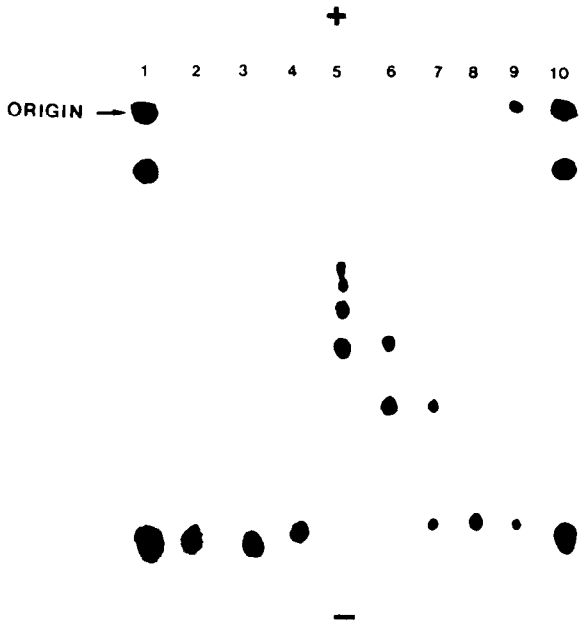

Fig.3. Paper electrophoresis and radioautographic examination of ^{14}C-labelled oligosaccharides released from *N*-methylated glycogen by α-amylase/glucoamylase and further treated with acid. (These are the radiolabelled components seen in fig.2D.) All control substances were also ^{14}C-labelled. Lanes: 1, 10, Glc, Gly, Me-GlcN; 2, 9, Me-GlcN; 3, GalN; 4, Me-GalN; 5, oligosaccharides; 6, oligosaccharides heated 1 h in 1 M HCl, 100°C; 7, heated 2 h in 4 M HCl, 110°C; 8, heated 20 h in 6 M HCl, 110°C.

family of substances migrating towards the cathode but less rapidly than authentic *N*-methylglucosamine and suggestive of a mixture of glucosamine-containing oligosaccharides (fig.3, lane 5). This supposition was confirmed when, after mild acid hydrolysis, the complexity of the mixture was reduced and a new major, faster-moving component (disaccharide?) was generated (fig.3, lane 6). Even stronger hydrolysis (4 M HCl, 2 h, 110°C) began to generate a compound migrating at the same rate as *N*-methylglucosamine (lane 7), while rigorous acid hydrolysis (6 M HCl, 20 h, 110°C) converted all the radiolabel into material migrating with *N*-methylglucosamine (lane 8). The migration on electrophoresis was not in itself an identification of the unknown compound as *N*-methylglucosamine. *N*-Methylgalactosamine, for example, migrates at the same rate as *N*-methylglucosamine (fig.3, lanes 2 and 4).

Table 1

Measurement of the glucosamine contents of rabbit- and pig-liver glycogens

Sample	Degree of purification[a]	Method used for glucosamine release[b]	Glucosamine content (nmol/g glycogen)
Rabbit 1	A	acid	266
Rabbit 1	A	enzyme	150
Rabbit 1	B	acid	122
Rabbit 1	B	enzyme	132
Rabbit 2	B	acid	128
Pig 1	A	enzyme	86

[a] Purification stage A was immediately before passing through DEAE-cellulose (see section 2.2); stage B was after DEAE-cellulose

[b] See section 2.2. The conditions of acid hydrolysis were 4 M HCl, 20 h, 110°C

Our interpretation of these findings, based on the assumption that the unknown compound was indeed *N*-methylglucosamine, is as follows: α-amylase and glucoamylase liberate a mixture of oligosaccharides from the labelled glycogen that contain *N*-methylglucosamine and 2 or more glucose residues linked 1,4 and possibly also containing the 1,6-branch linkage. Mild acid hydrolysis of these fragments, using conditions that split bonds between neutral hexoses, gives rise mainly to a compound which is probably a maltose analog in which *N*-methylglucosamine replaces the non-reducing glucose residue. The positive charge on the glucosamine moiety renders the glycosidic bond resistant to conditions of acid hydrolysis that cleave glycosidic links between neutral aldohexoses [8]. Progressively severe acid hydrolysis eventually converts this disaccharide into free methylglucosamine (fig.3, lane 8).

3.3. *Identification of the unknown radiolabelled compound as* N-*methylglucosamine*

A definitive identification of the radiolabelled liver glycogen component as *N*-methylglucosamine was obtained in the amino acid analyzer. Again, the enriched radiolabelled fraction collected from CM-Sephadex after α-amylase/glucoamylase digestion of glycogen was used. When hydrolyzed in 6 M HCl under conditions which (as shown by paper electrophoresis, fig.3, lane 8) converted all

the radiolabel into a compound migrating at the same rate as *N*-methylglucosamine, this material, when fractionated on the amino acid analyzer in a program appropriate for separation of *N*-methylglucosamine, *N*-methylgalactosamine and the corresponding sugar alcohols, revealed that the radiolabel migrated with *N*-methylglucosamine. When the same material was reduced with sodium borohydride and then fractionated in the analyzer, the majority of the radiolabel now migrated in the same position as an authentic sample of *N*-methylglucosaminitol. When a variation on these experiments was performed in which the radiolabelled fragments were reduced with borohydride before acid hydrolysis and then fractionated, the radiolabel was found to be essentially present all in *N*-methylglucosamine and not in *N*-methylglucosaminitol. This revealed that *N*-methylglucosamine is not found at the reducing chain end of oligosaccharides liberated by α-amylase/glucoamylase and also allows us to conclude that glucosamine is not present in any significant amount at the reducing terminus of the glycogen molecule.

3.4. *Identification and quantitation of the glucosamine component of liver glycogen*

The amino compound detected in liver glycogen by reductive methylation was glucosamine. If this glucosamine was simply acting as a substitute for the normal glucose residues of glycogen, then it should be liberated as free glucosamine by the actions of α-amylase and glucoamylase on the glycogen [5]. This result was observed with both a rabbit- and pig-liver glycogen preparation, the former yielding 150 and 132 nmol glucosamine (2 experiments) per g glycogen and the latter 86 nmol/g. [We have compared the yields of glucosamine obtained by successive (glucoamylase/α-amylase) or simultaneous treatment of glycogen with the 2 enzymes. Glucoamylase alone readily releases 90% of the eventual yield of glucosamine found when α-amylase is then added. When the enzymes are used together the yield of glucosamine is variable and may be much lower. It appears that glucoamylase does not readily release glucosamine from the small oligosaccharides formed by α-amylase.] The enzymatic yield of glucosamine was compared with that obtained by acid (table 1) under conditions we found optimal for the release of glucosamine from glycogen (i.e. 4 M HCl, 20 h, 110°C). It was noted that the yield by either method was about the same with glycogen purified by passage through DEAE-cellulose. With glycogen not passed through the ion-exchanger, enzymatic digestion released the same amount of glucosamine as from more purified glycogen; however, acid hydrolysis released about twice as much. This extra glucosamine resulted from a contaminating carbohydrate desorbed from the DEAE-cellulose by 1 M NaCl. Acid hydrolysis of this material liberated the extra glucosamine but enzymic hydrolysis liberated none. We therefore recommend enzymic hydrolysis for the quantitation of the glucosamine content of glycogen because of the specificity of the method in releasing glucosamine only from glycogen.

Rabbit-muscle glycogen, purified through DEAE-cellulose as for the liver glycogen, provided an interesting comparison since it was free from glucosamine; none was liberated by enzymes or acid. The small amount of glucosamine (0.065%) we had earlier seen associated with the muscle glycogen [9] proved to be a hyaluronic acid contaminant which could be removed by elution through DEAE-cellulose.

3.5. *Intramolecular location of the glucosamine component of liver glycogen*

In an attempt to determine the location of the glucosamine component of glycogen, the latter was subjected to hydrolysis with β-amylase, an exo enzyme that removes about half of the glucose residues in the form of maltose, with the remainder being protected behind the 1,6-branch linkages. The maltose (50%) was separated from the glycogen β-limit dextrin (50%) and both were analyzed for their content of glucosamine by enzymic digestion. Glucosamine was found in each in almost equal amounts, indicating an apparently random distribution of the sugar within the glycogen molecule.

4. DISCUSSION

While non-glucose monosaccharides have been incorporated into glycogen by parenteral administration of sugars, or with sugar derivatives in vitro, our discovery of the presence of glucosamine in native liver glycogen (rabbit and pig) appears to

be the first demonstration of the natural occurrence in glycogen of a sugar residue other than glucose or glucose 6-phosphate [10]. The amount of the glucosamine is rather small. Expressed relative to 10 mg glycogen, for the reason that the limiting M_r of glycogen appears to be 10^7 [11], there would appear to be on average about 1–2 molecular proportions of glucosamine in the glycogen molecule. The numerology might seem significant, as indicating a specific role for the glucosamine, perhaps at the point of origin of the glycogen molecule. However, we demonstrated that glucosamine was released from the glycogen by β-amylase in an amount equal to that which remained with the glycogen β-limit dextrin. This indicates that the glucosamine is randomly placed in the glycogen and suggests that its presence there is adventitious. As to the origin of the glucosamine, the discovery of its presence in glycogen points to the presence in the normal state in liver of a pool of free galactosamine, from which UDP-glucosamine can be generated by the metabolic pathway shown in fig.1.

In experiments which are still in progress we have administered [^{14}C]galactosamine to rats to learn whether glucosamine is incorporated into tissues other than liver. We have observed that liver and kidney glycogens take up an approximately equal percentage of ^{14}C label although none is found in skeletal muscle or heart glycogens. The presence of glucosamine in liver and kidney glycogens, but not in the other tissues, may therefore, reflect the composition of the pool of metabolic intermediates in the cell.

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