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Note

Low-molecular-weight metabolites of D-[1-^{14}C]glucosamine in cultured, normal and carcinomatous, human-mammary cells

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We recently reported the characterization of a mucin-type glycopeptide whose saccharide moiety contained a pentasaccharide having the structure NeuAc→Gal→GlcNAc→Gal→GalNAc. This structure was found[1] to be associated with the two human-mammary-carcinoma cell-lines, MDA-MB-231 and MCF-7, and not with a normal human-mammary cell-line, HBL-100. The relevant glycopeptide was isolated after exhaustive Pronase treatment, (diethylaminoethyl)cellulose chromatography, and wheat-germ agglutinin(WGA)–Sepharose chromatography. The WGA-binding glycopeptides contained clustered oligosaccharide chains having the sequence αNeuAc→βGal→βGlcNAc→βGal→αGalNAc→Ser(Thr). The low-molecular-weight metabolites of D-[1-^{14}C]glucosamine were further investigated to determine whether any free oligosaccharide products were found to be associated with the two carcinoma cell-lines and to define better the pathway of amino sugar utilization by cultured cells. Preliminary accounts of this study have been presented elsewhere[2].

EXPERIMENTAL

Materials. — DEAE-Sephadex A25 was purchased from Pharmacia, Piscataway, NJ 08854. ACS II cocktail was purchased from Amersham–Searle, Arlington Heights, IL 600005. All other chemicals were reagent grade. Lactate dehydrogenase was purchased from Boehringer–Mannheim, Indianapolis, IN 46250; D-[1-^{14}C]glucosamine from New England Nuclear, Boston, MA 02118; and NADH from Sigma Chemical Co., St. Louis, MO 63178.

Methods. — Paper chromatography was performed in the descending mode on Whatman No. 1 paper with the following solvents (all v/v): Solvent (*A*), 6:4:3 1-butanol–pyridine–water; solvent (*B*), 3:2:1 butyl acetate–acetic acid–water; and solvent (*C*), 4:1 2-propanol–water; and in the ascending mode: solvent (*D*) 12:3:5 1-butanol–acetic acid–water. Sugars were made visible with the alkaline silver nitrate

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reagent[3]; pyruvate and lactate ions with the aniline-D-xylose reagent[4]; and amino compounds with ninhydrin[5]. Radioactivity was located by cutting paper chromatograms into 1-cm segments that were shaken for 1 h in vials with water (1 mL), followed by addition of ACS II (10 mL) and counting in a liquid scintillation counter.

Paper electrophoresis was performed on Whatman 3 MM paper in 50mM pyridinium acetate, pH 5.4, at 20 V/cm for 2 h, and in 10% sodium borate, 26 V/cm for 2 h. Chromatograms were scanned on a Packard model 7201 Strip Scanner.

For column chromatography of the dialyzate, 25 mL, corresponding to 50 mL of spent medium, were applied to a DEAE-Sephadex A25 column (1.5 × 27 cm). The column was washed with water (100 mL), and then eluted with a linear gradient of pyridinium acetate consisting of water (400 mL) (starting solvent) and 0.5M pyridinium acetate (400 mL), pH 5.4 (limit solvent). Fractions (5 mL) were collected and 0.2-mL aliquots assayed for radioactivity. Appropriate fractions were pooled, made neutral with M sodium hydroxide, and evaporated to dryness at 40°. Recoveries were greater than 90%. The unadsorbed fraction (Peak I) was loaded onto a column (0.5 × 13 cm) of Dowex 50 (H^+) (100–200 mesh) ion-exchange resin, washed with water (25 mL), and then with M hydrochloric acid (25 mL). Fractions were collected and assayed for radioactivity as just described. Material not retained by either column was termed "neutral"; material bound to the DEAE-Sephadex column, which was eluted with pyridinium acetate, was termed "acidic"; and material eluted from Dowex 50 (H^+) with M hydrochloric acid was termed "basic".

High-pressure-liquid chromatography was performed with a Waters chromatograph equipped with a Model 6000 A pump, a U6K injector, and a u.v. monitor (Schoffel Model SF 770). The column (Chromega An-Wax, ES Industries, Marlton, NJ 08053) was eluted with appropriate concentrations of potassium phosphate, pH 3.4, at a flow rate of 1 mL/min. Lactate dehydrogenase was assayed in the direction of pyruvate to lactate by the method of Kornberg[6].

Amino acids and amino sugars were separated by ion-exchange chromatography with the amino acid analyzer, as previously described[7]. Amino sugar analysis was also performed on a column of Dowex 50 (H^+), equilibrated with 0.3M hydrochloric acid[8].

Cell labeling. — MCA-MB-231, MCF-7, and HBL-100 cells were labeled with D-[1-^{14}C]glucosamine for 48 h by use of the conditions previously described[9]. Media from the cell lines were pooled and dialyzed against de-ionized water. The dialyzate was concentrated about 10-fold *in vacuo* by rotary evaporation and was used for subsequent steps.

RESULTS

The fractionations, on DEAE-Sephadex, of the three dialyzates from the cell lines are illustrated in Fig. 1. The pattern was virtually identical for all three cases, with the exception that the MCF-7 cell line's breakthrough-peak constituted 70% (Table I) of the radioactivity. It was necessary to make Fractions II–IV neutral

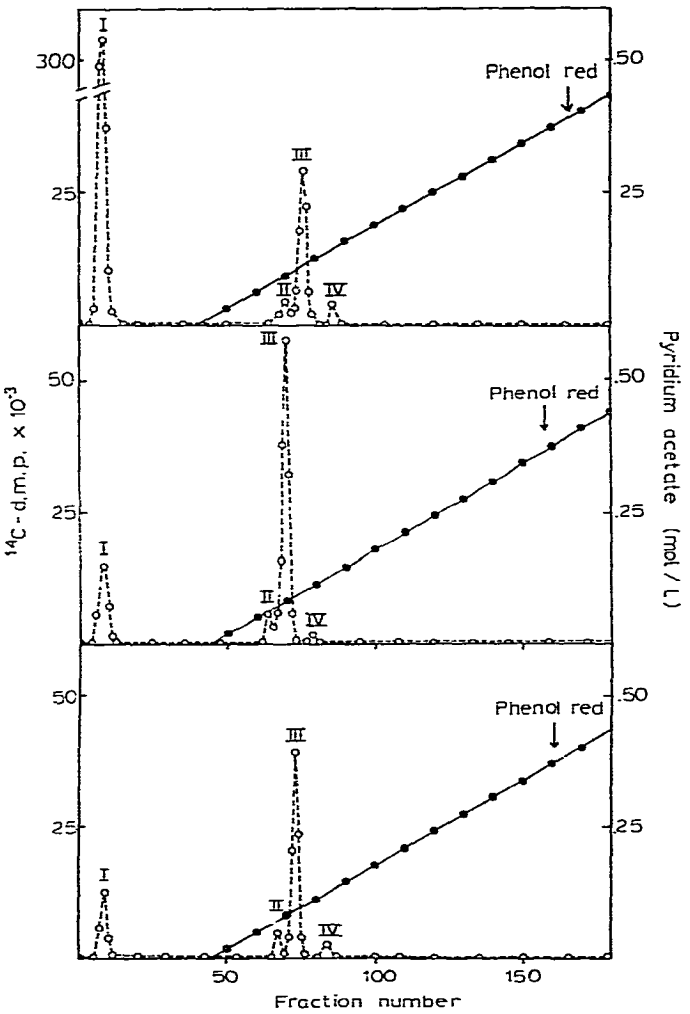

Fig. 1. Elution profile on DEAE–Sephadex A25 of the dialyzate obtained from cells grown in the presence of D-[1-^{14}C]glucosamine: material from MCF-7 (top), MDA-MB-231 (middle), and HBL-100 cells (bottom). Each dialyzate (10 mL) was applied to a column (1.5 × 27 cm) of DEAE-Sephadex A25, the column washed with water (100 mL) and then eluted with a linear gradient of 0–0.5M pyridinium acetate (800 mL, pH 5.4) (——●——●——). Fractions (5 mL) were collected and 0.2-mL aliquots assayed for radioactivity (--○--○--). The flow rate was ~20 mL/h.

with M sodium hydroxide, as pyridinium lactate and pyruvate are volatile under the conditions used to evaporate the samples. The first peak contained neutral or acidic compounds and was further fractionated on Dowex 50 (H^+) (Fig. 2). The bound material was eluted with M hydrochloric acid and subjected to amino acid analysis.

Lactic and pyruvic acids were identified by comigration with authentic standards in paper chromatography (solvent *D*) and in h.p.l.c. (50mM potassium phos-

TABLE I

DISTRIBUTION (%) OF RADIOACTIVE LABEL (D.P.M.) IN THREE CELL LINES

Cell line	*Fraction*							
	Neutral		*Basic*			*Acidic*		
	GlcNAc	*GalNAc*	*Ala*	*GlcN*	*Peptide*	*Glu*	*Lactic acid*	*Pyruvic acid*
MDA-MB-231	0.3	1.4	4.1	11.3	3.5	5.3	72.4	1.7
MCF-7	3.5	1.7	0	81.1	0	1.4	10.8	1.5
HBL-100	3.1	1.8	8.1	7.4	4.6	7.7	61.9	5.4

phate, pH 3.4) (Fig. 3). The latter acid was also converted into lactic acid by incubation with lactate dehydrogenase and NADH (Fig. 4). Glutamic acid was determined by ion-exchange chromatography and by h.p.l.c. in 25mM potassium phosphate, pH 3.4.

Amino acid analysis of the basic fraction showed the presence of alanine, glucosamine, and a poorly resolved shoulder near the glucosamine peak. Chromatography of the basic fraction on Dowex 50 in 0.3M hydrochloric acid showed that the

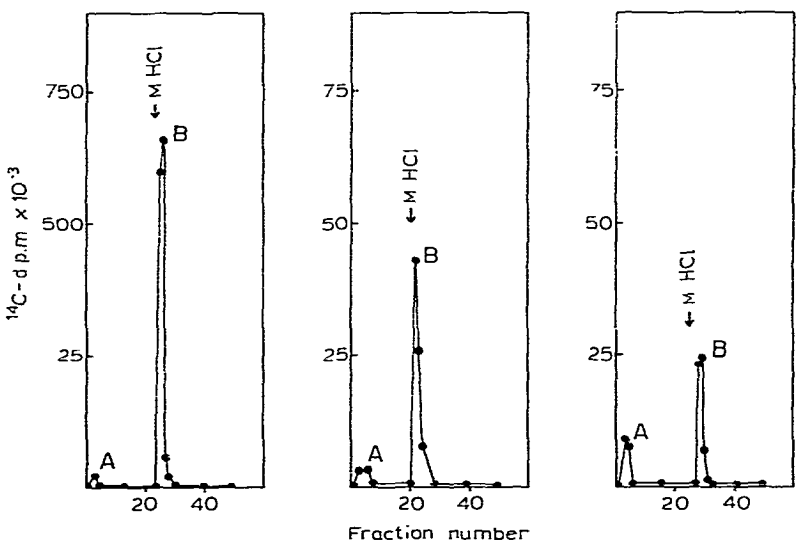

Fig. 2. Elution profile on Dowex 50-X8 (H^+) of Fraction I material eluted from DEAE–Sephadex column (Fig. 1): Fraction I material from MCF-7 (left profile), MDA-MB-231 (center profile), and HBL-100 cells (right profile). Fraction I was evaporated to dryness *in vacuo* and redissolved in water (5 mL), and the solution was applied to a Dowex 50-X8 (H^+, 200–400 mesh) column (0.5 × 14 cm). The column was washed with water and eluted with M hydrochloric acid as indicated. Fractions (2 mL) were collected, and 0.2-mL aliquots were assayed for radioactivity. The flow rate was approximately 15 mL/h.

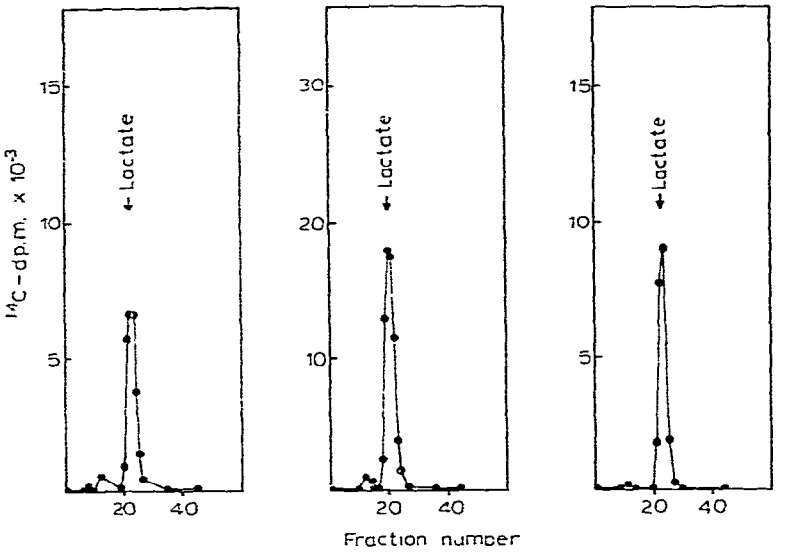

Fig. 3. High-pressure-liquid chromatography, on Chromega An-Wax equilibrated with 45mM KH_2PO_4, pH 3.4, of Fraction III eluted from DEAE-Sephadex A25 column (Fig. 1). Aliquots of neutralized Fraction III from MCF-7 (left profile), MDA-MB-231 (middle profile), and HBL-100 cells (right profile) were mixed with sodium lactate. Each mixture was applied to a Chromega An-Wax column (0.46 × 30 cm, 10-μm particle size) in 45mM KH_2PO_4, pH 3.4, at a flow rate of 1 mL/min. Fractions (0.5 mL) were collected and assayed for radioactivity. The elution volume of the sodium lactate standard was determined by absorbance at 210 nm.

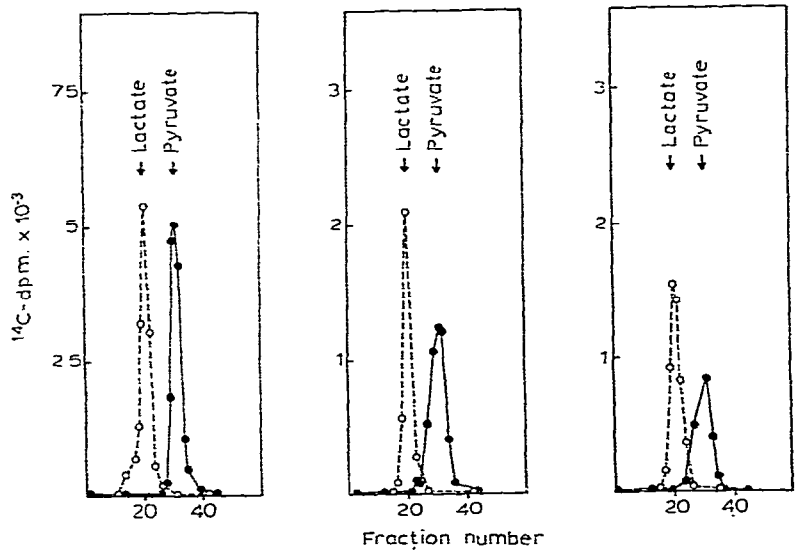

Fig. 4. High-pressure-liquid chromatography, on Chromega An-Wax, of Fraction IV eluted from DEAE-Sephadex A25 column (Fig. 1), before (—●—●—) and after (---○--○--) incubation with lactate dehydrogenase: MCF-7 (left profile), MDA-MB-231 (middle profile), and HBL-100 (right profile). The column conditions were: neutralized Fraction IV (50 μL), 0.2M KH_2PO_4, pH 7.4 (50 μL), M NADH (10 μL), and enzyme (5 μg) for 1 h at 37°.

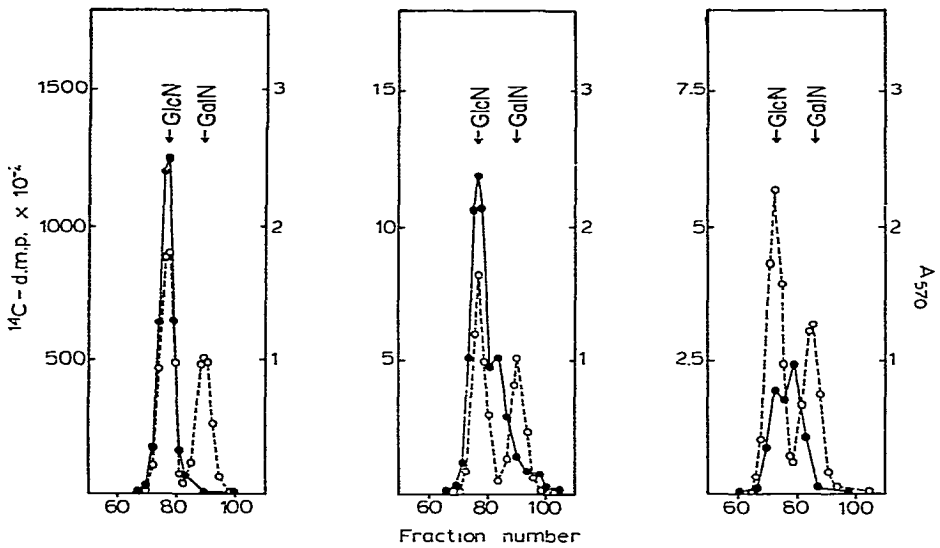

Fig. 5. Elution profile, on Dowex 50-X8 (H^+), of Fraction I-B eluted from Dowex 50 column (Fig. 2): Fractions I-B from MCF-7 (left profile), MDA-MB-231 (middle profile), and HBL-100 cells (right profile) were mixed with ~5 mg each of D-glucosamine and D-galactosamine. Each mixture was applied to a Dowex 50-X8 (H^+, 200–400 mesh) column (0.9 × 60 cm) equilibrated in 0.3M hydrochloric acid and developed with the same solvent. Fractions (2 mL) were collected, and aliquots of 0.2-mL were assayed for radioactivity (—●—●—), and of 0.1 mL with ninhydrin (--○--○--). The flow rate was 12 mL/h.

other component present was not galactosamine (Fig. 5). Hydrolysis of this material with 6M hydrochloric acid for 24 h at 100° liberated alanine, indicating that a peptide which contained alanine was present in the dialyzate. No mannosamine or galactosamine was found in the basic fraction upon paper electrophoresis[10,11].

The neutral fraction consisted mainly of *N*-acetylglucosamine and *N*-acetylgalactosamine as determined by paper chromatography in Solvents *A*, *B*, and *C*. After hydrolysis with 2M hydrochloric acid for 2 h at 100°, the radioactive material comigrated with glucosamine and galactosamine[8]; data are summarized in Table I.

DISCUSSION

D-Glucosamine labeling of cell cultures is a common technique used to incorporate radioactivity into glycoproteins and glycolipids. Our study has shown that from 10.4 to 20.9% of the added radioactivity is metabolized through the Embden–Meyerhof pathway. A substantial percentage of the radioactivity is also found in glutamic acid, alanine, and alanine-containing peptides.

The metabolism of D-glucosamine is well understood (for reviews, see Davidson[12], Schachter and Rodén[13], and Warren[14]). After phosphorylation, D-glucosamine 6-phosphate can either be *N*-acetylated or deaminated. The formation of *N*-acetylglucosamine 6-phosphate targets the amino sugar towards macromolecules[1,15],

whereas formation of D-fructose 6-phosphate allows the carbon atoms derived from D-glucosamine to enter other pathways, such as glycolysis.

Elevated levels of phosphoglucosamine isomerase, the enzyme responsible for conversion of D-glucosamine 6-phosphate to D-fructose 6-phosphate, have been found in the Yoshida sarcoma of rat liver, two ascites-hepatoma strains of rat, and a hypotetraploid-mouse Ehrlich ascites carcinoma[16]. The data suggest that the activity of this enzyme may be elevated in the MDA-MB-231 (human carcinoma) *vs.* the normal, mammary line (HBL-100). The other carcinoma line (MCF-7) is quite inefficient in utilization of exogenous D-glucosamine, as over 50% of the radioactive label is recovered as D-glucosamine; this may be due to impaired uptake of the free amino sugar.

The major metabolite of D-glucosamine is lactic acid; in addition, a small proportion of pyruvic acid is present and a substantial proportion of radioactivity was found in glutamic acid. The failure to observe a radioactive label in other metabolic intermediates (*e.g.*, those of the tricarboxylic acid cycle) is probably due to the components of the labeling medium, which contained ~20mM glutamine[9].

The presence of *N*-acetylgalactosamine, which is biosynthesized at the nucleotidyl (UDP) sugar level, makes it likely that the acetylated amino sugars are degradation products from glycoprotein or glycolipid structures[13]. It is interesting to note that free sialic acid was not detected in any of the fractions since, in the degradative pathways for glycoproteins containing complex oligosaccharides, sialic acid has to be removed before other glycosidases can function.

Under the labeling conditions, less than 1% of the radioactivity was found in either nucleoside diphosphate sugar fractions or glycogen (data not shown). The recovery of label from D-[1-^{14}C]glucosamine indicates that the hexose monophosphate shunt is not a significant pathway for D-glucosamine utilization.

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