GALACTOSYLTRANSFER IN MOUSE MASTOCYTOMA: SYNTHESIS OF A GALACTOSE-CONTAINING POLAR METABOLITE OF RETINOL

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Summary: A microsomal fraction from mouse mastocytoma catalyzed the transfer of galactose-^{14}C from UDP-galactose-^{14}C to endogenous lipid or unlabeled galactose from UDP-galactose-^{12}C to retinol-^{14}C. The galactoretinol was hydrogenolyzed to yield galactose-1-phosphate. Incubation of the particulate enzyme with galactoretinol labeled in the galactose moiety rendered the radioactivity insoluble in chloroform-methanol, indicating that the isolated substance might serve as an intermediate in glycoprotein biosynthesis.

Introduction

Recent investigations have focused attention on lipid intermediates in the biosynthesis of glycoproteins in mammalian systems (1-3). De Luca, Ross and Wolf (3) reported the synthesis by a membrane-rich fraction from rat liver of a mannolipid that contains a derivative of retinol. Furthermore, decreased synthesis in vitamin A deficient animals of a glycopeptide containing, in addition to fucose and hexosamine, a large proportion of galactose, was reported (4). These investigations prompted the search for similar intermediates in a cell-free system from mouse mastocytoma which incorporates large amounts of galactose from UDP-galactose (5). In this communication, the synthesis of a compound containing galactose and retinol, presumably linked to one another via a monophosphate bridge, is reported, and some evidence is also presented for its tentative role as intermediary metabolite in glycoprotein biosynthesis.

Experimental procedure

Materials. UDP-Galactose-^{14}C (190 μCi/μmole) was purchased from New England Nuclear, Frankfurt, Germany. Retinyl-^{3}H-acetate was kindly supplied by Professor O. Wiss, Hoffman La Roche Co., Basel, Switzerland, and retinol-^{14}C (6.8 μCi/μmole) was purchased from Radiochemical Centre, Amersham, England. Unlabeled sugar nucleotides were products of Sigma, St. Louis, Mo., USA.

A particulate enzyme fraction sedimenting at $1x10^5$ x g was obtained from FMS mastocytoma maintained on (AxLeaden)F_1 mice as described (5). Enzyme assay. After incubation of the particulate enzyme with the necessary co-factors (for exact conditions, see the legends to figures and Table 1), the reaction was stopped by addition of 10% TCA and the precipitate formed was washed three times with 5% TCA. The pellet was extracted with chloroform-methanol (2:1 by vol.) followed by analysis of the lipid extract by chromatography on DEAE-Sephadex, prepared as described by Dankert et al. (6).

Analytical methods. Protein was determined by the Lowry procedure (7). Thin layer chromatography on silica gels was performed in solvents (A), Benzene-$CHCl_3$-CH_3OH (50:12.5:12.5 by vol.) and (B), $CHCl_3$-CH_3OH-H_2O (65:25:4 by vol.). Paper chromatography was carried out in solvent C, ethyl acetate-acetic acid-water (3:1:1 by vol.). Paper electrophoresis was achieved in buffer D, 0.046 M acetic acid-0.08 M pyridine , pH 5.3, at 80 volts/cm for 45 minutes. Radioactive spots on paper strips were localized by a Packard strip scanner. Column eluates were analyzed for radioactivity with a Beckman LS 250 liquid scintillation spectrometer.

Reduction of galactoretinol in the presence of a platinum catalyst was performed by the method of Brown (8) as modified by Wright et al. (9).

Results

Synthesis of galactoretinol. Incubation of the particulate enzyme fraction with UDP-galactose-^{14}C followed by chromatography of the chloroform-methanol extract on DEAE-Sephadex gave a single peak of radioactivity, eluted slightly behind retinoic acid (Fig. 1a). A similar pattern of radioactivity was observed when retinol-^{14}C and UDP-galactose-^{3}H were incubated with the enzyme (Fig. 1b). Analysis of control incubations (Fig. 1c) using boiled enzyme or lacking UDP-galactose-^{12}C revealed that the commercial retinol-^{14}C contained some degradation products which were eluted as a broad peak in the position of retinoic acid. This low background radioactivity was, however, easily distinguishable from the product obtained in the presence of UDP-galactose and was separated from the product by thin layer chromatography (solvents A or B). When UDP-galactose-^{3}H was incubated with retinol-^{14}C, a single peak of radioactivity was obtained, in addition to the minor background peak containing ^{14}C-labeled isotopes. This material was further analyzed by chromatography on a column (20x1 cm) of silica gel, eluted with a linear gradient of methanol in chloroform (Fig. 1d). It is seen that the background radioactivity from the retinol preparation was eluted first, together with unlabeled marker retinoic acid, followed by a

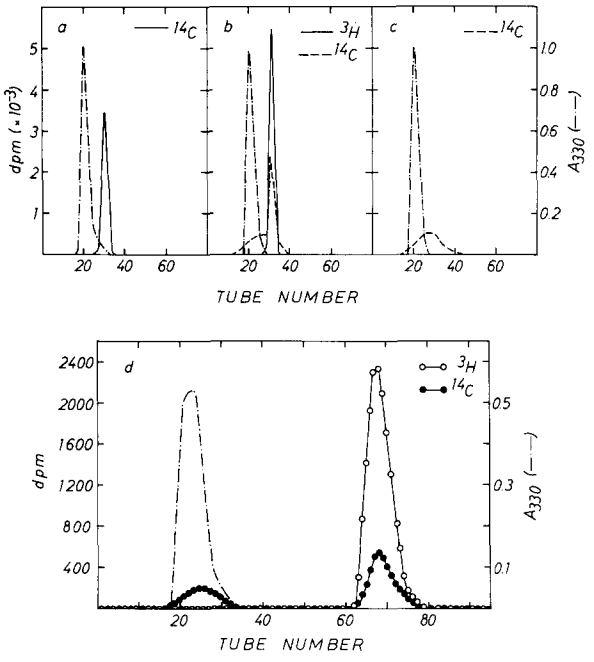

Fig. 1a-c: Analysis on DEAE-Sephadex of chloroform-methanol soluble products labeled with galactose. For incubation conditions, see Table 1. The chloroform-methanol extract was dissolved in 10 ml of 99% methanol and applied to a column (0.8x20 cm) of DEAE-Sephadex which was rinsed with 60 ml of 99% methanol. The adsorbed material was eluted by applying a 200 ml linear gradient of ammonium acetate (0-0.1M) in 99% methanol. Unlabeled retinoic acid used as an internal marker, was determined at 330 nm. (a) Pattern obtained with UDP-galactose-^{14}C and endogenous lipid. (b) Radioactive pattern after incubation with UDP-galactose-^{3}H (4 µCi; 32 µ Ci/µmole) and retinol-^{14}C (0.2 µCi; 6.8 µCi/µmole). Control incubation with retinol-^{14}C but with UDP-galactose excluded from the reaction mixture.

Fig. 1d: Analysis of doubly labeled galactoretinol on silicagel. Part of the material obtained from the chromatogram depicted in Fig. 1 b was desalted and applied to a column (1x20 cm) of silica gel, eluted with a 300 ml-linear gradient (20-50%) of methanol in chloroform.

single peak, containing ^{14}C as well as ^{3}H. If it is assumed that the endogenous retinol which may be present in the particulate fraction is small compared to the amounts of exogenously added substrates, the relative proportion of the two isotopes in the product corresponds well with the ratio expected for a compound containing equimolar amounts of galactose and retinol (cf. the specific activities of the added substrates, Fig. 1d).

In order to obtain information concerning the linkage between galactose and retinol, the isolated substance was subjected to catalytic

hydrogenation. Since retinol is an allylic alcohol and since sugar esters or glycosides linked to such groups may be liberated by hydrogenolysis (cf. ref. 8-9) a sample (1500 cpm) of the isolated compound labeled with galactose-^{14}C was subjected to treatment with reduced hexachloroplatinic acid in the presence of hydrogen gas. On analysis of the reaction mixture (paper electrophoresis, buffer D), essentially all of the water-soluble radioactivity migrated as did galactose-1-phosphate (Fig. 2). Treatment of the hydrogenolytic fragment with alkaline phosphatase followed by paper electrophoresis caused the radioactivity to shift from the position of galactose-1-phosphate to that of galactose. Finally, the product obtained after digestion with phosphatase showed a chromatographic mobility (solvent C) similar to that of galactose. These data seem to suggest the presence in the DEAE-Sephadex eluate of a compound with the proposed structure, retinol phosphate galactose, in analogy with the previously isolated dolichol monophosphate glucose from rat liver (2). Furthermore, the stability towards acid hydrolysis of the isolated compound makes the presence of a pyrophosphate bridge less probable. When the galactoretinol was mixed with tritium-labeled UDP-galactose and treated with 0.1 M HCl at 30°C, essentially all of the tritium was recovered as a neutral product (galactose or methyl

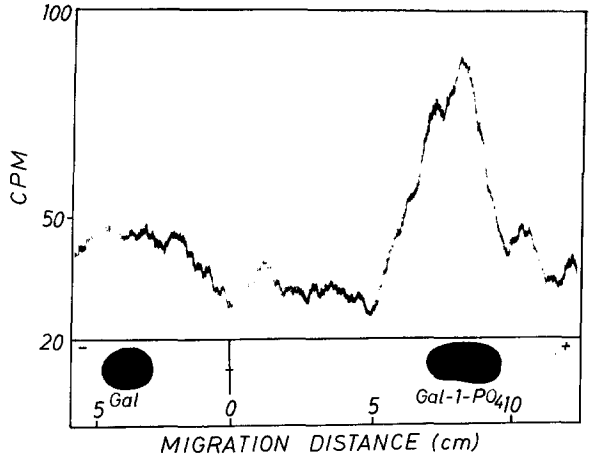

Fig. 2: Conversion of galactoretinol to galactose-1-phosphate. A sample (1500 cpm) of galactoretinol was hydrogenolyzed as described in the text, dissolved in 1 ml of chloroform-methanol and extracted with water. The water extract (600 cpm; 40%) was applied to Whatman no. 3MM paper and subjected to electrophoresis in buffer D (80 volts/cm; 45 minutes). The figure illustrates the distribution of radioactivity with the position of galactose and of galactose-1-phosphate indicated on the guide strip below the tracing.

galactoside; paper electrophoresis, buffer D). In contrast, no radioactivity from the ^{14}C-labeled galactoretinol was released after 40 minutes of incubation under the same conditions. After 2 hours at 60° in 0.1 M HCl, approximately half of the galactoretinol had been hydrolyzed to yield galactose.

Properties. Table I summarizes some kinetic properties of the system that were determined. As is seen, the reaction was strongly inhibited by the presence of UDP or of UMP, and this inhibitory effect was particularly evident when ATP was included in the reaction mixture. These findings are at present being investigated further. About 85% of the

Table 1

Properties of the galactosyltransferase system

Incubation conditions	cpm product
Complete	3120
-ATP	3490
-ATP + UMP (1 mM)	1720
-ATP + UDP (1 mM)	1650
+ UMP (1 mM)	720
+ UDP (1 mM)	735
-$MnCl_2$ + EDTA (25 mM)	250
incubated for 0 minutes	18
incubated for 90 minutes	4700
incubated for 3 hours	6250
-UDP-Gal-^{14}C + GDP-mannose-^{14}C (0.1 µCi; 143 µCi/µmole)	1760
-UDP-Gal-^{14}C + UDP-xylose-^{14}C (0.1 µCi; 168 µCi/µmole)	35
-UDP-Gal-^{14}C + UDP-GlcUA-^{14}C (0.1 µCi; 302 µCi/µmole)	20

The complete system, in a total volume of 0.075 ml included particulate enzyme from mouse mastocytoma (0.45 mg of protein suspended in 0.05 ml of tris-acetate (50 mM), pH 7.4; KCl (70 mM) and EDTA (1 mM)), UDP-galactose-^{14}C (0.1 µCi; 190 µCi/µmole), $MnCl_2$ (28 mM) and ATP, (3 mM). The tubes were kept at 30°C for 60 minutes and assayed as described in "Experimental procedure".

enzymatic activity resided in the microsomal fraction which had a specific activity exceeding that of the crude homogenate about fourfold.

Specificity. In addition to the synthesis of galactoretinol, the mastocytoma preparation also catalyzed the transfer of mannose-^{14}C from GDP-mannose-^{14}C to an endogenous lipid. The product which yielded mannose as the only radioactive substance on acid hydrolysis (2N HCl at 100°C for 3 hours; paper chromatography, solvent C) was eluted at a position

similar to that of galactoretinol from the DEAE-Sephadex column. Further, in the presence of retinol-^{14}C and GDP-mannose-^{12}C a small peak of radioactivity above the background was observed on analysis of the products by chromatography on DEAE-Sephadex. Under the conditions tested, no sugar lipids with similar properties were present in incubation mixtures containing UDP-xylose-^{14}C or UDP-glucuronic acid-^{14}C (Table 1).

Transfer of galactose from galactoretinol. Incubation of the particulate enzyme preparation with galactose-^{14}C labeled galactoretinol resulted in a rapid fall of radioactivity in the chloroform-methanol soluble extract, synchronized with the appearence of radioactivity in chloroform-methanol insoluble substances (Fig. 3). Although the latter product has not yet been characterized further, it appeared to be stable in the incubation mixture since no significant amount of radioactivity was released into the water-soluble phase (Fig. 3).

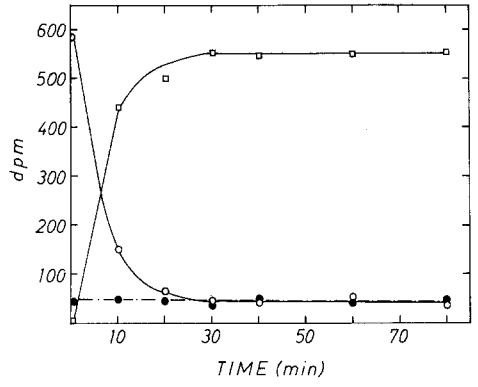

Fig. 3: Transfer of galactose-^{14}C from galactoretinol to the 100 000 x g particulate enzyme fraction. Galactoretinol (labeled with ^{14}C in galactose) was incubated at 30° with particulate enzyme (1.8 mg of protein) in a total volume of 200 µl containing tris-acetate (50 mM), pH 7.4, KCl (70 mM) EDTA, (6 mM), β-mercaptoethanol (70 mM) and 1% Triton X-100. At various time intervals, aliquots were withdrawn and added to 1 ml of chloroform-methanol and 0.25 ml of 4 mM $MgCl_2$. After centrifugation, the precipitate appeared at the chloroform-water interphase. The chloroform phase, the water phase and the interphase material (the latter after washing with butanol) were counted separately in liquid scintillation spectrometer. (o —o), choroform phase: (□ —□), precipitate; and (• — •), water phase.

Discussion

Recent work has implicated sugar lipids containing glucose (2) or mannose (1,3) as intermediates in glycosyltransferase reactions leading to the biosynthesis of (secreted) glycoproteins. The isolation of a

compound with the tentative structure, retinol monophosphate galactose described in this communication may provide yet another example of this class of compounds. The suggested structure is based on the following considerations: (A) Product formation is dependent on UDP-galactose and endogenous lipid or exogenously supplied retinol-^{14}C; (B) Elution characteristics are similar to those expected for compounds having analogous structures with the one proposed (cf. ref. 1,2); (C) The stability of the substance towards acid hydrolysis is inconsistent with the presence of a pyrophosphate bridge between galactose and retinol; (D) Hydrogenolysis of the product resulted in partial conversion to a substance with the properties of galactose-1-phosphate. It should be noted that quantitative hydrogenolysis of the presumed retinol monophosphate galactose may be difficult to realize since saturation of the proximal double bond in the structural unit, $-C(CH_3)=CH-CH_2-O-R$, prior to cleavage may be a competing reaction (10).

The present work supports recent communications (3,4) implicating vitamin A in the biosynthesis of glycoproteins on a molecular level. In analogy with lipid carriers such as undecaprenol phosphates involved in polysaccharide synthesis in bacterial systems, it is conceivable that vitamin A may fulfill a similar role in mammalian cell membrane biosynthesis, in particular since the effects, on a molecular basis, of the vitamin have so far been recorded for three tissues (liver, intestine, and mastocytoma) and for two monosaccharides (mannose and galactose). A detailed investigation of compounds such as galactoretinol would seem to provide a useful approach in attempts to define a molecular function of vitamin A.

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