FORMATION OF RETINOL GLYCOSIDES WITH NUCLEOTIDE SUGARS IN THE PRESENCE OF WHOLE HOMOGENATES OF RAT THYROID

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1. Introduction

Recent results achieved with undecaisoprenol and dolichol phosphate in glycosyl transfer reactions (for ref. see [1]) prompted us in our studies related to the thyroidal metabolism of sugar nucleotides [2–4] to investigate another natural polyisoprenol, retinol (vitamin A), for its ability to form lipid–carbohydrate intermediates. In a review of the literature accomplished during the course of our work it was found that De Lucca et al. [5] had already observed an enzymatic reaction between retinol and sugars. They found that after incubation of GDPmannose and retinol with a liver microsomal fraction, a compound was formed containing both retinol and mannose. Recently Helting and Peterson [6] described the formation of retinol monophosphate galactose, catalyzed by a particulate fraction from mouse mastocytoma.

We report here the separation and tentative identification of the main reaction products obtained from retinol after incubation with homogenates of rat thyroid in the presence of UDPglucose, UDPgalactose or GDPmannose. A preliminary report of part of the studies has been given previously [7].

2. Materials

UDPglucose, UDPgalactose, GDPmannose, *trans*-retinol and Triton X-100 were purchased from Sigma Chemical Company, St. Louis, Mo., USA, and UDP-[^{14}C]glucose from New England Nuclear, Boston, Mass., USA. n-Butanol was obtained from J.T. Baker Chemical Co., Phillipsburg, N.J., USA; Whatman cellulose powder, Standard Grade from W. and R. Balston Ltd., Maidstone, Kent, England; Kieselgur G was purchased from E. Merck A.G., Darmstadt, Germany, and [6,7-^{14}C]retinol (specific activity 47 μCi/mg) was a kind gift of Prof. O. Wiss of Hoffman La Roche and Co., Basel, Switzerland.

3. Experimental

3.1. *Homogenate preparation and incubation*

Thyroid glands from 20 rats, briefly washed with 0.9% NaCl solution, were minced with scissors, suspended in 0.7 ml of 0.05 M Tris-HCl buffer, pH 7.5, and homogenized at 4° in a Potter–Elvehjem homogenizer. To this preparation the components of the incubation mixture were added. The concentrations in a final volume of 1.0 ml were: 0.5 mM nucleotide sugar – either UDPglucose, UDPgalactose, or GDPmannose – 0.5 mM *trans*-retinol, 1.2% (v/v) Triton X-100 and 5 mM $Mg(CH_3COO)_2$. As radioactive markers 0.47 μCi [6,7-^{14}C]retinol or 1.0 μCi UDP-[^{14}C]glucose were added. After 60 min incubation at 37°, 20 ml of chloroform:methanol (6:4, v/v) were added, and mixed during 10 min. The resulting protein precipitate was separated by centrifugation, and the chloroform phase was evaporated under nitrogen to about 1 ml.

3.2. *Separation of reaction products*

A column (26 X 1.7 cm) was prepared with

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n-butanol-washed cellulose. The 1 ml sample described was applied on the top of the column, and eluted with n-butanol:water (78:17); all run at 4° in the cold room. Fractions of 1 ml each were collected, and aliquots counted for radioactivity. Descending chromatography on Whatman No. 3 paper has been applied also for the identification of reaction products. The solvent system consisted of n-butanol:acetic acid:water (78:5:17). In this case UDP-[^{14}C]glucose instead of [^{14}C]retinol was the radioactive marker. All procedures were performed in subdued light in order to prevent changes of the retinol molecule by irradiation.

3.3. *Radioactivity determinations*

^{14}C-Labeled compounds in the column eluate were determined in the usual way with a Packard Tri-Carb liquid scintillation spectrometer. Paper chromatograms were scanned for radioactivity with a Vanguard strip scanner.

3.4. *Identification of saccharides and phosphate determination*

The dry residues obtained after the evaporation of the pooled fractions of the column eluate corresponding to the [^{14}C]retinol peaks were hydrolyzed for 1 hr by heating in a boiling water bath with 0.01 N HCl. Aliquots of the hydrolysate were placed on Kieselgur G plates impregnated previously with phosphate buffer (pH 5) and analyzed for saccharides. It was found indispensable to keep the plates for at least 1 hr before use at 140° in order to get reproducible results. The solvent system used was n-butanol: acetone:phosphate buffer of pH 5 (40:50:10) [8]. The sugars were detected with aniline–diphenylamine–phosphoric acid [9]. Total phosphate was determined in another aliquot of the hydrolysate after digestion with approximately 15% perchloric acid as by Beveridge and Johnson [10].

4. Results and discussion

When UDP-[^{14}C]glucose and retinol were incubated with a whole homogenate of rat thyroid tissue, and a chloroform–methanol extract of the incubation mixture was submitted to paper chromatography, the formation of a glucose-containing retinol compound was observed (fig. 1). The upper part of the figure represents the scanning of the chromatogram. The lower part shows the chromatogram after the spray with the $SbCl_3$ reagent [11]. It is shown in fig. 1 that

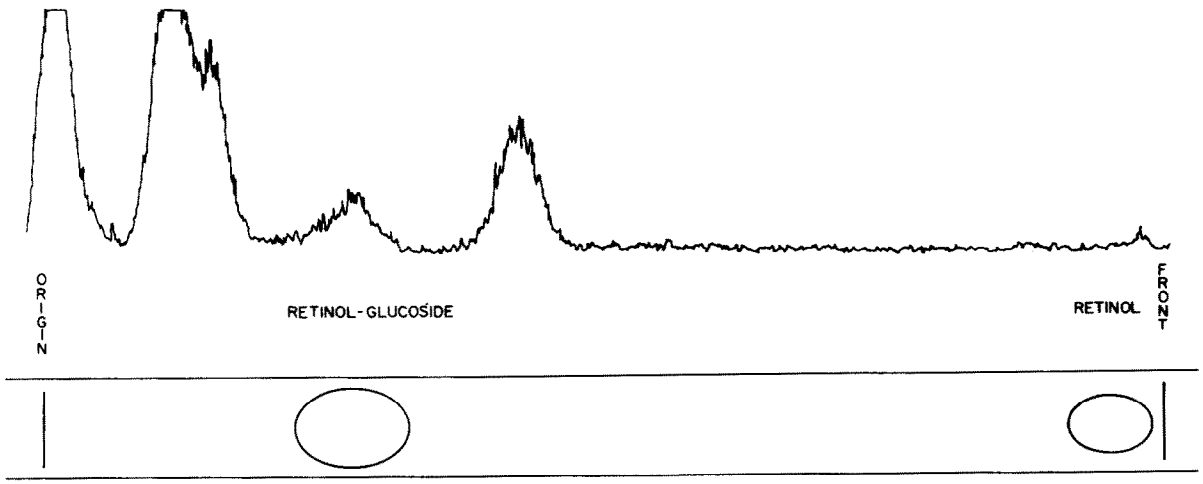

Fig. 1. Formation of a retinol–glucose compound. The reaction mixture contained 0.5 μmoles of *trans*-retinol, 5 μmoles of $Mg(CH_3COO)_2$, 0.5 μmoles of UDPglucose, 0.5 μCi of UDP-[^{14}C]glucose, 1.2% Triton X-100, and thyroid homogenate in a final vol of 1.0 ml. After incubation the reaction mixture was treated as described in the text under sect. 3.1. The evaporated sample was subjected to paper chromatography in n-butanol:acetic acid:water (78:5:17). Compounds have been identified by scanning for radioactivity (upper part) and by spraying with $SbCl_3$ reagent (lower part).

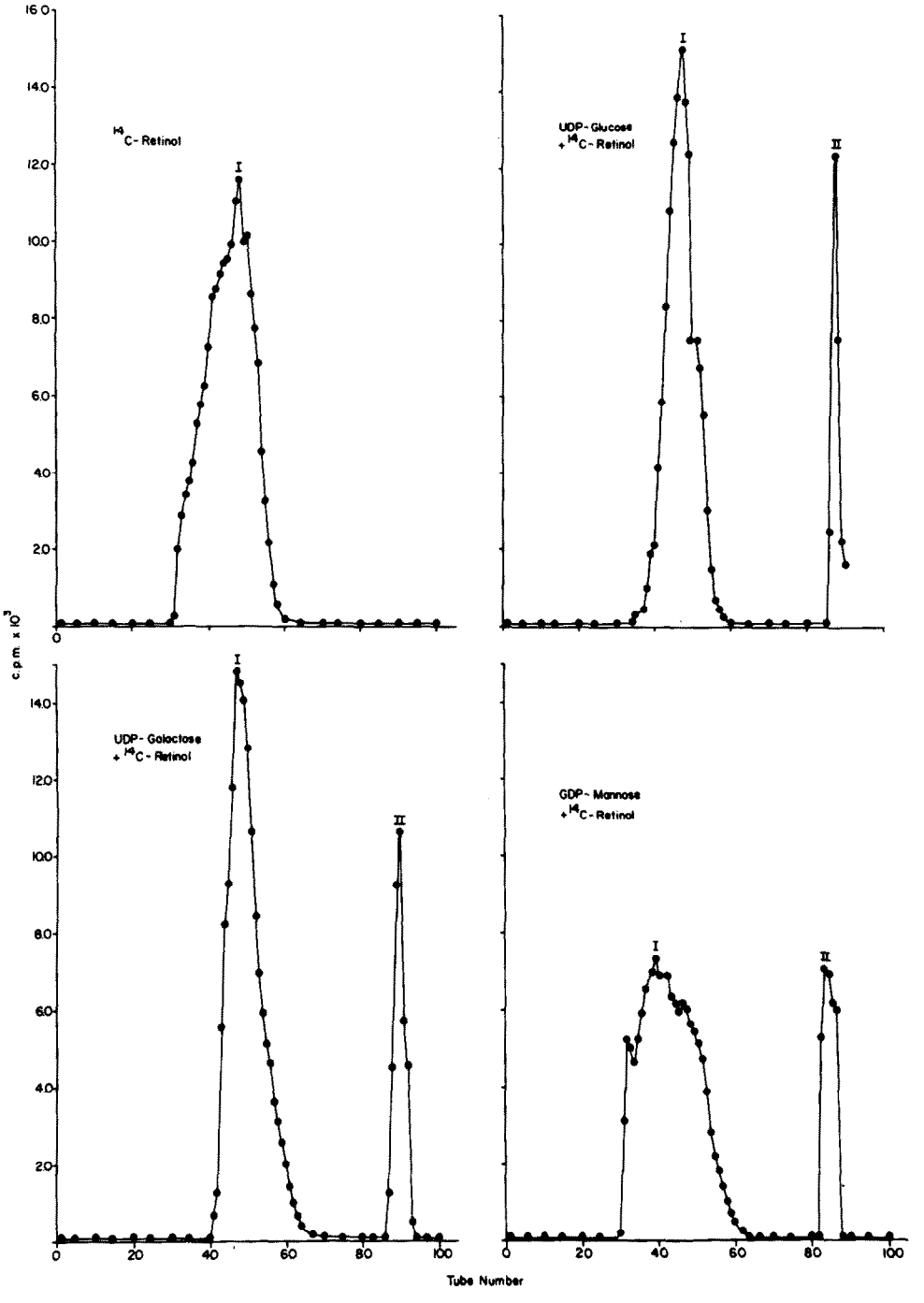

Fig. 2. Cellulose-column chromatography of the retinol–saccharide compounds. The incubation was performed as in fig. 1, but [^{14}C]retinol was the radioactive marker. The compounds were eluted with n-butanol:water (78:17). Fractions of 1 ml were collected. Radioactivity is plotted as cpm per 0.5 ml aliquot (●—●—●). Upper left is the elution pattern of [^{14}C]retinol, following incubation and extraction as described under sect. 3.1. Elution pattern of the chloroform–methanol extract of the incubation mixture containing [^{14}C]retinol and UDPglucose (upper right), [^{14}C]retinol and UDPgalactose (lower left) and [^{14}C]retinol with GDPmannose (lower right) are also shown.

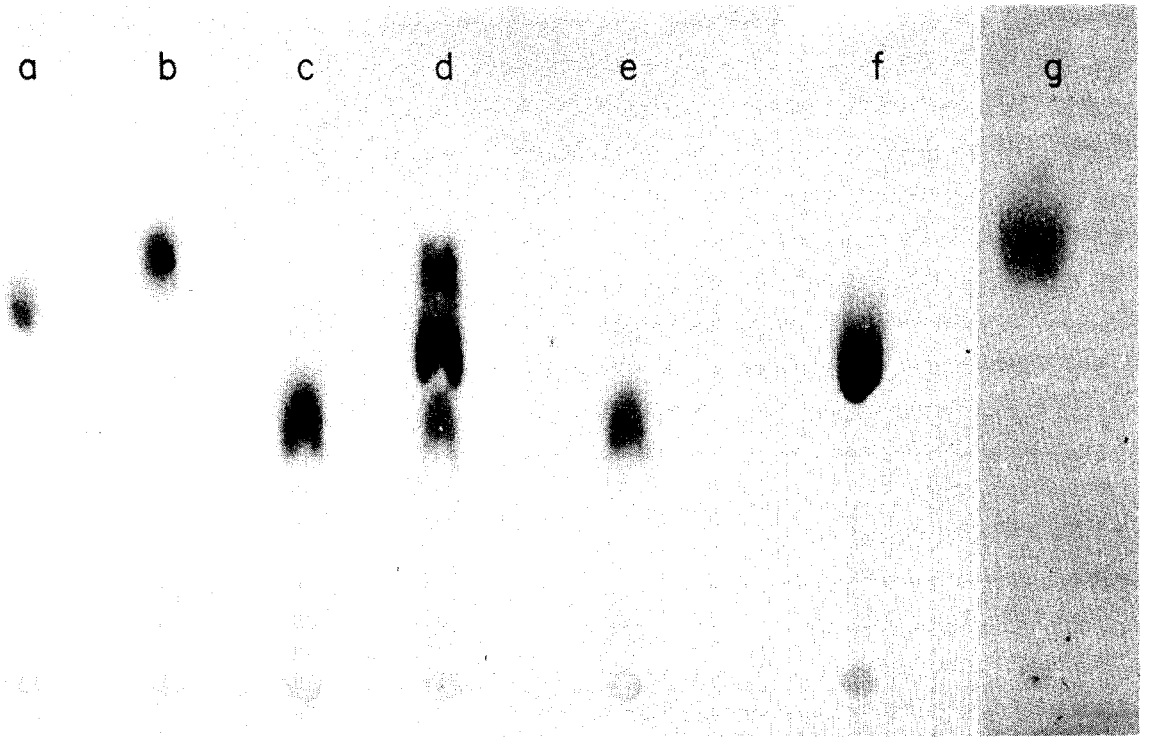

Fig. 3. Thin-layer chromatography of monosaccharides obtained by hydrolysis of the retinol saccharide compounds. Fractions corresponding to each retinol–saccharide compound (peak II) obtained by column chromatography were evaporated to dryness. The residues were hydrolyzed (0.01 N HCl, boiling water bath) for 60 min and the neutralized hydrolysates evaporated to dryness. The samples were chromatographed on Kieselgur G in n-butanol:acetone:phosphate buffer pH 5 (40:50:10), along with 10 μg samples of authentic glucose (a), mannose (b), galactose (c) and a mixture of these sugars (d). The plates were sprayed with aniline:diphenylamine:phosphoric acid. Sugars obtained from incubation experiments using UDPgalactose, UDPglucose and GDPmannose are designated e, f, and g, respectively.

Table 1

Yield and composition of retinol saccharide compounds isolated as products from the reaction* of retinol with nucleotide sugars.

Nucleotide sugar (0.5 μmole)	Recovery as retinol–saccharide (%)	(μmoles)	Analyzed saccharide	Total phosphorous (μequiv.)
UDPglucose	17.6	0.09	Glucose	0.7
	22.1	0.11	Glucose	1.2
UDPgalactose	16.75	0.08	Galactose	Not detectable
	22.5	0.11	Galactose	Not detectable
GDPmannose	21.3	0.11	Mannose	Not detectable
	21.7	0.11	Mannose	Not detectable

* Experimental details described under sect. 3.1.

there are two retinol containing, Carr–Price positive spots on the paper. As revealed in control experiments, the compound which migrates shortly behind the solvent front is free retinol. The other spot of a R_f of 0.27 coincides with a radioactive peak of the upper scan. This spot did not appear when an authentic sample of retinol alone, or extracts of the whole reaction mixture, stopped at zero time by the addition of chloroform–methanol, were submitted to chromatography. The new compound appeared only in the presence of a detergent (Triton X-100) and Mg ions. Addition of ATP to the incubation mixture was without effect on the results. It is reasonable to assume that the second spot was produced by a compound which contained glucose and retinol. The other radioactive peaks are given by metabolic products of UDPglucose which we have not identified.

When the retinol–glucose compound was eluted from the paper it was partly hydrolyzed. This was concluded from the results obtained by rechromatography of the eluted compound since free retinol was obtained besides a spot which corresponded to the eluted glucose–retinol compound.

When retinol was omitted and UDP-[^{14}C]glucose alone was submitted to incubation, a small peak of radioactivity was observed which coincided with the migration of the glucose–retinol compound. It is assumed that the formation of this compound was due to endogenous retinol present in the tissue preparation.

When the incubation products from retinol and sugar nucleotides were subjected to column chromatography using cellulose powder and aqueous butanol as an eluent, results of the same kind have been obtained. Authentic [^{14}C]retinol, with and without incubation with the tissue homogenate, formed a single peak of radioactivity (fig. 2, upper left). However, when the labelled retinol had been incubated with the tissue homogenate in the presence of nucleotide sugars, two peaks of radioactivity were observed (fig. 2), indicating the production of derivatives of retinol. So far, UDPgalactose and GDPmannose, besides UDPglucose, have been found to react with retinol. The yields of the new retinol compounds varied only slightly from each other (table 1). With excess retinol and sugar nucleotides (0.5 μmole each), about 0.1 μmole of the newly formed compounds has been obtained, as judged from the recovery of the added [^{14}C]retinol.

The separation procedure gave material in sufficient amount for a tentative identification of the compound. The fractions of each peak were combined, the solvent evaporated, and the residue submitted to hydrolysis with 0.01 N HCl in a boiling water bath for 1 hr. Analysis for monosaccharides and phosphate gave the following results. The first peak corresponded to a compound which did not contain sugars. It was free retinol, as concluded also from control experiments. The analysis of the pooled fractions corresponding to the second, and smaller, retinol-containing peak revealed that it was made up by a compound which contained monosaccharides (fig. 3) besides the polyprenol, more precisely, those saccharides corresponding to the sugar moiety of the nucleotide added to the reaction mixture.

Phosphate determinations revealed the complete absence of the element in the retinol–mannose and retinol–galactose compounds. The fractions corresponding to the retinol–glucose compound were, as must be assumed, contaminated with a phosphorous-containing lipid since the analytical values were inconstant and much higher than would correspond to one phosphate or pyrophosphate group per mole of retinol saccharide (table 1).

It is tentatively concluded from the analytical results and the fact that the compounds, submitted to mild acid hydrolysis, render retinol and the sugar to be expected, that the novel compounds are retinol glycosides which have been formed during the tissue-enzyme catalyzed reaction of retinol with sugar nucleotides according to the following equation:

$$\text{retinol} + \text{XDP–sugar} \rightarrow \text{retinyl–glycoside} + \text{XDP} \quad (1)$$

It is interesting to note that with this equation a third type of reaction in which polyprenols are involved has been found. With bacterial preparations [12, 13] the following reactions (eq. 2, 3) have been observed:

$$\text{polyprenol-P} + \text{XDP–sugar} \rightarrow \rightarrow \text{polyprenyl-pyrophosphate–sugar} + \text{XMP} \quad (2)$$

and

$$\text{polyprenol-P} + \text{XDP–sugar} \rightarrow \rightarrow \text{polyprenyl-phosphate–sugar} + \text{XDP} \quad (3)$$

The latter reaction (eq. 3) has also been shown in plant [14], yeast [15] and mammalian systems [6, 16].

Finally, it should be mentioned that the present results may have a special significance for thyroid physiology, since they indicate that in the metabolism of the gland another environmental factor, vitamin A, may play a certain role besides iodine. The exact nature of the retinol interaction, however, has to be established in future investigations.

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