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VITAMIN A AND GALACTOSYL TRANSFERASE OF TRACHEAL EPITHELIUM

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Summary

We developed an assay for galactosyl transferase (uridine diphosphategalactose:glycoprotein galactosyltransferase, EC 2.4.1.22) in microsomes of rat tracheal epithelium and characterized the properties of this enzyme system. We found that vitamin A deficiency greatly depressed galactosyl transferase activity. Activity could be restored within 48 h by dosing the animal with retinyl acetate. Adding retinol to the microsomes from tracheal epithelium of vitamin A-deficient rats also restored galactosyl transferase activity to some extent. Full restoration was achieved by pre-incubating retinol with the microsomal preparation for 30 min. Optimal concentration of pre-incubated retinol was 10^{-8} M. Pre-incubation with retinyl phosphate and retinoic acid stimulated galactosyl transferase activity in microsomes from tracheas of deficient rats; pre-incubation with dolichyl phosphate, anhydroretinol and the dimethylacetylcyclopentenyl analog of retinoic acid did not. We concluded that vitamin A is involved in the galactosyl transferase system of rat tracheal epithelium, possibly by being linked to galactose.

Introduction

The enzyme galactosyl transferase (uridine diphosphategalactose:glycoprotein galactosyltransferase, EC 2.4.1.22), which resides on the cell surface, appears to be involved in several cell surface phenomena, such as cell differentiation, adhesion, and recognition [1]. The enzyme seems to increase in

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some cases of cell transformation (for instance, in fibroblasts) [2]. Podolsky et al. [3] found that an isozyme of galactosyl transferase occurs on tumor cell surfaces and is readily shed from these surfaces into the surrounding medium. In rats bearing chemically induced bladder tumors, we found that galactosyl transferase shed from bladder tumor cells into urine had a 10-fold greater specific enzymatic activity than galactosyl transferase from normal bladder cells [4]. Furthermore, biopsies of human bladder transitional cell carcinoma show greatly increased galactosyl transferase activity compared with non-malignant human bladder epithelium [5]. The enzyme appears to reside on the cell surface.

Compounds with vitamin A activity can inhibit induction of several epithelial tissue tumors, among them tracheal, mammary and bladder [6]. Vitamin A depletion has been shown to increase susceptibility to respiratory carcinogenesis [7]. In epithelial tissues, the early stages of carcinogenesis resemble vitamin A deficiency morphologically (for example, squamous metphasia appears in both cases), although differences have also been noted [8].

Since it has been established that vitamin A functions in glycoprotein synthesis, particularly in mannosyl but also in galactosyl transfer reactions [9], we wanted to determine whether the action of vitamin A on epithelial carcinogenesis is mediated through participation in the galactosyl transfer enzyme system. This paper describes the effect of retinol on tracheal epithelium galactosyl transferase.

Materials and Methods

Materials. UDP[^{3}H]galactose (specific activity 16.3 mCi per μmol) was obtained from New England Nuclear, Boston, MA. Unlabeled nucleotides and nucleotide sugars, retinoic acid, dolichyl phosphate and sialidase (*Vibrio cholerae*) were purchased from Sigma Corp., St. Louis, MO. Ovine submaxillary mucin was prepared from sheep submaxillary glands by the method of Gottschalk and Bhargava [10] and was de-sialilated by the method of Spiro and Spiro [11]. Retinyl phosphate was prepared by the method of Ross et al. [12].

Animals. Weanling, 21-day-old male Holtzman albino rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained on a Vitamin A-free diet formulated by Wolf et al. [13] and prepared by Teklad Mills, Madison, WI. Rats designated as controls received the same diet plus 2000 I.U. vitamin A acetate/week. After 4 weeks, rats were paired according to weight (control with deficient) and pair-fed from then on. The deficient rats usually reached the weight plateau stage, which corresponds to the onset of deficiency, after 6—7 weeks on the diet. All animals with obvious signs of respiratory illness were eliminated, and apparently healthy pairs were killed at the stage of zero weight gain.

Preparation of microsomes. Animals were killed by an intrahepatic injection of pentobarbital. Tracheas were opened along their length, excised, cleaned of connective tissue and cut into small pieces. They were homogenized lightly, to avoid contamination with underlying connective tissue, by a Potter-Elvehjem

homogenizer, with six strokes of a loose-fitting pestle at medium speed (800 rev./min). Homogenization took place in Tris-HCl buffer (0.01 M, pH 7.4) containing 0.25 M sucrose, with 4 ml buffer per trachea. A typical preparation contained tissue from three tracheas. The homogenate was centrifuged at 7000 × *g* for 15 min and the supernatant fraction was then centrifuged at 20 000 × *g* for 30 min; the supernatant fraction of this was then centrifuged at 104 000 × *g* for 1 h. The pellet so obtained represents the microsomal fraction used in the incubation. Protein was assayed by the method of Lowry et al. [14].

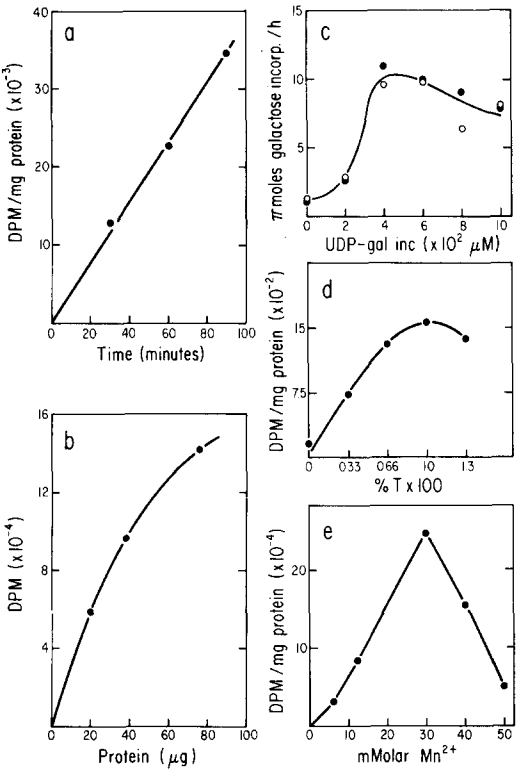
Assay for galactosyl transferase. This assay was adapted from that of Baker and Munro [15], as used later in our laboratory [5]. For the standard assay, unless otherwise stated, the above microsomes (76 μg protein) were incubated with 400 μg de-sialilated ovine submaxillary mucin, 100 μg ATP, 20 μl of a 4% solution of Triton X-100, $MnCl_2$ (final concentration, 20 mM) and 1 μCi UDP-[^{3}H]galactose (final concentration, 0.6 μM) in a final volume of 100 μl 0.1 M Mes * buffer at pH 6.8. Incubation was for 1 h at 37°C. The reaction mixture was cooled in ice, and 2 ml 10% tricholoroacetic acid were added; the mixture was then filtered through a 0.3 μm pore size Millipore filter (Millipore Corp, Bedford, MA), washed with 50 ml cold 10% tricholoroacetic acid, heat-dried, transferred to a liquid scintillation vial and assayed for radioactivity in toluene/0.5% diphenyloxazole.

Results

Validation of the assay. (a) To ascertain that the homogenate was free from connective tissue and contained only tracheal epithelium, the supernatant fraction was assayed for bound uronic acid by the method of Bitter and Muir [16], after homogenization and centrifugation of three tracheas at 7000 × *g*. No uronic acid was detected. Sialic acid assays [17] showed that almost all the sialic acid of the whole homogenate could be recovered in the 7000 × *g* supernatant fraction. (b) To show that all the galactose that was transferred to submaxillary mucin was bound to this acceptor in the standard galactosyl transferase assay, the mixture was centrifuged at the end of the incubation period to remove microsomes. The mixture was then passed through a column of Sephadex G-200. The labeled peak in the void volume coincided with the protein peak of submaxillary mucin. No transfer of galactose to endogenous protein occurred. When the labeled submaxillary mucin, obtained from the column, was hydrolyzed, and the hydrolysate was re-chromatographed, radioactivity disappeared from the void volume and appeared in the bed volume as galactose.

Characterization of the galactosyl transferase reaction. The reactions' dependence on time (Fig. 1a), protein (Fig. 1b), substrate (Fig. 1c) and detergent (Fig. 1d) is shown. The effect of Mn^{2+} is illustrated (Fig. 1e). Mg^{2+} could not substitute for this ion, and there was no reaction in its absence. Surprisingly, there was a partial dependence on ATP (Fig. 2) which may have served in part to inhibit destruction of UDP-galactose. The reaction was

* Mes, 2-(*N*-morpholino)ethanesulfonic acid.



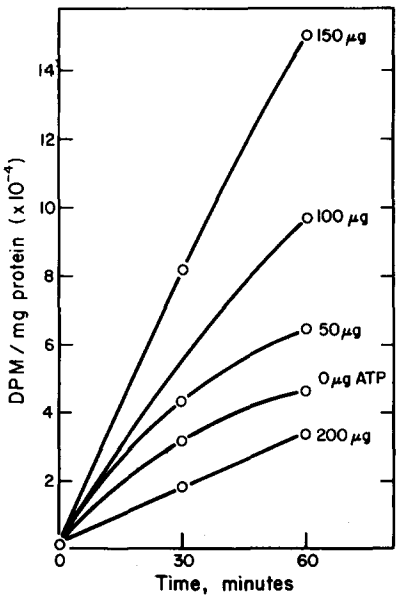


Fig. 1. Transfer of galactose from UDP-galactose (UDP-gal) to submaxillary mucin as a function of: a, time; b, protein concentration; c, substrate concentration; d, detergent (Triton X-100, T X 100) concentration; e, Mn^{2+} concentration. Incubations were carried out with the amounts of microsomal protein, substrate, detergent and metal concentrations indicated, as described in Materials and Methods. In (c), a fixed amount of labeled UDP-galactose (1 μCi) was incubated with increasing amounts of unlabeled UDP-galactose, giving the molarities indicated and the pmol galactose transferred.

Fig. 2. Transfer of galactose from UDP-galactose to submaxillary mucin as a function of ATP concentration. Incubations were carried out with the amounts of ATP indicated, as described in Materials and Methods.

dependent on the presence of submaxillary mucin, with optimal concentration at 400 μg per 0.1 ml (data not shown).

Effect of vitamin A on galactosyl transferase. Preparation from trachea of vitamin A-deficient rats showed depressed galactosyl transferase activity (Fig. 3). Galactosyl transferase activity declined as the vitamin A deficiency developed (Fig. 4), but recovered within 48 h of an intragastric dose of the vitamin (Fig. 5). Addition of retinol to the incubation medium resulted in increased transfer (Fig. 6), but pre-incubation of the retinol with microsomal preparation brought the activity to a nearly normal level (Fig. 3).

To determine the effect of retinol level on galactosyl transferase activity, incubations were prepared with submaxillary mucin to which molar logarithmic increments of retinol were added over a span of 6 decades. After pre-incubation for 30 min, incubations were conducted for 1 h in the presence of submaxillary mucin and analyzed in the usual fashion. Although the reproducibility of the

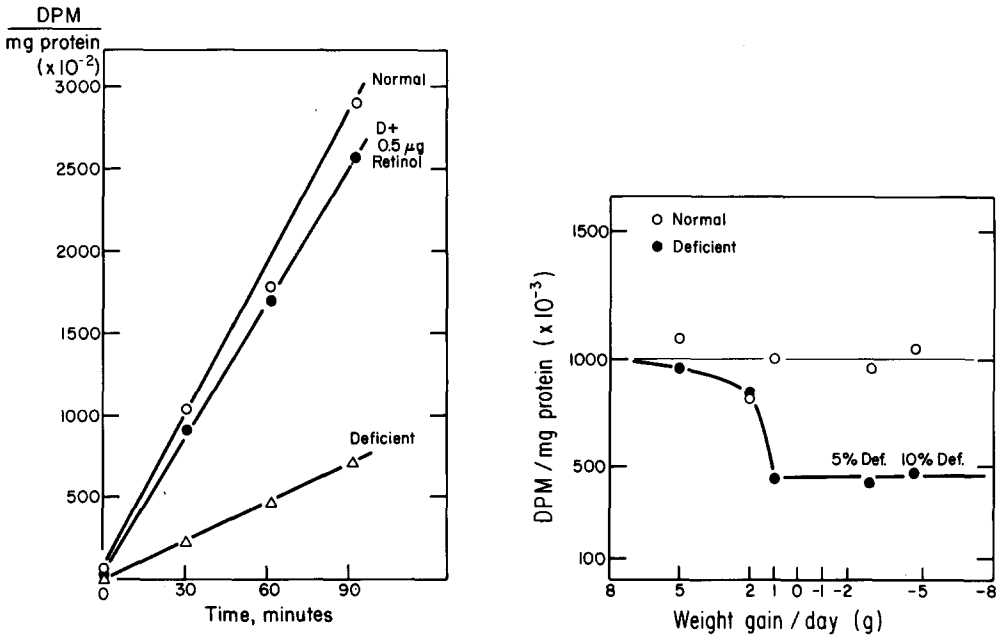


Fig. 3. Transfer of galactose from UDP-galactose to submaxillary mucin as a function of added retinol. Incubations were carried out with microsomes from tracheal epithelium of vitamin A-deficient (△———△) and pair-fed normal (○———○) control rats as described in Materials and Methods. Retinol (0.5 μg), previously dispersed in the Triton X-100, was incubated with the microsomes from six vitamin A-deficient (D) rats, in the absence of submaxillary mucin, for 30 min. The acceptor was then added and the incubationss continued for 90 min, resulting in transfer as shown (●———●).

Fig. 4. Transfer of galactose from UDP-galactose to submaxillary mucin during development of vitamin A deficiency. Incubations were carried out as described in Materials and Methods, using microsomes from tracheal epithelium of deficient rats at progressive stages of the deficiency, starting at the weight-plateau stage. These microsomes were compared with those of pair-fed normal control rats. The scale of the x-axis is expressed to demonstrate daily weight gains or losses, as indicators of developing vitamin A deficiency. Pair-fed control rats, because they were fed identical amounts of diet, show the same weight gains or losses.

reaction was poor at low retinol concentrations, probably because of the instability of this vitamin, one could nonetheless observe a trend showing (Fig. 7a) that retinol at physiological dose levels stimulated transferase activity. At pharmacological dose levels it appeared to have little or no effect.

A specificity test showed that retinoic acid activated galactosyl transferase reaction, whereas the dimethylacetylcyclopentenyl analog of retinoic acid and anhydroretinol did not (Fig. 7b). Pre-incubation with retinyl phosphate activated the reaction considerably, whereas no activation was produced by pre-incubation with dolichyl phosphate.

Solubilization of galactosyl transferase. Microsomes from normal rat trachea, prepared as described, were dispersed in a solution of Mes (0.1 M, pH 6.5) containing 0.5% Triton X-100, at 4° C with a loose-fitted dounce homogenizer. The preparation was maintained at 4° C for 1 h after which it was dispersed in an additional volume of pH 6.5 Mes (without sucrose) and centrifuged at 104 000 × g_{av}. We examined the pellet and supernatant obtained for galactosyl

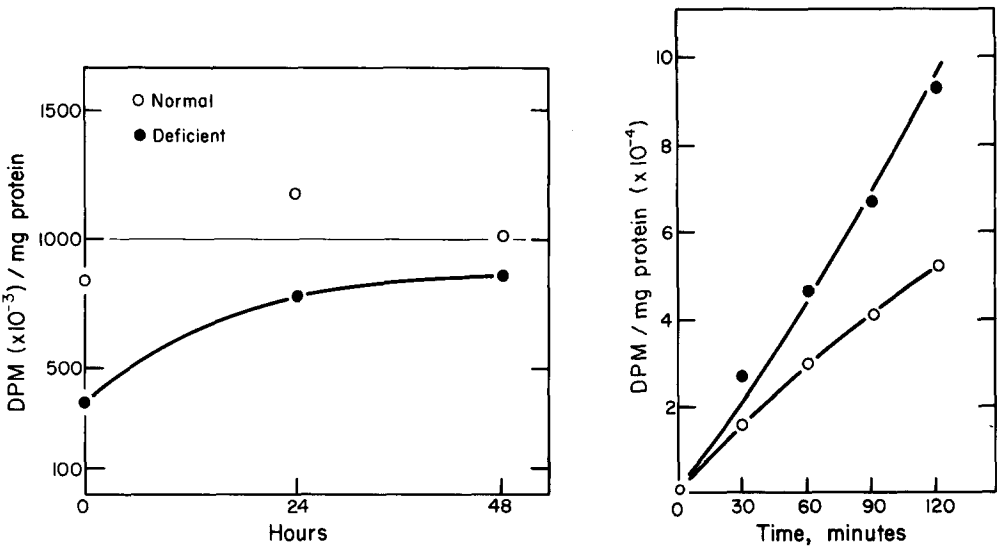


Fig. 5. Transfer of galactose from UDP-galactose to submaxillary mucin during recovery from vitamin A deficiency. Incubations were carried out as described in Materials and Methods, comparing microsomes from tracheal epithelium of two rats at the weight-plateau stage with those of two pair-fed control rats. At zero time, the rats were injected intraperitoneally with 1.0 mg retinyl acetate in 0.5 ml propyleneglycol, and intragastrically with 1.0 mg retinyl acetate in 0.2 ml cottonseed oil [22]. Two deficient and two control rats were then killed, and their tracheal microsomes were incubated over a period of 48 h. Two pairs were killed every 24 h. Transfer of galactose is shown.

Fig. 6. In vitro stimulation of transfer of galactose to submaxillary mucin in tracheas from vitamin A-deficient rats. Incubations were carried out as described in Materials and Methods, with microsomes from tracheal epithelium of vitamin A-deficient rats at the weight-plateau stage. One set of incubations contained 0.5 μg retinol previously dispersed in Triton X-100 per incubatiion (●———●), the other contained a standard incubation solution (○———○).

transferase activity, as previously outlined, and found that 80% of the activity resided in the supernatant fraction.

Galactosyl transferase activity obtained from co-incubation of soluble and pellet preparations was not greater than the sum of that obtained from separate incubations of the two preparations. When the solubilized enzyme system derived from vitamin A-deficient rats was pre-incubated with retinol, enhancement of galactosyl transferase activity similar to that observed with microsomal preparations resulted (Fig. 8).

Discussion

The important enzyme that mediates transfer of galactose to glycoprotein, galactosyl transferase, has not hitherto been described for tracheal epithelium. We found that its properties resemble those of galactosyl transferase from serum [18] or from human bladder epithelium [5]. The effect of substrate concentration on the rate of galactose transfer (Fig. 1c) is unusual, and may be related to the presence of hydrolytic enzyme(s) in the membrane system used; these enzymes may destroy some of the substrate at low substrate concentrations. Protection from the action of hydrolytic enzymes may account for the activa-

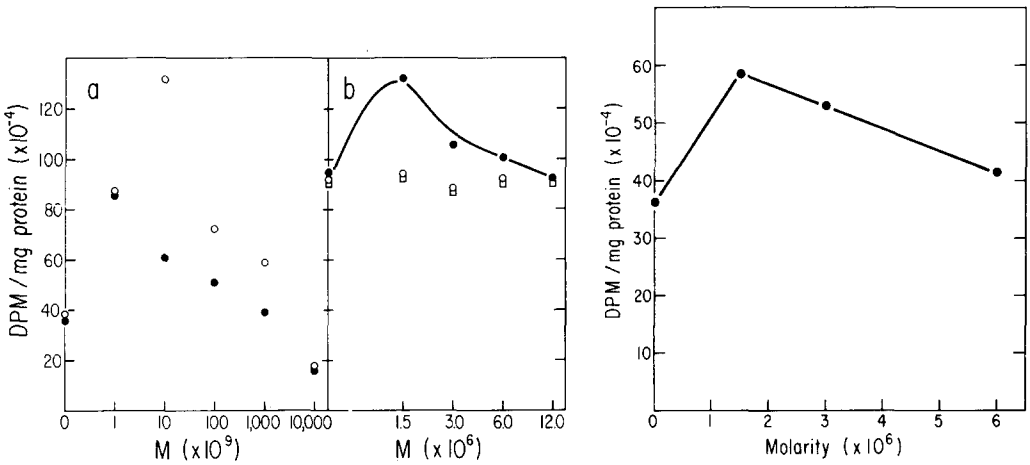


Fig. 7. In vitro stimulation of transfer of galactose from UDP-galactose to submaxillary mucin in tracheas from vitamin A-deficient rats as a function of concentration of retinol or analogs: a, effect of pre-incubation. Microsomes from vitamin A-deficient rats (weight-plateau stage) were uncubated without submaxillary mucin, as described in Materials and Methods, but with retinol (as described in Fig. 3) for 30 min. Submaxillary mucin was then added and incubations continued for 1 h. Points (●———●) and (○———○) illustrate two separate experiments, done at different times with different sets of deficient rats. b, Microsomes from vitamin A-deficient rats (weight-plateau stage) were pre-incubated with retinoic acid (●———●), anydroretinol (□———□), and the dimethylacetylcyclopentenyl analog of retinoic acid (○———○). Incubations were then carried out as described in Materials and Methods.

Fig. 8. In vitro stimulation of transfer of galactose from UDP-galactose for submaxillary mucin by solubilized galactosyl transferase. Microsomes from tracheas of vitamin A-deficient rats were treated as described under Results to solubilize the enzyme. The solution was then pre-incubated with the concentrations of retinol indicated, as described under Fig. 7.

tion of the enzyme reaction by ATP (Fig. 2), previously observed for the bladder enzyme [5]. A large excess of ATP, on the other hand, may compete with the substrate, UDP-galactose, for sites on the enzyme. High concentrations of detergent [19] or of metal ions [20] are known to inhibit glycosyl transferases, as we observed with the tracheal galactosyl transferase (Fig. 1d and e).

The results presented suggest that vitamin A has a function in the galactosyl transferase system of tracheal epithelium. The concentration of added retinol (10^{-8} M) which gives maximum reactivation of galactosyl transferase differs only slightly from that which reactivates glycoprotein synthesis in vitamin A-deficient cornea (10^{-7} M) [21], or from that which causes reversal of keratinization of tracheas from vitamin A-deficient hamsters in organ culture (10^{-9} M) [6]. The restoration time for in vivo galactosyl transferase activity upon feeding retinol to deficient rats (Fig. 5) matches that for glycoprotein synthesis in tracheas of vitamin A-deficient hamsters (48 h) [22], and compares favorably with that for complete restoration of normal RNA synthesis (2 weeks) [23].

Lipid-linked sugars play an important part as intermediates in glycoprotein synthesis. Generally, dolichol-linked sugars function as intermediates [24]; however, in examining a large variety of tissues, dolichol-linked galactose was not detected [24]. Therefore it is possible that galactosylretinyl phosphate (similar to the well known mannosylretinyl phosphate [9]), functions as a

component of the galactosyl transferase system, transferring galactose from UDP-galactose to glycoprotein. The fact that pre-incubation of retinol gives greater galactosyl transferase activition (Fig. 3) than direct addition (Fig. 6) might suggest that retinol is converted into a derivative before it functions. Retinol-linked galactose involvement in the tracheal galactosyl transferase system is indicated by in vitro stimulation of deficient tracheal microsomes by pre-incubation with retinol, stimulation by ATP, and the fact that retinyl phosphate was found to be as active as retinol. Preliminary data indicate that galactosylretinyl phosphate is formed from retinyl phosphate and UDP-galactose by tracheal microsomes. It is significant that these microsomes do not catalyze the reaction of dolichyl phosphate with UDP-galactose.

The galactose acceptor is an exogenously added artificial glycoprotein (desialilated submaxillary mucin). We do not known what the endogenous acceptor is, nor what type of linkage binds the galactose.

We do not as yet known why the galactosyl transferase system declines in activity in epithelium from vitamin A-deficient tracheas, but increases greatly in cancerous bladder epithelium. Since early events in epithelial carcinogenesis resemble the effects of vitamin A deficiency morphologically [8], one would expect to find a decrease of galactosyl transferase activity in epithelial carcinogenesis. We have found in preliminary experiments that in the early stages of carcinogenesis galactosyl transferase activity actually declines. Vitamin A, acting as an anti-carcinogen, could then be expected to reverse this decline and stimulate galactosyl transferase activity, as presently observed. In that case, only rapidly proliferating epithelial turmos would show increased galactosyl transferase activity, possibly as a result of galactosyl transferase isoenzyme formation.

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