

# Isolation, characterization, and biological activity of retinyl phosphate from hamster intestinal epithelium

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**Abstract** Epithelial cells from hamster small intestine, in short term culture, incorporate [carbinol-<sup>14</sup>C]retinol into a compound that is identical to synthetic retinyl phosphate, as judged by chromatography on DEAE-cellulose, silicic acid, and thin layers of silica gel. The biological compound displays the same absorption spectrum as does synthetic retinyl phosphate with a maximum at 325 nm.

Hydrolysis with mild alkali yields anhydroretinol, as it does for synthetic retinyl phosphate, with absorption maxima at 388, 368, and 346 nm. Enzymic hydrolysis by alkaline phosphatase releases 9% of the radioactivity as [<sup>14</sup>C]retinol. Under the same conditions, 9% of synthetic retinyl phosphate is hydrolyzed to retinol.

The biological compound was tested for biological activity. At a concentration of  $5.5 \times 10^{-8}$  M it was as active as retinol and retinyl phosphate in reversing keratinization induced in hamster tracheal epithelium by vitamin A deficiency.

It is concluded that hamster intestinal cells synthesize retinyl phosphate.

**Supplementary key words** retinol · retinyl phosphate · manose · dolichyl phosphate · tracheal cultures

In liver, incorporation of [<sup>14</sup>C]mannose into glycopeptides and glycolipids of the membranes is dramatically affected by deficiency of vitamin A<sup>1</sup> (1). Conversely, excess vitamin A enhances the incorporation of [<sup>14</sup>C]mannose into liver mannosyl lipids and mannoproteins<sup>1</sup>.

Studies on the incorporation of [1-<sup>14</sup>C]glucosamine into intestinal (2–4) and respiratory (5, 6) epithelial glycoproteins have revealed that some, but not all, glycoproteins are affected by deficiency of the vitamin. These and other observations led us to test the hypothesis that retinol may be involved as a carrier of some monosaccharides within the membrane (7, 8) in a fashion similar to other polyprenols in bacteria (9–12).

In vivo studies in hamster liver with [carbinol-<sup>14</sup>C]retinol and [3R,4S-<sup>3</sup>H]mevalonic acid have shown that these compounds are incorporated into

phosphorylated derivatives of retinol and dolichol (13). Further characterization was difficult because of the low incorporation of [carbinol-<sup>14</sup>C]retinol when injected in vivo in the whole animal. Similar results were reported by Martin and Thorne (14).

Here we report the isolation, characterization and biological activity of retinyl phosphate from intestinal cells in a short term culture.

## MATERIALS AND METHODS

### Materials

Culture medium L-15 of Leibovitz, with glutamine and antibiotics (penicillin: 100 U/ml of incubation mixture, and streptomycin: 100 μg/ml of incubation mixture), was obtained from Grand Island Biological Company, Grand Island, N. Y. DEAE-cellulose, all-*trans* retinol, and trichloroacetonitrile were obtained from Eastman Kodak Company, Rochester, N. Y. Silicic acid (Bio-Sil A, 200–325 mesh) was obtained from Bio-Rad Laboratories, Richmond, California; acetonitrile from J. T. Baker Chemical Company, Phillipsburg, N. J.; thin-layer plates, 0.250 mm thick, precoated with silica gel with or without fluorescent indicator (F 254) from E. Merck A. G., Darmstadt, Germany; chicken intestinal alkaline phosphatase (0.95 U/mg) from Worthington Biochemical Corporation, Freehold, N. J.; *p*-nitrophenol and *p*-nitrophenylphosphate di-(cyclohexylammonium) salt from Calbiochem, San Diego, California; [carbinol-<sup>14</sup>C]-retinol (10 mCi/mMole) from Amersham-Searle, Arlington Heights, Ill; and H<sub>3</sub><sup>32</sup>PO<sub>4</sub> (carrier-free) and guanosine-diphosphate-[<sup>14</sup>C]mannose (221

Abbreviations: BHT, butylated hydroxytoluene; TLC, thin-layer chromatography.

<sup>1</sup> Hassell, J. R., C. S. Silverman-Jones, and L. M. De Luca, unpublished data.

mCi/mMole) from New England Nuclear Corporation, Boston, Mass.

## Methods

*Preparation of a suspension of hamster intestinal epithelial cells.* Male Syrian golden hamsters were fed ad libitum and killed by decapitation after anesthesia with ether. Their average body weight was 80 g, unless stated otherwise. The small intestine was removed from the ligament of Treitz to the caecum, flushed with about 30 ml of cold saline, cut open on a cold plate, and the epithelial cells scraped as previously described (2). From this point, all operations were conducted in red light. Scraped mucosal cells from each hamster (usually about 1 g wet wt per animal) were immersed in 3 ml of L-15 medium, at room temperature. Labeled [carbinol- $^{14}\text{C}$ ]retinol was added as a solution of 5  $\mu\text{Ci}$  (sp act 10 mCi/mMole) in 50  $\mu\text{l}$  of dimethyl sulfoxide. This basic incubation system was used for most studies unless stated otherwise.

*Extraction.* At the end of incubation, extraction proceeded according to Ito et al. (15). Vitamin C was added as an antioxidant to a final concentration of 0.5%. The total incubation was then frozen and lyophilized. Lyophilization was never allowed overnight, since samples were dried within 2–3 hours. To each flask containing the mucosal scrapings from one hamster, 5 ml of 99% methanol and 1% water containing 0.05% butylated hydroxytoluene (BHT) was added and the samples were allowed to stand overnight at 4°C.

*DEAE-cellulose chromatography.* This was performed essentially as previously described (16), except that a concave gradient of ammonium acetate was applied to separate retinyl phosphate saccharide from retinyl phosphate with an Ultrograd gradient maker (LKB Instruments, Rockville, Md.). Alternately, in the kinetic studies, short columns (1  $\times$  6 cm) were used and were eluted batchwise, first with 50 ml of 99% methanol and then with 25 ml of 0.1 M ammonium acetate, which elutes retinyl phosphate and derivatives. The ammonium acetate was removed in the water phase by addition of two volumes of chloroform to the eluate and then 0.2 volume of distilled water. The organic phase was dried under nitrogen after adding BHT to a final concentration of 0.05%.

*Silicic acid chromatography.* Silicic acid was activated at 100°C for 45 min and stored in a desiccator. Columns (1  $\times$  6 cm) were packed with a slurry of silicic acid in chloroform. The sample was usually dissolved in chloroform–methanol 8:1 and applied to the column. Elution started with chloroform–methanol 8:1 and continued batchwise to chloroform–methanol 1:2, which eluted retinyl phosphate and derivatives.

*Thin-layer chromatography.* This was done on silica gel plates prewashed with the developing solvent unless plates with fluorescent indicator were used. The most common solvent systems were: (A) chloroform–methanol–water 60:25:4 as developed by Tkacz et al. (17) for the separation of different chain length prenyl phosphates, and (B), toluene–chloroform–methanol 50:12.5:12.5 to separate retinol from anhydroretinol, retinoic acid, and retinyl phosphate.

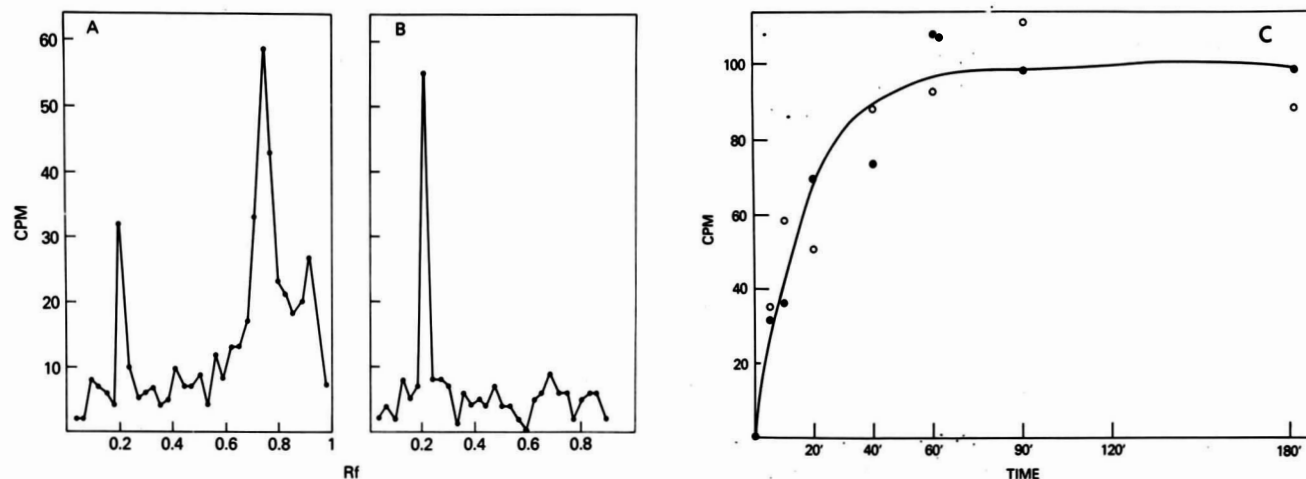
*Radioautography.* Thin-layer plates were radioautographed with Kodak medical X-ray film, NS 2T 5  $\times$  7 inches, for different lengths of time according to the energy of the incident radiation.

*Chemical synthesis of retinyl phosphate.* This was achieved as previously described (16). The chemically synthesized product was stored in the presence of 0.05% BHT.

*Preparation of anhydroretinol.* The procedure of Dunagin and Olson (18) was followed. The ultraviolet spectrum of the product showed maxima at 346, 368 and 388 as shown in Fig. 4, similar to values reported by Shautz, Cowley, and Embree (19). The yield of the reaction was about 15%.

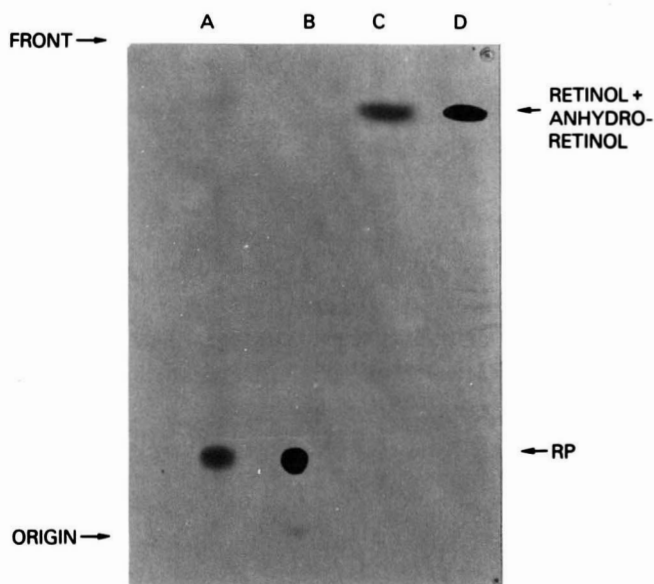
*Mild alkaline hydrolysis of retinyl phosphate.* The compound was dissolved in 250  $\mu\text{l}$  of chloroform–methanol 1:4 to which 25  $\mu\text{l}$  of 1 N NaOH (aqueous) was added. The hydrolysis proceeded for 20 min at 37°C. The solution was cooled in ice and neutralized with 1 N acetic acid. Chloroform was added to a final concentration of 2:1 and the solution was extracted with 0.2 volumes of water. The organic phase was then rechromatographed as explained under Results.

*Hydrolysis of purified retinyl phosphate by alkaline phosphatase.* The incubation mixture consisted of 1 ml of 0.2 M Tris-HCl, pH 7.5, containing  $\text{MgCl}_2$  (50 mM), 0.1 ml of 1 mg of chicken intestinal alkaline phosphatase per ml of saline [the enzyme had been tested before use with a solution of 0.004 M *p*-nitrophenylphosphate-di-(cyclohexylammonium) and gave 0.0062 mole of *p*-nitrophenol per minute], purified retinol phosphate dissolved in 25  $\mu\text{l}$  of dimethylsulfoxide, and 0.5 ml of distilled water. Incubations were performed at room temperature (26°C) in the dark and stopped after 45 min by addition of 5 volumes of chloroform–methanol 2:1. The same incubation mixture without enzyme was used as a standard. The organic phases were dried under nitrogen and kept in the nitrogen freezer. Then the reaction products contained in the lipid phase were applied to silica gel plates F254; standard retinol, anhydroretinol, and retinylphosphate were used as chromatographic standard and localized by ultraviolet light. Chromatography in solvent B took place in a tank previously flushed with nitrogen.



**Fig. 1.** Time course study on the synthesis of [carbinol- $^{14}\text{C}$ ]retinyl phosphate by intestinal cells. A. 1,100 cpm of  $^{14}\text{C}$ -labeled lipid off DEAE-cellulose from the 20 min time point was chromatographed in solvent A. Bands of 0.5 cm were scraped with an automatic zonal scraper, suspended in 0.1 ml of 99% methanol and counted. 10% of the total radioactive product was recovered as a sharp peak in the area of chemically synthesized retinyl phosphate,  $R_f$  0.2. B. 400 cpm of the  $^{14}\text{C}$ -labeled lipid after DEAE-cellulose and silicic acid chromatography from the 20 min time point was chromatographed and processed as in A. C. Uptake of [carbinol- $^{14}\text{C}$ ]retinol into the lipid at  $R_f$  0.2 after DEAE-cellulose (○ — ○) and silicic acid chromatography (● — ●).

*Enzymatic synthesis of retinyl phosphate [ $^{14}\text{C}$ ]mannose.* A mixture of retinyl phosphate [ $^{14}\text{C}$ ]mannose and dolichylphosphate [ $^{14}\text{C}$ ]mannose was obtained according to Rosso et al. (16), except that zonyl A was



**Fig. 2.** Chromatography in solvent A before and after mild alkaline hydrolysis of the  $^{14}\text{C}$ -labeled lipid obtained by DEAE-cellulose and silicic acid. 1,350 cpm of purified  $^{14}\text{C}$ -labeled lipid and 22  $\mu\text{g}$  of chemically synthesized retinyl phosphate were hydrolyzed with mild alkali as described under Methods. One-half of the hydrolysis products were chromatographed in solvent A and one-half in solvent B (Fig. 3), along with 11  $\mu\text{g}$  of intact chemically synthesized retinyl phosphate and 675 cpm of  $^{14}\text{C}$ -labeled lipid. Plates were radioautographed for two months and viewed under a UV lamp. A. Intact  $^{14}\text{C}$ -labeled lipid; B. Intact chemically made retinyl phosphate; C. Hydrolyzed  $^{14}\text{C}$ -labeled lipid; D. Hydrolyzed chemically made retinyl phosphate. The positions of retinol and anhydroretinol and of retinyl phosphate (RP) are indicated.

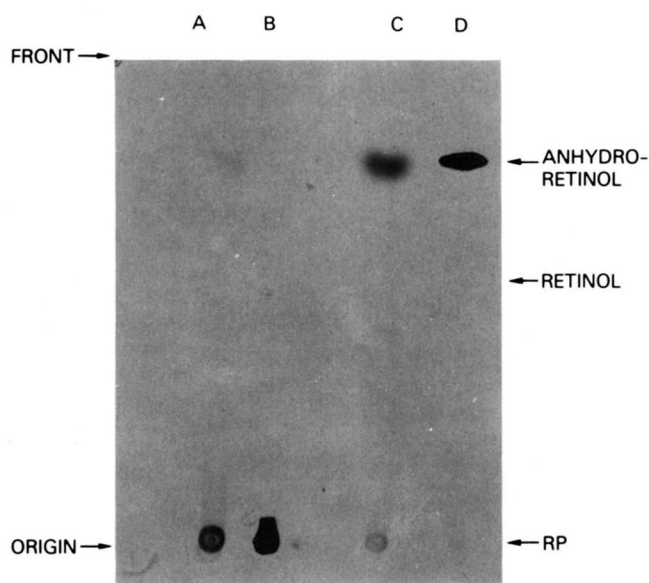
replaced by dimethyl sulfoxide and the incubation was for 30 min at 37°C.

*Characterization by UV absorption.* To obtain a UV spectrum of retinyl phosphate, mucosal scrapings were obtained from thirty Syrian golden hamsters, 70–80 g body weight. Fifty  $\mu\text{Ci}$  of [carbinol- $^{14}\text{C}$ ]retinol was dissolved in a volume of 18 ml of medium L-15, as described. Six vessels were used for incubation with 3 ml of medium in each one. The mucosal scrapings from the first six animals were incubated separately for 30 min each at 20°C in the dark. The cellular material was collected on filter paper and the medium was recycled. [ $^{14}\text{C}$ ]retinyl phosphate was purified as usual. 80,000 cpm (50% efficiency) was obtained in the ammonium acetate eluate. This material was chromatographed in solvent A on a preparative silica gel plate, without fluorescent indicator, and the area corresponding to the radioactivity and to chemically synthesized retinyl phosphate was eluted with 99% methanol containing BHT (0.05%). About 42,000 cpm of the original 80,000 could be extracted from the gel. This extract was chromatographed on DEAE-cellulose (0.5  $\times$  4 cm) to remove impurities present in methanolic extracts of scraped silica gel eluted by 99% methanol. 21,000 cpm were recovered after this procedure.

## RESULTS

### Time course study of the incorporation of [carbinol- $^{14}\text{C}$ ]retinol into [ $^{14}\text{C}$ ]retinyl phosphate by intestinal cells

Approximately 1 g of mucosal cells was obtained from each hamster and incubated as described under



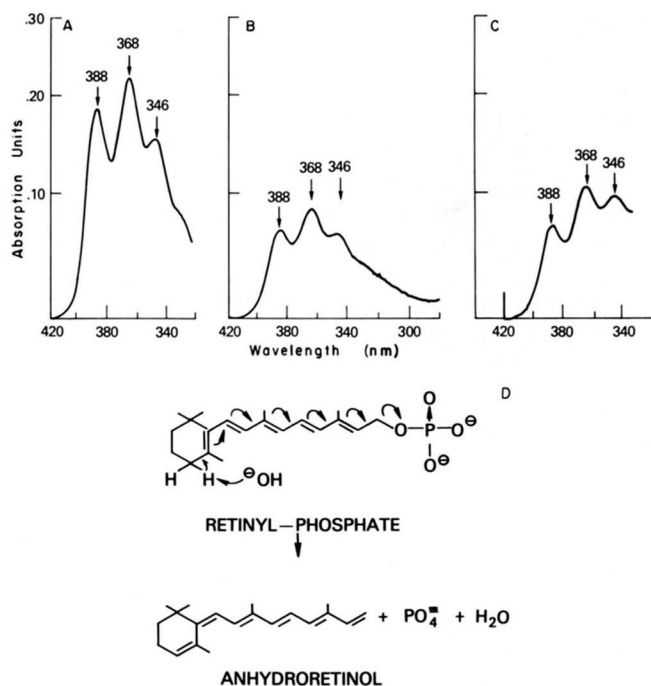
**Fig. 3.** Chromatography in solvent B of intact and hydrolyzed  $^{14}\text{C}$ -labeled lipid and standards. The equivalent of 675 cpm of intact and hydrolyzed  $^{14}\text{C}$ -labeled lipid and 11  $\mu\text{g}$  of intact and hydrolyzed chemically made retinyl phosphate were chromatographed in solvent B. A. Intact  $^{14}\text{C}$ -labeled lipid; B. Intact retinyl phosphate; C. Hydrolyzed  $^{14}\text{C}$ -labeled lipid; D. Hydrolyzed retinyl phosphate. Shaded spots (Bands B and D) represent UV absorption. The positions of retinol and anhydroretinol are indicated. Radioautographs were exposed for two months.

Methods for the following times: 0, 5, 10, 20, 40, 60, 90, and 180 min. The lipid extract was processed through short columns ( $1 \times 6$  cm) of DEAE-cellulose and silicic acid and the eluates, containing retinyl phosphate and derivatives, from each chromatography were chromatographed on thin layers of silica gel in solvent A. A typical chromatographic behavior of the ammonium acetate eluate from DEAE-cellulose is shown in **Fig. 1A** with three main products, only one of which has the same  $R_f$  as chemically synthesized retinyl phosphate ( $R_f$  0.15–0.2). This component constitutes about 10% of the total eluted radioactivity. **Fig. 1B** shows the behavior of the labeled compound after elution from silicic acid. The only radioactive product was detected in the area of chemically synthesized retinyl phosphate. This was confirmed by radioautography (**Fig. 2A**). Uptake of [ $^{14}\text{C}$ ]retinol into [ $^{14}\text{C}$ ]retinol phosphate with time was the same, whether calculated after DEAE-cellulose and thin-layer chromatography or with an additional chromatography on silicic acid (**Fig. 1C**). This was due to the effective separation of [ $^{14}\text{C}$ ]retinyl phosphate from the other two labeled compounds in solvent A.

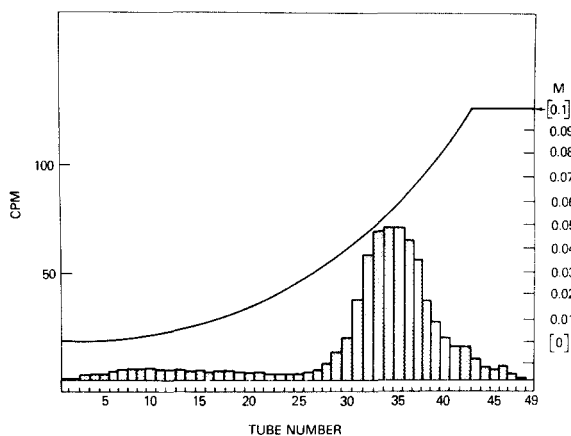
Studies of mild alkaline hydrolysis were conducted on the  $^{14}\text{C}$ -labeled lipid obtained after both DEAE-cellulose and silicic acid chromatography. The lipid soluble products were analyzed in solvents A and B

along with the intact  $^{14}\text{C}$ -labeled lipid, standard retinyl phosphate, retinol, and anhydroretinol.

**Fig. 2** shows the results for solvent A. The  $^{14}\text{C}$ -labeled lipid was hydrolyzed to a compound (**Fig. 2C**) with the same mobility as retinol and anhydroretinol, which cochromatographed in this solvent system (**2D**). The intact compound (**2A**) had the same  $R_f$  as retinyl phosphate (**2B**). Chromatography in solvent B before (**Fig. 3A**) and after (**Fig. 3C**) mild alkaline hydrolysis showed that the hydrolyzed radioactive compound behaved as anhydroretinol. The same spot (**3D**) was obtained by hydrolysis of synthetic retinyl phosphate. The hydrolysate from 44  $\mu\text{g}$  of retinyl phosphate was rerun, the area corresponding to anhydroretinol was scraped off the plate and eluted with 5% acetone in ethanol, and the absorption spectrum was found to be in good agreement with that of chemically prepared anhydroretinol (**Fig. 4A,B,C**), thus unequivocally confirming that this compound is the main product of alkaline hydrolysis of retinyl phosphate by a mechanism consistent with the scheme shown in **Fig. 4D**. It should be emphasized that no TLC system has as yet been



**Fig. 4.** Absorption spectrum of the product of mild alkaline hydrolysis of synthetic retinyl phosphate. 44  $\mu\text{g}$  of retinyl phosphate was hydrolyzed by mild alkali and the product was recovered from thin-layer plates after chromatography in solvent B. The UV-absorbing material in the area of standard anhydroretinol was eluted from the silica gel as described under Results and the absorption spectrum was recorded. A. Standard anhydroretinol; B. Standard anhydroretinol recovered from silica gel after chromatography in solvent B; C. Product of mild alkali hydrolysis of synthetic retinyl phosphate; D. Scheme of mechanism of formation of anhydroretinol from retinyl phosphate in mild alkali.



**Fig. 5.** Concave ammonium acetate gradient of biosynthetic  $^{14}\text{C}$ -labeled lipid. 3,100 cpm of the purified  $^{14}\text{C}$ -labeled lipid was applied to a  $1.3 \times 7$  cm column (Chromatronics) which had been packed by manual pressure to obtain a uniform bed. A 16 hr concave gradient eluted 48 fractions of 2.2 ml each with an automatic LKB ultragrad gradient maker. All operations were conducted in dim light in a coldroom. The same gradient was run on synthetic retinyl phosphate with essentially the same elution at 0.06 M ammonium acetate.

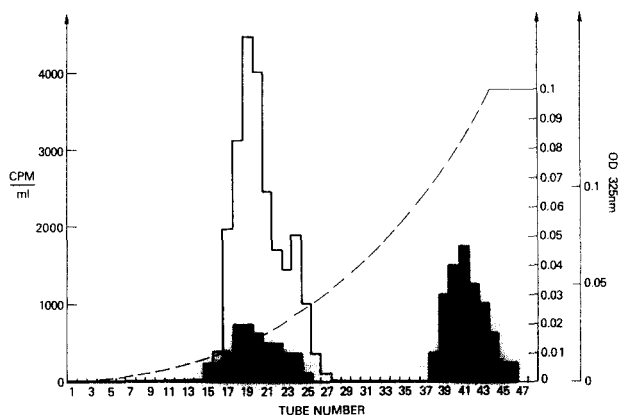
found to separate retinyl phosphate from retinyl phosphate mannose effectively and consistently. Thus it is likely that the compound at  $R_f$  0.15–0.2 contains retinyl phosphate and its saccharide derivatives. However, we have reported that an ammonium acetate gradient can effectively separate these two compounds (16). To study the relative proportion of retinyl phosphate and its derivatives the separation on a gradient was performed.

#### Concave gradient of retinyl phosphate derivatives on DEAE-cellulose

Two male golden hamsters were kept on a vitamin A-deficient diet for 35 days to decrease their body pool of vitamin A. No weight loss due to the deficiency occurred. The two intestinal mucosa were incubated for 20 min at  $20^\circ\text{C}$  in the presence of  $100 \mu\text{Ci}$  of [ $^{14}\text{C}$ ]retinol. The [ $^{14}\text{C}$ ]retinyl phosphate fraction was extracted, purified and tested in solvents A and B. Thin-layer chromatography was also run on plates of aluminum oxide F-254 type T, 0.250 mm thick, with methanol as solvent. The radioactive compound stayed at the origin, as expected for a negatively charged molecule. Moreover, when the  $^{14}\text{C}$ -labeled lipid was partitioned between 5 volumes of chloroform–methanol 2:1 and 1 volume of water, one-third of the radioactivity was consistently recovered in the water phase.

A portion of the  $^{14}\text{C}$ -labeled lipid (3100 cpm) was applied to a column of DEAE-cellulose. **Fig. 5** shows the shape of the gradient (0  $\rightarrow$  0.10 M ammonium acetate). For comparison, the same gradient was

run on chemically synthesized retinyl phosphate. This compound behaved very similarly to the main radioactive compound which contained 90% of the radioactivity. Both were eluted with approximately 0.06–0.07 M ammonium acetate. As reported previously for synthetic retinyl phosphate (16),  $^{14}\text{C}$ -labeled retinol-lipid gave only one spot on TLC in solvent A at  $R_f$  0.15–0.2, the same as chemically synthesized retinyl phosphate. About 10% of the radioactivity was eluted in the area of retinyl phosphate mannose (0.025 M ammonium acetate). **Fig. 6** shows a typical separation of [ $^{14}\text{C}$ ]mannolipid prepared as described under Methods from [ $^{14}\text{C}$ ]retinyl phosphate by DEAE-cellulose chromatography. As demonstrated previously (16), both dolichyl phosphate [ $^{14}\text{C}$ ]mannose and retinyl phosphate [ $^{14}\text{C}$ ]mannose were eluted as a single peak at 0.025 M ammonium acetate. Of the two peaks absorbing at 325 nm, one was eluted in the area of retinyl phosphate mannose and dolichyl phosphate mannose, and the other in the area of retinyl phosphate. In conclusion the data show unequivocally that the majority of the radioactivity in the polar derivative (90%) is [ $^{14}\text{C}$ ]retinyl phosphate, at least at 20 min incubation of the intestinal cells. This compound had



**Fig. 6.** Separation by DEAE-cellulose chromatography of retinyl phosphate mannose and dolichyl phosphate mannose from retinyl phosphate. A mixture of retinyl phosphate [ $^{14}\text{C}$ ]mannose and dolichyl phosphate [ $^{14}\text{C}$ ]mannose was obtained as described under Methods. An aliquot of about 2,300 cpm out of 300,000 dpm of the extractable lipid was chromatographed in solvent A. About 39% of the radioactivity was recovered in the area of mannose, mannosyl-1-phosphate, and guanosine diphosphate mannose [ $R_f$  values lower than 0.15 (16)]. 910 cpm was in retinyl phosphate [ $^{14}\text{C}$ ]mannose and 4% cpm in dolichyl phosphate [ $^{14}\text{C}$ ]mannose. The column ( $1.3 \times 7$  cm) was eluted as described for Fig. 5, except that 3.5-ml fractions were collected. The absorption at 325 nm (black blocks) and the radioactivity (solid line) on 0.1 ml aliquots were monitored. 55% of the initial radioactivity (165,000 dpm out of 300,000 dpm) was associated with the mannosyl lipids, as expected from chromatography in solvent A. The calculated amount of retinol recovered from DEAE-cellulose as the sum of retinyl phosphate and retinyl phosphate mannose was about  $7 \mu\text{g}$ . The calculated molar ratio of retinol to mannose was very close to unity.

a  $R_f$  of 0.15–0.2 on TLC in solvent A and could be recovered from the silica gel by extraction of the gel with 99% methanol. Recovery from the plate was usually about 40%, as for chemically synthesized retinyl phosphate.

#### Characterization by UV absorption

The equivalent of 16,000 cpm (34,500 dpm) of  $^{14}\text{C}$ -labeled lipid, purified as described under Methods, was dried, dissolved in 1 ml of dehydrated ethanol, and the spectrum was then recorded (Fig. 7). The spectrum of chemically synthesized retinyl phosphate, obtained through the same procedure, is also shown in Fig. 7. The two compounds display identical spectra. Since  $\text{OD}_{325\text{nm}} = 0.12$ , we calculated a total of 1  $\mu\text{g}$ , and approximately 0.035  $\mu\text{g}$  of retinyl phosphate derivatives for each small intestinal lining of the hamster. On the basis of specific radioactivity (10 mCi/mMole) the amount of retinol equivalent to 16,000 cpm (34,500 dpm) is 0.49  $\mu\text{g}$ . Hence there is a dilution of 1.5-fold by endogenous retinyl phosphate.

#### Hydrolysis of purified [ $^{14}\text{C}$ ]retinyl phosphate by alkaline phosphatase

About 4,000 cpm of the purified [ $^{14}\text{C}$ ]retinyl phosphate was used for enzymatic hydrolysis with alkaline

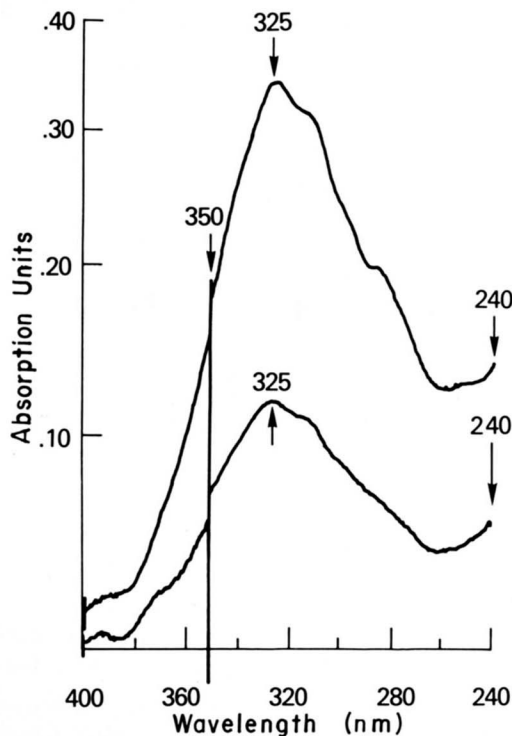


Fig. 7. Ultraviolet absorption spectrum of synthetic and biosynthetic retinyl phosphate. The ordinate represents absorbance units; the abscissa represents the absorption wavelength in nanometers.

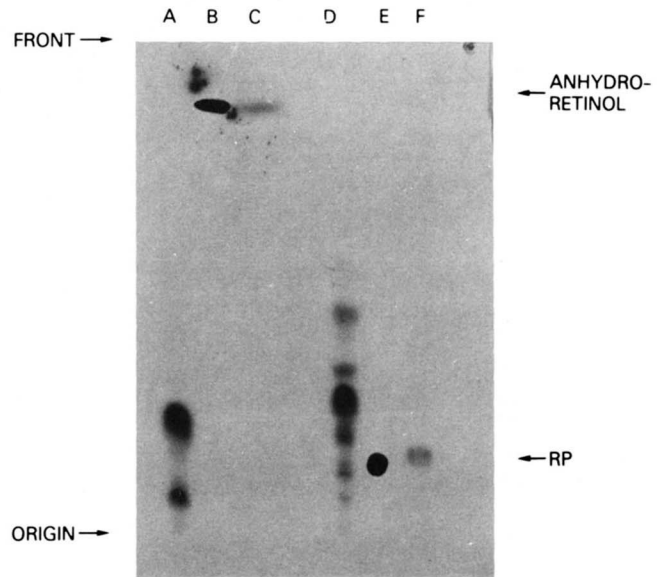
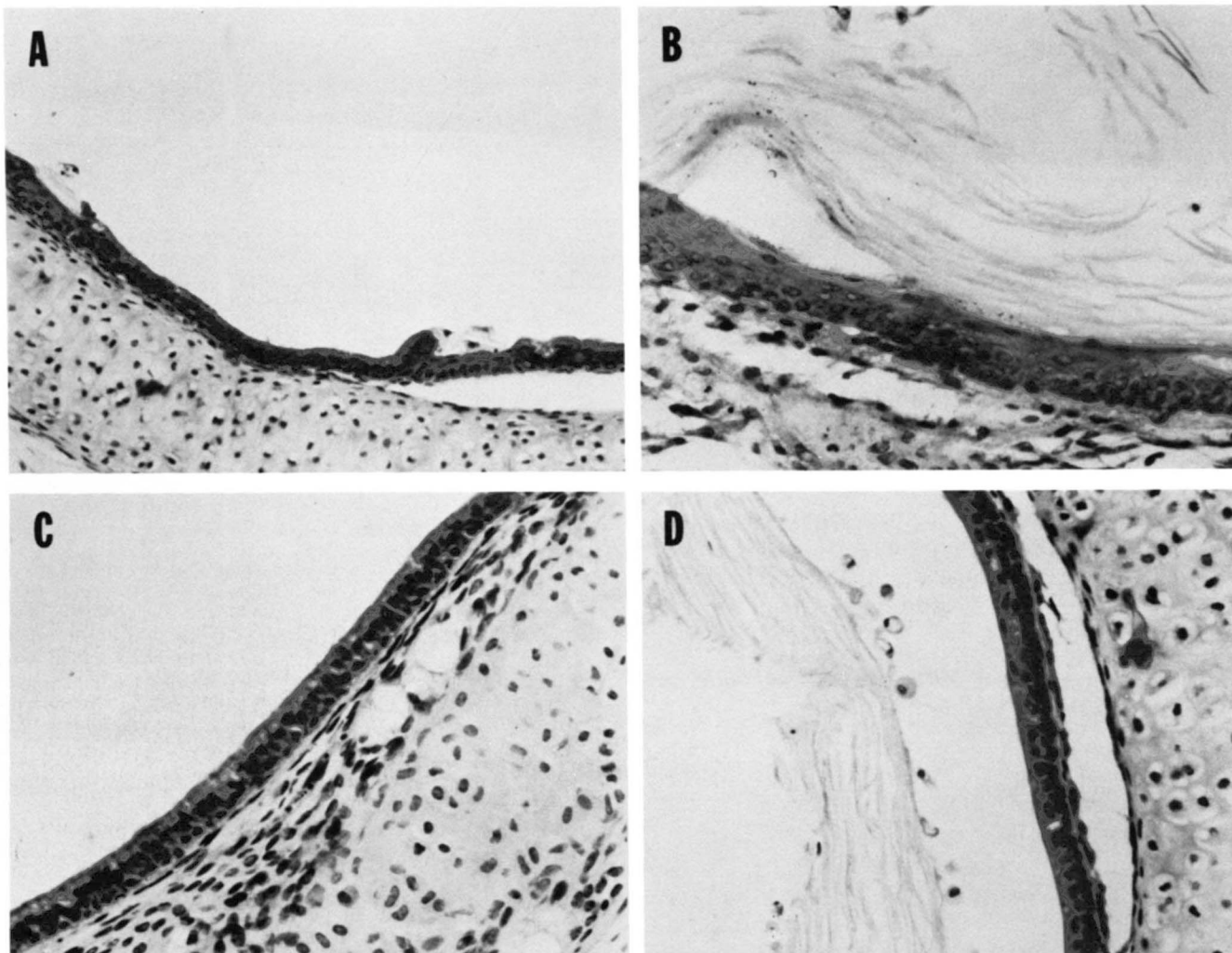


Fig. 8. Comparative thin-layer chromatography before and after mild alkali hydrolysis of  $^{14}\text{C}$ -labeled lipid,  $^{32}\text{P}$ -labeled lipid, and synthetic retinyl phosphate. 335 cpm of  $^{32}\text{P}$ -labeled lipid, 675 cpm of  $^{14}\text{C}$ -labeled lipid, and 11  $\mu\text{g}$  of retinyl phosphate after and before mild alkali hydrolysis were chromatographed on thin-layer plates (F254) in solvent A. A.  $^{32}\text{P}$ -labeled lipid after alkaline hydrolysis; B. anhydroretinol standard; C.  $^{14}\text{C}$ -labeled lipid after alkaline hydrolysis; D. Intact  $^{32}\text{P}$ -labeled lipid; E. Intact retinyl phosphate; F. Intact  $^{14}\text{C}$ -labeled lipid.

phosphatase from chicken intestine as described under Methods. Only 9% of the radioactivity was released as retinol, as for chemically synthesized retinyl phosphate.

#### [ $^{32}\text{P}$ ]phosphate incorporation into phosphorylated retinol by intestinal epithelial cells

Essentially the same conditions of culture, extraction, and purification as for the time course study were used. The medium contained 43  $\mu\text{g}$  of [ $^{14}\text{C}$ ]retinol/ml as well as 41.5  $\mu\text{Ci}/\text{ml}$  of  $\text{H}_3^{32}\text{PO}_4$  (carrier-free). Eight hamsters were killed and the cells were incubated for 90 min at 37°C. The silicic acid-purified lipids and 45  $\mu\text{g}$  of chemically synthesized retinyl phosphate were, before and after mild alkali hydrolysis, chromatographed on silica gel plates that were developed in solvent A. Radioautography (Fig. 8) shows that the  $^{32}\text{P}$ -labeled fraction after DEAE-cellulose and silicic acid chromatography contained at least seven main components. Two minor spots corresponded to chemically made retinyl phosphate and biosynthetic [ $^{14}\text{C}$ ]retinyl phosphate. It is clear from this chromatogram that direct scraping of the [ $^{14}\text{C}$ ]retinyl phosphate removes all extraneous phospholipids more effectively than any other procedure known. Fig. 8A illustrates the behavior in solvent A of the  $^{32}\text{P}$ -labeled lipid after mild alkaline hydrolysis. Only two main products remained lipid soluble.

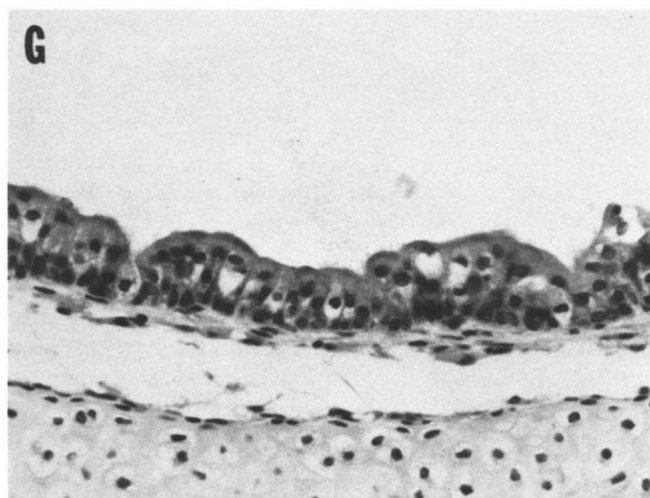
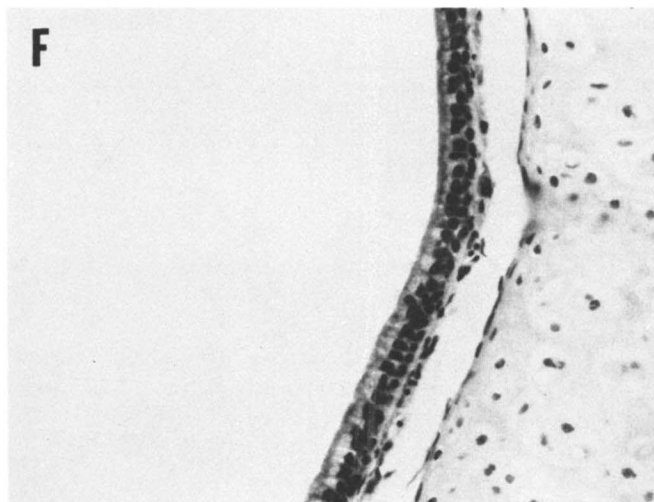
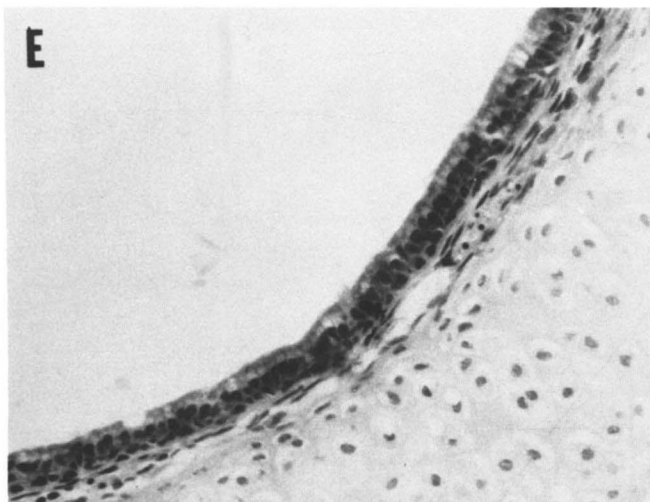


**Fig. 9.** Biological activity of  $\beta$ -retinol, chemical retinyl phosphate, and biosynthetic [ $^{14}\text{C}$ ]retinyl phosphate in the tracheal organ culture assay. Treatments are explained in the Results section and in Table 1. A. Tracheal section from group 1 (Table 1), kept for 6 days in a vitamin A-deficient medium, showing mild squamous metaplasia; B. Tracheal section from group 2, kept 13 days in vitamin A-deficient medium, with severe squamous metaplasia; C. Tracheal section from group 3, with cultures exposed to  $1 \times 10^{-7}$  M  $\beta$ -retinol for 24 hr, showing regeneration of normal epithelium; D. Same as C, except that tracheas were exposed to  $1 \times 10^{-8}$  M retinol. The epithelium is undergoing a healing process. There is no production of keratohyaline granules. E. Section from cultures of group 5, treated for 24 hr with  $1 \times 10^{-7}$  M retinyl phosphate, chemically synthesized. The biological activity is similar to retinol; F. A different section from group 5; G. Section from group 6 kept in the presence of biosynthetic [ $^{14}\text{C}$ ]retinyl phosphate at a concentration of  $5.5 \times 10^{-8}$  M. No keratinization is visible. This section is from one of two tracheas (out of 5 in group 6) that presented squamoid epithelium on a healing stage with disorientation; H. The same treatment as G. Prominent areas of glandular formation are shown. Magnification  $\times 320$ .

### Biological activity of the retinyl phosphate derivatives

Six male golden Syrian hamsters were kept on a vitamin A-deficient diet from birth to the day of killing (age 30 days). At the time of killing the animals were still growing and no overt signs of deficiency were visible. [ $^{14}\text{C}$ ]retinyl phosphate was prepared as usual from intestinal cells from [carbinol- $^{14}\text{C}$ ]retinol. The total amount of radioactivity was 60,000 cpm dissolved in 25  $\mu\text{l}$ . Of this solution 5  $\mu\text{l}$  was used for each dish. The biological test system developed by Sporn et al. (20) was used for the study of

the biological activity of retinoic acid, retinol, and other retinol derivatives. Tracheal rings from 30-day-old hamsters fed a vitamin A-deficient diet since birth were placed in culture before overt symptoms of deficiency were evident in the animals. The tracheas were cultured in a chemically defined, serum-free medium: CMRL-1066; with crystalline bovine insulin, 1.0  $\mu\text{g}/\text{ml}$ ; hydrocortisone hemisuccinate, 0.1  $\mu\text{g}/\text{ml}$ ; glutamine 2 mM; penicillin 100 U/ml and streptomycin 100  $\mu\text{g}/\text{ml}$ . Cultures were aerated with 50% oxygen, 45% nitrogen and 5% carbon dioxide. The dishes were rocked to allow contact with



the gas and liquid phases. All the tracheas were grown in medium containing no vitamin A for 6 days. Seven of them were harvested and analyzed at this time (group 1). Five other groups were included. Group 2 was cultured for 13 days without vitamin A. Group 3 was exposed for only 24 hr (day 7 of culture) to  $1 \times 10^{-7}$  M retinol. Group 4 was exposed for only 24 hr (day 7 of culture) to  $1 \times 10^{-8}$  M retinol. Group 5 was exposed for 24 hr to synthetic retinyl phosphate at  $1 \times 10^{-7}$  M and group 6 was exposed for 24 hr to [ $^{14}\text{C}$ ]retinyl phosphate from intestinal cells at  $5.5 \times 10^{-8}$  M as calculated from radioactivity; if the dilution from endogenous retinyl phosphate is 1.5, then the actual concentration would have been  $8.2 \times 10^{-8}$  M.

Of the 7 cultures analyzed at day 6 without vitamin A (group 1), three had mild (2–10% of epithelial surface) squamous metaplasia; two had moderate (10–40% of the epithelial surface) squamous metaplasia; and two had severe (more than 40% of the epithelial surface) squamous metaplasia. All cultures

in group 1 had signs of keratinization and keratohyaline granules. **Fig. 9A** shows a tracheal culture from group 1 displaying mild squamous metaplasia.

Group 2 showed a more severe pattern of squamous metaplasia (4 out of 7 with moderate squamous metaplasia). **Fig. 9B** shows a section from this group with severe squamous metaplasia.

Group 3 contained 8 tracheas which were exposed to  $1 \times 10^{-7}$  M  $\beta$ -retinol for 24 hr (day 7) and cultured in fresh medium up to day 13. Out of 8 tracheas, none had keratohyaline granules (KHG) and the squamous metaplasia was less severe. **Fig. 9C** shows a section emphasizing the normal appearance of these cultures.  $\beta$ -Retinol was not as effective at  $1 \times 10^{-8}$  M, group 4. In this group, five out of seven cultures had keratohyaline granules. Moreover, the extent of squamous metaplasia was much less than for groups 1 and 2 (**Table 1**). **Fig. 9D** shows a section from group 4. The keratin had been shed off, there was no production of new keratohyaline



TABLE 1. Biological activity of chemically and biologically synthesized retinyl phosphate<sup>a</sup>

Group	Treatment of Cultures	Total Tracheas Per Group	Columnar	Cuboidal	Minimal Squamoid	Mild 2–10%	Moderate 10–40%	Severe 40%	K	KHG
1	No Vitamin A harvested on day 6	7				3 out of 7	2 out of 7	2 out of 7	7 out of 7	7 out of 7
2	No Vitamin A harvested on day 13	7				1 out of 7	4 out of 7	2 out of 7	7 out of 7	7 out of 7
3	$\beta$ -Retinol ( $1 \times 10^{-7}$ M) 1 day exposure harvested on day 13	8		1 out of 8	3 out of 8	2 out of 8		2 out of 8	3 out of 8	none out of 8
4	$\beta$ -Retinol ( $1 \times 10^{-8}$ M) 1 day, day 13	7				5 out of 7	1 out of 7	1 out of 7	5 out of 7	5 out of 7
5	$\beta$ -Retinyl-phosphate Synthetic ( $1 \times 10^{-7}$ M) 1 day exposure harvested on day 13	5			1 out of 5	2 out of 5	2 out of 5		1 out of 5	none out of 5
6	[ <sup>14</sup> C]Retinyl-phosphate Biosynthetic ( $5.5 \times 10^{-8}$ M) harvested on day 13	5 <sup>b</sup>		1 out of 5	1 out of 5	1 out of 5	none out of 5	none out of 5	none out of 5	none out of 5

The treatment for each group is evident from the column on Treatment of Cultures and from the Results section. Each trachea was fixed and stained with hematoxylin and eosin. Cultures were graded as to percentage of their total epithelium showing squamous metaplasia on eight cross sections from the middle of each trachea. Squamous metaplasia was defined as severe when it involved more than 40% of the total epithelial section; moderate when it involved 10–40% of the total surface; mild when it involved 2–10% of the total surface; and minimal for less than 2%, as by Sporn et al (20). K, keratin; KHG, keratohyaline granules.

<sup>a</sup> [<sup>14</sup>C]Retinyl phosphate was prepared as detailed under Results.

<sup>b</sup> Two out of five presented squamoid epithelia at a healing stage with disorientation and with glandular structures.

granules, and the epithelium was undergoing a healing process, although it was still squamoid.

Chemically synthesized retinyl phosphate (group 5) at  $1 \times 10^{-7}$  M was effective in suppressing the formation of keratohyaline granules in all five cultures and its activity was similar to that of retinol. Fig. 9E and F show tracheal sections from group 5.

The biological compound was tested in five cultures, group 6, at a concentration of  $5.5 \times 10^{-8}$  M. The compound was as active as retinol and retinyl phosphate in suppressing the formation of keratohyaline granules and more active than retinol in reversing keratinization. None of the cultures showed signs of keratinization. None of the five tracheas had severe or moderate squamous metaplasia. One out of five tracheas had mild metaplasia, one was squamoid, and one was cuboidal. In addition, two out of five cultures presented squamoid epithelium at a healing stage with disorientation (Fig. 9G and H) and with very prominent glandular structures in some areas. One of the most prominent areas is shown in Fig. 9H. The glands appear to be filled with proteinaceous material.

It was of interest to determine whether breakdown of the biological compound had occurred during 24 hr of exposure to the tracheal cells. The media from the 5 incubations were pooled (approximately 25 ml in toto) and 5 volumes of chloroform–methanol 2:1

were added. The organic phases were dried and applied to silica gel plates for TLC in toluene–chloroform–methanol 50:12.5:12.5; retinyl phosphate stayed at the origin and retinol had an  $R_f$  of 0.65. All the radioactivity behaved as retinyl phosphate. Surprisingly, only 1.5% of the original compound was recovered in the medium; thus more than 95% of the biological compound had been utilized by the cultures in 24 hr.

## DISCUSSION

The finding that incorporation of [<sup>14</sup>C]mannose into glycolipids and glycoproteins is dramatically (95%) decreased in vitamin A deficiency (1) and elevated (5–6-fold) in excess vitamin A<sup>1</sup> led us to formulate the hypothesis that retinol is molecularly involved in the transfer of mannose and possibly other monosaccharides. To test this hypothesis we have synthesized retinyl phosphate and demonstrated that it functions as an acceptor of mannose from guanosine diphosphate mannose in an in vitro system with rat liver membranes, which also synthesize dolichyl phosphate mannose (16). The addition of chemical retinyl phosphate stimulated the synthesis of retinyl phosphate mannose, a compound synthesized by normal rat liver membranes even in the absence

of exogenously supplied retinyl phosphate (16). The amount of radioactivity incorporated into the endogenous acceptor was only 5–10% of the total mannanolipid. The rest was associated with dolichyl phosphate mannose (16). The syntheses of retinyl pyrophosphate (21) and retinyl phosphate galactose (22–24) have also been reported.

If retinyl phosphate is an intermediate in the synthesis of retinyl phosphate glycosides, it should be possible to isolate it, if the proper biological system is used. Attempts to isolate enough [carbinol-<sup>14</sup>C]-retinyl phosphate from hamster liver were not very successful because of the enormous dilution of labeled [<sup>14</sup>C]retinol by the endogenous pool of [<sup>12</sup>C]retinol. A compound with chromatographic properties similar to retinyl phosphate, but not characterized further, was isolated by Barr and De Luca<sup>2</sup>. The use of higher specific radioactivity [1-<sup>3</sup>H]retinol in the whole animal gave a variety of radioactive products, probably due to exchange of tritium, and this approach was abandoned.

The intestinal cell systems of partially depleted and normal hamsters proved rewarding. After 60 min of incubation with [carbinol-<sup>14</sup>C]retinol, 90% of the radioactivity incorporated into phosphorylated derivatives of retinol was a [<sup>14</sup>C]retinyl phosphate. This compound was shown to behave like synthetic retinyl phosphate by chromatography on silicic acid and DEAE-cellulose and by TLC; to be labile to mild alkaline conditions, giving a compound identical with anhydroretinol (Fig. 4D) as for chemically made retinyl phosphate; and to possess the same absorption spectrum as synthetic retinyl phosphate with a maximum at 325 nm. This last finding and the formation of anhydroretinol by mild alkali strongly suggest the retention of the five conjugated double bonds of retinol in the phosphorylated compound. The biological compound, like synthetic retinyl phosphate, is not a good substrate for alkaline phosphatase, which releases only 9% of the original compound as retinol. The biological compound is as active as retinol and synthetic retinyl phosphate in suppressing the formation of keratohyaline granules in cultures of hamster tracheas at a concentration of at least  $5.5 \times 10^{-8}$  M. Only 1.5% of the compound is recovered in the medium, all of it as retinyl phosphate, suggesting that 98.5% had been taken up by the vitamin A-deficient tracheas.

A concentration of about 0.035  $\mu$ g of retinyl phosphate per small intestinal lining was calculated. We have demonstrated that retinyl phosphate can be

separated from retinyl phosphate mannose by a linear (16) and a concave gradient of ammonium acetate (Fig. 6). About 90% of the radioactivity from [carbinol-<sup>14</sup>C]retinol is associated with retinyl phosphate and only 10% is eluted in the area of its glycosylated derivatives (Fig. 5), at least after 60 min of incubation of the intestinal cells.

In previous work (13), [carbinol-<sup>14</sup>C]retinol was injected intraperitoneally into Syrian golden hamsters 3.5 hr before they were killed. Extraction and analysis of liver lipids by DEAE-cellulose and silicic acid chromatography showed that 90% of the radioactivity was eluted by 0.025 M ammonium acetate as for retinyl phosphate mannose and only 10%, or less, in the area of retinyl phosphate. Hence, it is evident that the relative proportions of [<sup>14</sup>C]retinyl phosphate and its glycosylated derivatives depend on the particular conditions of the assay.

In conclusion, we have demonstrated that retinol is phosphorylated to retinyl phosphate and that this compound accepts mannose from guanosine diphosphate mannose to form retinyl phosphate mannose. ■

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