Chemical Synthesis of all-trans- β -Retinoyl Phosphate

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(Received 2 September 1976)

all-trans- β -Retinoic acid is phosphorylated to retinoyl phosphate by bis(triethylamine) phosphate with yields of 10–15%. The product is soluble in methanol and is eluted from DEAE-cellulose acetate at a concentration of 0.1M-ammonium acetate in 99% (v/v) methanol. Its phosphate/retinoic acid molar ratio is 1. Retinoyl phosphate has an absorption maximum at 360nm in methanol, whereas retinoic acid has a maximum at 350nm. The compound is hydrolysed at pH2 and pH13 for 20min at 37°C, but is relatively stable under the same conditions at pH4, 6, 8 and 10. Retinoyl phosphate (R_F 0.1) can be separated from retinyl phosphate (R_F 0.2) by chromatography on thin layers of silica gel in chloroform/methanol/water (60:25:4, by vol.).

Retinoic acid (Arens & Van Dorp, 1946a) is not reduced to retinol (Arens & Van Dorp, 1946b; Dowling & Wald, 1960) and cannot maintain vision (Dowling & Wald, 1960) and reproduction (Thompson, 1969), but is active in growth (Sharman, 1949; Howell *et al.*, 1967; Juneja *et al.*, 1969) and maintenance of normal epithelial phenotype (Sporn *et al.*, 1975).

Phosphoryl (Frot-Coutaz *et al.*, 1976) and glycosyl-phosphoryl (De Luca *et al.*, 1970b, 1973; Rosso *et al.*, 1975) derivatives of retinol are synthesized by normal epithelial tissue and the synthesis of epithelial glycoproteins has been shown to depend on vitamin A (De Luca *et al.*, 1970a, 1971, 1975).

It is therefore of interest to investigate whether retinoic acid is involved in a similar pathway. We report here the chemical synthesis and characterization of retinoyl phosphate.

Materials

DEAE-cellulose, all-trans-retinoic acid and trichloroacetonitrile were obtained from Eastman-Kodak, Rochester, NY, U.S.A. all-trans-[carboxyl- 14 C]Retinoic acid was from Amersham-Searle, Arlington Heights, IL, U.S.A., acetonitrile was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A., phosphoric acid was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., and t.l.c. plates (0.250 mm thick), precoated with silica gel 60 F254, were from E. Merck A.G., Darmstadt, Germany. Bis(triethylamine) phosphate was synthesized as described by Popják *et al.* (1962).

Experimental and Results

Synthesis and purification

Retinoyl phosphate was prepared by a modification of the method of Popják *et al.* (1962) for farnesyl phosphate and pyrophosphate. all-trans- β -Retinoic acid (40mg) was completely dissolved in 10ml of acetonitrile, and 1 ml of trichloroacetonitrile and 10ml of acetonitrile containing 100mg of bis(triethylamine) phosphate was added to the retinoic acid solution. The mixture was stirred at room temperature (25°C) in a stoppered flask under N_2 for 1 h in red light (650nm). The mixture was neutralized by the addition of small portions of concentrated (29%) NH_3 with continuous stirring, and the resulting mixture was filtered to remove the precipitate. The filtrate was applied to a column (1.3 cm $\times$ 13 cm) of DEAE-cellulose acetate (Frot-Coutaz *et al.*, 1976) equilibrated with 99% (v/v) methanol. The column was first eluted with 100ml of 0.01M-ammonium acetate to remove unesterified retinoic acid and then with a concave gradient of ammonium acetate as shown in Fig. 1. Retinoyl phosphate was eluted at 0.1M-ammonium acetate (Fig. 1); its absorption spectrum (Fig. 2b) has a maximum at 360nm, a shift of 10nm from the maximum absorption of retinoic acid (350nm; Fig. 2a).

The phosphorylated product was pooled (169ml), and 2vol. of chloroform (338 ml) and 100ml of water were added to study the partition of retinoyl phosphate between the upper and lower phases. The volume of the lower organic phase was 370ml and that of the upper phase 250ml. A sample (0.1 ml) was taken

Fig. 1. Purification of retinoyl phosphate by chromatography on DEAE-cellulose

The filtrate of the neutralized incubation mixture was applied to a column (1.3 cm × 13 cm) of DEAE-cellulose acetate, as described under 'Synthesis and purification'. The column was first eluted with 100 ml of 0.01 M-ammonium acetate, which removed retinoic acid. A gradient (●) of 0.01–0.1 M-ammonium acetate was run over a period of 87 h to elute retinoyl phosphate at a flow rate of 5.7 ml/h. Fractions were collected every 20 min. The elution was monitored at 360 nm (○).

Fig. 2. Absorption spectra of retinoic acid (a) and of retinoyl phosphate (b)

(a) A solution of 15 nmol of all-*trans*-β-retinoic acid/ml of methanol was used. (b) A solution of 205 nmol of synthetic retinoyl phosphate/ml of methanol was used.

out of each phase and the absorption spectrum recorded. Both phases contained the phosphorylated product. The lower organic phase contained 2.8 mg of retinoyl phosphate (32%) and the upper phase 5.8 mg (68%).

Determination of the extinction coefficient of retinoyl phosphate

Retinoic acid (29.1 mg) was dissolved in 10 ml of acetonitrile; to this was added 10 μCi of radiochemically pure all-*trans*-[carboxyl-¹⁴C]retinoic acid of specific radioactivity 9.5 mCi/mmol, 1 ml of trichloroacetonitrile and 10 ml of acetonitrile containing 100 mg of bis(triethylamine) phosphate. The reaction and purification of retinoyl phosphate were as described above.

[carboxyl-¹⁴C]Retinoyl phosphate was obtained of specific radioactivity 0.102 mCi/mmol. The absorption spectrum showed a maximum at 360 nm. The extinction coefficient calculated from the specific radioactivity is 34000 at 360 nm. This was measured as follows. A portion (1 ml) of the radioactive methanolic solution of the product purified by chromatography on DEAE-cellulose was assayed for radioactivity as described in Table 1. From the specific radioactivity of retinoic acid (0.102 mCi/mmol) a value of 0.225 μmol of [carboxyl-¹⁴C]retinoyl phosphate was obtained in 1 ml of methanolic solution. The absorption of the same sample at 360 nm was determined (7.6 E units/ml of methanolic solution). Thus:

$$\epsilon = \frac{7.6 \times 10}{0.225 \times 10} = 34000$$

Phosphate assay

Retinoyl phosphate (0.225 μmol) was dissolved in 1 ml of 99% methanol, to which 1 ml of NH₃ (final concentration 15%, 3.5 M) was added, and the solution was left overnight at room temperature (25°C). The solution was dried at 100°C. The dry residue was dissolved in 2 ml of deionized water and assayed for phosphate by the method of Chen *et al.* (1956) along with standards. The molar ratio of retinoic acid (measured from the E₃₆₀) to phosphate was 1.09.

Chromatographic properties on thin layers of silica gel

Retinoyl phosphate was chromatographed in chloroform/methanol/water (60:25:4, by vol.) along with standard retinoic acid and retinyl phosphate. Retinoyl phosphate had *R_F* 0.1, retinyl phosphate *R_F* 0.2 and retinoic acid *R_F* 0.8. Chromatography in toluene/chloroform/methanol (4:1:1, by vol.) gave the following values: retinyl phosphate and retinoyl phosphate, *R_F* 0; retinoic acid, *R_F* 0.5.

Hydrolyses

These were carried out at different pH values as

Table 1. *Hydrolysis of retinoyl phosphate at different pH values*

[carboxyl- ^{14}C]Retinoyl phosphate was prepared as described in the Experimental and Results section. Portions containing 3000 d.p.m. were dissolved in 250 μl of chloroform/methanol/water (3:48:47, by vol.) as described by Folch *et al.* (1957), and kept at different pH values in the dark at 37°C for 20 min. pH2 was obtained by adding 75 μl of 1M-acetic acid in water and 0.175 ml of water. pH4 was obtained by adding 250 μl of 0.05M-potassium biphthalate buffer, pH4. pH6 was obtained by adding 250 μl of 0.05M- KH_2PO_4 /NaOH buffer, pH6. pH8 was obtained by adding 250 μl of 0.025M- KH_2PO_4 buffer, pH8. pH10 was obtained by adding 25 μl of 1M-NaOH and 175 μl of water. pH13 was obtained by adding 75 μl of 1M-NaOH and 175 μl of water. Hydrolysis was stopped by cooling to 4°C and partitioning (as usual) between 5 vol. of chloroform/methanol (2:1, v/v) and 1 vol. of water. Solutions were not neutralized because of the high rates of hydrolysis for mixed anhydrides at pH7 (Isaacs, 1974). The organic phase was applied to thin layers of silica gel, and chromatographed in toluene/methanol/chloroform (4:1:1, by vol.). In this system retinoyl phosphate has R_F 0.0, retinoic acid has R_F 0.45 and methyl retinoate R_F 0.9. The plates were scraped by an automatic zonal scaper, obtained from New England Nuclear, Cambridge, MA, U.S.A. Portions (0.5 cm) were collected directly into counting vials. Methanol (0.2 ml or a 99%, v/v, solution) was added to each vial and the vials were counted for radioactivity after addition of 15 ml of scintillant, as previously described (Frot-Coutaz *et al.*, 1976). The Table shows the percentage of radioactivity found as retinoyl phosphate, retinoic acid and methyl retinoate at different pH values.

pH	Percentage as intact retinoyl phosphate	Percentage as retinoic acid	Percentage as methyl retinoate
2	21	59	20
4	93	7	0
6	94	6	0
8	86	14	0
10	93	7	0
13	10	38	52

described in Table 1. The compound was labile at pH2 and pH13 when kept for 20 min at 37°C. It yielded 59% retinoic acid and 20% methyl retinoate at pH2. At pH13 38% was as retinoic acid and 52% as methyl retinoate.

Discussion

Initially this method was designed by Popják *et al.* (1962) for the phosphorylation of farnesol to the monophosphate and the pyrophosphate derivatives.

Here we demonstrate that bis(triethylamine) phosphate in the presence of trichloroacetonitrile is effective in yielding a mixed anhydride of retinoic acid

and phosphoric acid without the formation of a pyrophosphate derivative and with yields of 10–15%.

Moreover, this method is more effective for preparing retinyl phosphate, with consistent 4–5-fold higher yields than our previous method (Rosso *et al.*, 1975). This is due to the omission of the alkaline extraction step used previously (Rosso *et al.*, 1975); indeed retinyl phosphate has been reported to be very sensitive to alkali, with the formation of anhydroretinol (Frot-Coutaz *et al.*, 1976).

Since our main concern is to find out whether retinoic acid undergoes phosphorylation and subsequent mannosylation, as for retinol (Frot-Coutaz *et al.*, 1976; Rosso *et al.*, 1975), the availability of authentic retinoyl phosphate will allow us to determine whether such a pathway occurs.

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