The conversion of exogenous retinol and related compounds into retinyl phosphate mannose by adult *Brugia pahangi* *in vitro*

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(Received 3 February 1983/Accepted 5 May 1983)

Adult *Brugia pahangi* took up and incorporated β -carotene and free retinol *in vitro*. The uptake of retinol was 50 times greater than that of β -carotene under similar incubation conditions. β -Carotene was almost entirely metabolized, primarily to retinol. The metabolism of retinol by *B. pahangi in vitro* was less extensive, with a variety of retinoids tentatively identified, including retinyl phosphate (Ret-P), retinyl phosphate mannose (Ret-P-Man) and anhydroretinol as minor metabolites. *B. pahangi* microsomes were also shown to biosynthesize Ret-P-Man from exogenous Ret-P and GDP-mannose, but not from endogenous lipid acceptors alone. In this circumstance an unidentified lipid appeared to be mannosylated by *B. pahangi*. The rate of mannose transfer to exogenous Ret-P by *B. pahangi* microsomes was $150 \text{ pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$. Ret-P-Man synthetase activity from both *B. pahangi* and rat liver microsomes had an absolute requirement for bovine serum albumin and MnCl_2 , and occurred in the absence of detergent. The results suggest a biochemical role for vitamin A in *B. pahangi*, possibly in filarial glycoprotein synthesis.

Recently two attempts were made to investigate interactions of vitamin A between the human host and the causative organism of African river blindness, the nematode *Onchocerca volvulus* (Joseph *et al.*, 1979; Haller & Lauber, 1980). The results of these separate clinical and epidemiological studies in which blood vitamin A levels were determined in onchocerciasis-infected populations showed only slight trends, confusingly opposite in effect. Perhaps the most significant finding was that of Struchler *et al.* (1981), who observed that the vitamin A alcohol (retinol) concentration of adult *O. volvulus* was eight times higher than that of the surrounding host tissue. This was the first demonstration of a vitamin A derivative in a filarial nematode, although the literature contains several publications (Leutskaya, 1961, 1971, 1974; Juhasz & Babos, 1969) on the gastrointestinal nematode *Ascaris lumbricoides*, detailing not only the occurrence of retinol, but its

uptake from the gut environment and its conversion from β -carotene by the parasite.

The role of vitamin A in *A. lumbricoides* and *O. volvulus* remains to be elucidated, although there is some evidence (Leutskaya & Fais, 1977; Leutskaya & Prokhorova, 1979) to indicate that vitamin A may influence the host-parasite interaction by modulating the immune response. In this paper we should like to suggest that vitamin A in its phosphorylated form (Ret-P) may function as an intermediate lipid carrier in filarial glycoprotein synthesis. A similar function in mammalian glycoprotein biosynthesis has been indicated (De Luca *et al.*, 1979a), and a metabolite of retinol, Ret-P-Man, has been shown to function directly as a substrate for mannosyltransferases of rat liver microsomal membranes (Quill & Wolf, 1981). In addition we have recently demonstrated that glycoprotein synthesis in the dog heartworm *Dirofilaria immitis* (a related filarial nematode) proceeds via a prenyl phosphate-stimulated glycosyltransferase system (Comley *et al.*, 1982).

We now report our most recent finding that the adult filarial nematode *Brugia pahangi in vitro* took up and incorporated β -carotene and retinol. We have also shown the conversion of β -carotene into retinol *in vitro* by *B. pahangi* and the metabolism of retinol to Ret-P and Ret-P-Man. In addition we have

Abbreviations used: BSA, bovine serum albumin; Dol-P-Man, dolichyl phosphate mannose; MEM, minimum essential medium; Ret-P-Man, retinyl phosphate mannose; Ret-P, retinyl phosphate; r.p.t.l.c., reversed-phase thin-layer chromatography.

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demonstrated the biosynthesis of Ret-*P*-Man from GDP-mannose and exogenous Ret-*P* in *B. pahangi* microsomes. These findings are discussed with reference to the potential involvement of Ret-*P* in filarial glycoprotein synthesis.

Materials and methods

Materials

Alumina, AMP, ATP, β -carotene, Carr-Price spray reagent, DEAE-Sephacel, dolichyl monophosphate (grade III), GDP-mannose, retinal, retinol, retinoic acid, retinyl acetate, retinyl palmitate, silicic acid (100–300 mesh) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Prenol C₅₅ phosphate (undecaprenyl phosphate) and BSA (fraction V) were obtained from Calbiochem–Behring Corporation, La Jolla, CA, U.S.A. All culture media were obtained from Gibco, Grand Island, NY, U.S.A. Scintasol cocktail was purchased from Isolab Inc., Akron, OH, U.S.A. KC₁₈F r.p.t.l.c. plates were from Whatman Inc., Clifton, NJ, U.S.A. Silica gel 60F-254 (Merck) t.l.c. plates were from Scientific Products, Boston, MA, U.S.A. GDP-[U-¹⁴C]mannose [sp. radioactivity 203 Ci/mol (7.51 GBq/mmol)] and [11,12(n)-³H]-vitamin A [free alcohol; sp. radioactivity 45 Ci/mol (1.57 TBq/mmol)] were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. β -[15,15'-³H]Carotene [sp. radioactivity 0.08 mCi/mg (2.96 MBq/mmol); lot no. Wh. IV/117] was supplied by Dr. W. E. Scott, Hoffmann–La Roche, Nutley, NJ, U.S.A. Standard Ret-*P*-[¹⁴C]Man and [¹⁴C]-Dol-*P*-Man were biosynthesized and purified by the procedures of Sasak *et al.* (1979) from rat liver microsomes utilizing GDP-[U-¹⁴C]mannose as the radioactive substrate. Ret-*P* was synthesized by the procedure of Bhat *et al.* (1980) and kindly provided by Dr. Luigi M. De Luca, N.I.H., Bethesda, MD, U.S.A. The Ret-*P*-[¹⁴C]Man, [¹⁴C]Dol-*P*-Man and Ret-*P* were stored protected from light in methanolic solutions, under N₂ at –20°C and usually used within 4 weeks of preparation. The Ret-*P* concentration was periodically checked spectrophotometrically on the basis of $A_{1\text{ cm}, 325\text{ nm}}^{1\%}$ (in methanol) = 1440. En³Hance was obtained from New England Nuclear, Boston, MA, U.S.A.

Adult *B. pahangi* were obtained from laboratory-infected jirds (gerbils) provided by Dr. J. M. McCall, University of Georgia, Athens, GA, U.S.A., under the auspices of the U.S.–Japan Co-operative Medical Science Program–N.I.A.I.D.

Preparation of crude microsomal vesicles

Crude microsomal vesicles were prepared from fresh adult *B. pahangi* (mixed sexes) and from the livers of male albino Sprague–Dawley rats (weighing 200–300 g) by the methods of Shidoji & De Luca

(1981). The *B. pahangi* extracts were always assayed on the day of the preparation, whereas some rat preparations were stored for up to 1 month at –70°C without apparent loss of activity.

Assay for mannosyltransferase activity (Ret-*P*-Man synthetase)

Mannosyltransferase activity to the lipid intermediate was measured in the absence of detergent in the presence of BSA by using the filter binding technique of Shidoji & De Luca (1981) developed specifically for the assay of Ret-*P*-Man synthesis. The procedure was followed exactly as those authors described, except that Scintasol was substituted as the scintillation cocktail. The contents of the assay are given in the legend to Table 1.

In other experiments, the equivalent of up to 0.5 mg of *B. pahangi* or 1 mg of rat liver microsomal protein were used in the above BSA-containing assays, except that 0.2 μ Ci of GDP-[U-¹⁴C]mannose was now added. Assays were run in the presence or in the absence of exogenous Ret-*P* at 37°C, and stopped after 10 min by the addition of 5 vol. of chloroform/methanol (2:1, v/v). The tubes were then cooled, vortex-mixed and two phases were allowed to separate by low-speed centrifugation at 3000 g for 15 min. The lower phase was removed, dried under a stream of N₂ and the residue immediately redissolved in 50 μ l of chloroform/methanol (2:1, v/v) containing 25 μ g of Ret-*P*. The resulting material was divided into two portions and each was spotted on to separate silica gel 60F-254 plates, along with authentic standards. Plates were developed in system 1 (described below) and the standards were visually detected under u.v. light or by exposure to I₂ vapour. One of the plates was then scraped for determination of the radioactive content and fluorography was carried out on the other (at –70°C) using Kodak X-OMAT-RP film after previously spraying the plate with En³Hance.

Incubation conditions

Filariae were maintained *in vitro* in Eagle's MEM, containing 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer and Earle's salts, to which was added MEM non-essential amino acids, antibiotic/antimycotic mixtures, L-glutamine and 3% (w/v) BSA.

B. pahangi of both sexes, in numbers equivalent to 0.2 g wet wt., were placed in 50 ml of the above culture medium to which was added 125 μ Ci (4625 kBq) of [11,12(n)-³H]vitamin A alcohol or 125 μ Ci (4625 kBq) of β -[15,15'-³H]carotene. The former was added to the culture medium directly from the manufacturer's sterile vial in 250 μ l of ethanol/water (9:1, v/v). β -Carotene was stored dissolved in a stock solution of toluene, in the dark under N₂ at 4°C. Just before the incubation, the re-

quired amount of β -carotene was transferred to a sterile tube and solvent was evaporated under a stream of N_2 . The β -carotene was resuspended in 500 μ l of sterile-filtered dimethyl sulphoxide, which was then added directly to the culture medium. Worms were incubated at 37°C for 24 h in the dark. At the end of the incubation period, worms were removed from the medium, pooled and then washed thoroughly with sterile Hepes/KOH buffer (pH 7.2, 10 mM) containing 0.9% (w/w) NaCl, then briefly rinsed with 99% methanol for about 1 min to remove any surface-adhering radioactivity, blotted dry and weighed.

Extraction of radioactive lipids derived from [11,12(n)- 3H] vitamin A alcohol

Extraction of the radioactive lipids derived from [11,13(n)- 3H]vitamin A alcohol proceeded immediately upon termination of the incubation in a semi-darkened laboratory. The worms were completely homogenized with 2 ml of 99% methanol and a small portion was removed for measurement of the radioactive content. The extract was then reduced to 0.5 ml under a stream of N_2 and diluted with 4 ml of chloroform to give chloroform/methanol (8:1, v/v).

Silicic acid chromatography

Silicic acid chromatography was then performed on columns (1 cm $\times$ 5 cm) packed with a slurry of silicic acid in chloroform/methanol (8:1, v/v) that was run into the column, and the free retinol and retinyl esters were eluted with 9 ml of chloroform/methanol (8:1, v/v); the flow rate was about 1.5 ml/h (Frot-Coutaz *et al.*, 1976). Ret-*P*, Ret-*P*-Man and any unrelated oxidation products were then eluted overnight from the column at 5°C, in the dark, with 18 ml of chloroform/methanol (1:1, v/v). Resulting eluates were rotary-evaporated to dryness under reduced pressure and immediately redissolved in 0.5 ml of 99% methanol, at which point a known portion was removed for measurement of the radioactive content.

DEAE-Sephacel chromatography

DEAE-Sephacel chromatography to separate Ret-*P* from Ret-*P*-Man in the fraction eluting with chloroform/methanol (1:1, v/v) on the silicic acid column was carried out by the method outlined by Sasak *et al.* (1979), the only difference being that carrier Ret-*P* (in 50 μ l of 99% methanol/30 mM-ammonium acetate, containing 25 μ g of Ret-*P*) was added to the sample before chromatography, and the first five fractions (5 ml each) were eluted with 99% methanol. The column was then eluted with 100 ml of 99% methanol/10 mM-ammonium acetate, followed by 100 ml of 99% methanol/50 mM-ammonium acetate. The radioactivity eluting in each fraction was measured by liquid-scintillation count-

ing of a known portion in 10 ml of Scintasol. Elution of Ret-*P* from the column was monitored by u.v. absorption spectroscopy at 325 nm.

T.l.c.

Known portions of the various eluates collected from the silicic acid and DEAE-Sephacel columns were separated by t.l.c.

System 1 used silica gel 60F-254 (0.25 mm thickness) and was developed with chloroform/methanol/water (45:35:6, by vol.) to a height of 14 cm (Shidoji & De Luca, 1981). System 2 used a $KC_{18}F$ reversed-phase plate (0.2 mm). This latter system was designed to repeat the conditions of the r.p.t.l.c. separation of retinoids achieved by Bhat *et al.* (1980). The greatest separation of the various retinoids was achieved by allowing the chromatogram to develop with ethanol/water (4:1, v/v) for an additional 3 h after the solvent had reached the top of the plate (i.e. a total development time of 6 h). Under these conditions, a good separation was achieved between Ret-*P* and Ret-*P*-Man; the former compound was visually detected by using a u.v. lamp or I_2 vapour. The position of Ret-*P*-Man was determined by using biosynthetic Ret-*P*-[^{14}C]Man (see Fig. 3, IV). Other authentic retinoids were visually detected either by using a u.v. lamp or by spraying with the Carr-Price reagent. The radioactivity in the plates was detected by scraping sections (0.5 cm) of the silica gel into counting vials, followed by the addition of 0.25 ml of methanol and 10 ml of Scintasol.

Alumina chromatography

Known portions of the fractions eluting with chloroform/methanol (8:1, v/v) on silicic acid and 0–10 mM-ammonium acetate in 99% methanol on DEAE-Sephacel were further separated by alumina chromatography (Sundaresan & De Luca, 1977). Columns (1 cm $\times$ 3 cm) of water-deactivated alumina (Brookmann grade III) were prepared in n-hexane. Samples were applied to the column in n-hexane, and anhydroretinol was eluted from the column with 9 ml of n-hexane. The resulting eluates were rotary-evaporated to dryness under reduced pressure, transferred to scintillation vials with n-hexane, evaporated under a stream of N_2 and 10 ml of Scintasol was added before scintillation counting.

Extraction of radioactive lipids derived from β -[15,15'- 3H]carotene

The initial extraction of radioactive lipids proceeded as for the [11,12(n)- 3H]vitamin A alcohol incubation except that the 99% methanol homogenate was divided into two equal portions. One-half was subjected to silicic acid, DEAE-Sephacel and

silica gel 60F-254 chromatography to reveal incorporation into Ret-*P* and Ret-*P*-Man as described above.

Alumina chromatography was utilized to separate unchanged incorporated β -carotene and anhydroretinol from the retinyl esters and from the free alcohol (Sundaresan & De Luca, 1977) in the remaining portion of the extract. Columns (5.5 cm $\times$ 2.2 cm; volume 20 ml) of water-deactivated alumina (Brookmann grade III) were prepared in *n*-hexane. The samples dissolved in 99% methanol was dried under a stream of N_2 , redissolved in 25 μ l of acetone, to which was added 1 ml of *n*-hexane, before application to the equilibrated column. β -Carotene and any anhydroretinol were eluted with 60 ml of *n*-hexane esters were eluted with 60 ml of 2% acetone in *n*-hexane and the free alcohol (retinol) was eluted with 60 ml of 10% acetone in *n*-hexane. The flow rate was adjusted to 3 ml/min. The resulting eluates were rotary-evaporated to dryness under reduced pressure, redissolved in a known volume of *n*-hexane and portions were taken for scintillation counting.

Radiochemical methods

Radioactivity was measured in 10 ml of Scintasol cocktail, and the samples counted in a Beckman L.S. 7000 liquid-scintillation spectrometer. The counting efficiency in Scintasol for 3H was 46% and for ^{14}C was 93%.

Results

Mannosyl transfer from GDP-mannose to microsomal vesicles

The transfer of mannose to microsomes by both rat liver and *B. pahangi* was markedly stimulated by the addition of Ret-*P*, as indicated by the increased retention of radioactive mannosylipid on the filters. In the absence of exogenous Ret-*P*, 7% and 43% of the transfer activity was detectable in rat liver and *B. pahangi* (respectively) at 37°C (Table 1). After subtraction of this endogenous component rat liver microsomal activity ranged from 0.275 to 0.370 nmol of mannose transferred/min per mg of protein, compared with 0.124–0.150 nmol of mannose transferred/min per mg of protein by the *B. pahangi* microsomal preparation. The reaction in both rat liver and *B. pahangi* required BSA, and respectively showed only 20 and 25% of the control activity when BSA was removed from the assay (Table 1). Removal of manganese from the assay [in actuality only reducing the final bivalent metal concentration in the assay from 5 to 2.5 mM, since $MgCl_2$ is present at 5 mM in medium A in which the microsomes were suspended (Shidoji & De Luca, 1981)], greatly reduced transfer activity, particularly in the *B. pahangi* enzyme, where it would appear to inhibit the endogenous activity as well. Exogenous ATP and AMP stimulated the transfer reaction by both enzymes. The removal of NaF resulted in only a slight reduction (4% in rat liver and 16% in *B. pahangi*) of maximal activity (Table 1).

Table 1. *Effect of cofactors on Ret-P-mediated mannosyl transfer to the microsomes of rat liver and Brugia pahangi*
The complete assay mixture consisted of the following components: GDP-[U- ^{14}C]mannose, 0.1 μ Ci; unlabelled GDP-D-mannose, 20 μ M; Ret-*P*, 10 μ g (150 μ M); BSA, 4 mg/ml (final concentration); Tris/HCl buffer, 30 mM (pH 8.0); $MnCl_2$, 2.5 mM; NaF, 8.0 mM; ATP, 5 mM; and the microsomal preparation (0.1 ml; protein content as indicated) in a total volume of 0.2 ml. Dolichyl phosphate or undecaprenyl phosphate was substituted for retinyl phosphate in the complete assay mixture at a final concentration of 150 μ M, assuming a mol.wt. of 1410 for dolichyl phosphate (i.e. a C_{100} molecule). Incubations were for 2 min at 37°C, with those assay components omitted or added as indicated. The results represent the average of two determinations from two separate experiments, after subtraction of background and appropriate control values.

Conditions	^{14}C radioactivity			
	Rat liver		<i>Brugia pahangi</i>	
	(c.p.m./2 min per 1.3 mg of protein)	(% of control activity)	(c.p.m./2 min per 150 μ g of protein)	(% of control activity)
Complete assay mixture (control)	34 721	100	3611	100
No retinyl phosphate	2329	7	1537	43
No BSA	7112	20	890	25
No $MnCl_2$	9660	28	477	13
No NaF	33 199	96	3042	84
No ATP	26 361	76	1353	37
No AMP	20 535	60	2056	57
Plus dolichyl phosphate	2140	6	1201	33
Plus undecaprenyl phosphate	3210	15	1599	44

No stimulation over basal levels of mannosyl transfer was observed when equimolar exogenous dolichyl phosphate replaced Ret-*P* in the rat liver and *B. pahangi* incubation mixture, or when undecaprenyl phosphate replaced Ret-*P* in the filarial incubation mixture (Table 1). However, undecaprenyl phosphate did slightly stimulate mannosyl transfer by the rat system, in the absence of Ret-*P* (Table 1).

T.l.c. analysis of the products of the mannosyl transfer process

T.l.c. analysis of the lower-phase lipid extracts derived from incubations containing exogenous Ret-*P* are shown in Fig. 1(a) (rat liver) and Fig. 2(a) (*B. pahangi*). Most of the radioactivity in both

Fig. 1. T.l.c. distribution of [^{14}C]mannose-labelled lipid acceptors synthesized by rat liver microsomes as described in the Materials and methods section and separated using t.l.c. system 1 [silica gel 60F, developed with chloroform/methanol/water (45:35:6, by vol.)] (a) In the presence of exogenous $150\text{ }\mu\text{M}$ -Ret-*P*; (b) endogenous lipid acceptors alone. Fluorograms of replicate t.l.c. separations are shown beneath the radiohistograms. The positions of authentic standards are as follows: G, GDP-mannose or mannose 1-phosphate; M, mannose; R, Ret-*P*; C, undecaprenyl phosphate; D, Dol-*P*-Man. The arrows indicate the centres of the respective bands of standards.

Fig. 2. T.l.c. distribution of [^{14}C]mannose-labelled lipid acceptors synthesized by *Brugia pahangi* microsomes as described in the Materials and methods section and separated using t.l.c. system 1 [Silica gel 60F, developed with chloroform/methanol/water (45:35:6, by vol.)] (a) In the presence of exogenous $150\text{ }\mu\text{M}$ -Ret-*P*; (b) endogenous lipid acceptors alone. Fluorograms of replicate t.l.c. separations are shown beneath the radiohistograms. The position of authentic standards are as follows: G, GDP-mannose or mannose 1-phosphate; M, mannose; R, Ret-*P*; C, undecaprenyl phosphate; D, Dol-*P*-Man. The arrows indicate the centres of the respective bands of standards.

preparations chromatographed as Ret-*P*-Man (R_F 0.32). Radioactivity was also found in the area of mannose (R_F 0.2–0.25) and mannose 1-phosphate (R_F 0), the breakdown products of Ret-*P*-Man on t.l.c. (Sasak *et al.*, 1973). The radioactivity near the origin could also represent unchanged GDP-mannose (R_F 0) carried through by the partition. Rat liver, but not *B. pahangi*, microsomes also synthesized small amounts of Dol-*P*-Man (R_F 0.63) in the presence of exogenous Ret-*P*.

T.l.c. analysis of the radioactive products derived from endogenous lipid acceptors are shown in Fig. 1(b) (rat liver) and Fig. 2(b) (*B. pahangi*). In the absence of exogenous Ret-*P* rat liver microsomes synthesized both Ret-*P*-Man and Dol-*P*-Man in approximately equal amounts as judged by the t.l.c.

Table 2. *Incorporation of radioactivity from [11,12(n)-³H]vitamin A alcohol into the retinoids of Brugia pahangi in vitro*

Approx. 0.2g wet wt. of *B. pahangi* (both sexes) was incubated in the presence of 125 μ Ci (4625 kBq) of [11,12(n)-³H]vitamin A alcohol under conditions described in the Materials and methods section.

Total incorporation

5432200 c.p.m. (4.3% incorporation)

Silicic acid chromatography

Eluting with chloroform/methanol (8:1, v/v),
4954350 c.p.m. (I)

Eluting with chloroform/methanol (1:1, v/v),
400510 c.p.m. (II)

DEAE-Sephacel chromatography of II

Eluting with 0–10 mm-ammonium acetate,
364130 c.p.m. (III)

Eluting with 50 mm-ammonium acetate, and with
carrier Ret-P, 27560 c.p.m. (IV)

Silica-gel 60F-254 t.l.c. of III and IV

III: co-chromatographing with Ret-P-[¹⁴C]Man
(R_F 0.43), 13170 c.p.m.

IV: co-chromatographing with Ret-P (R_F 0.38),
9270 c.p.m.

Whatman KC₁₈F r.p.t.l.c. of III and IV

III: co-chromatographing with Ret-P-[¹⁴C]Man
(R_F 0.66), 17830 c.p.m.

IV: co-chromatographing with Ret-P (R_F 0.53),
13670 c.p.m.

Alumina chromatography of I and III

I: eluting with n-hexane (anhydroretinol), 468170 c.p.m.

III: eluting with n-hexane (anhydroretinol), 2020 c.p.m.

scrapings. Fluorographically the Ret-P-Man peak appears as a very narrow band associated with the Ret-P standard, whereas the Dol-P-Man peak is more dispersed (Fig. 1b). *B. pahangi* microsomes incubated in the absence of Ret-P appeared to synthesize only one radioactive product that co-chromatographed with undecaprenyl phosphate (R_F 0.54).

Uptake and incorporation of [11,12(n)-³H]vitamin A alcohol

Adult *B. pahangi* avidly took up [³H]retinol, incorporating some 4.4% of the available exogenous label during the 24 h incubation *in vitro* (Table 2). Silicic acid chromatography demonstrated that greater than 90% of the incorporated radioactivity was in the non-phosphorylated retinoid fraction. Reversed-phase and absorption t.l.c. separations of this fraction tentatively indicated that as much as one-third of this radioactivity is unchanged retinol (Fig. 3, I), with radioactivity distributed among other retinoids including retinal, retinoic acid, retinyl acetate and retinyl palmitate.

DEAE-Sephacel chromatography (Fig. 4) demonstrated that 27560 c.p.m. (0.51% of the total incorporation) was eluted with 50 mm-ammonium acetate and carrier Ret-P. Subsequent t.l.c. of this phosphorylated retinoid fraction (Fig. 3, III) again indicated that most of this radioactivity co-migrated with Ret-P. The radioactivity that was eluted beyond the area of Ret-P absorption (fractions 32–38 in Fig. 4) may possibly be from the same unidentified material as described by Sasak *et al.* (1979). The 0–10 mm-ammonium acetate radioactive eluate on DEAE-Sephacel did not yield a clearly defined Ret-P-Man peak as was expected (Sasak *et al.*, 1979), perhaps because of the very low mass of Ret-P-Man recovered from the worms or because of the presence of a large portion of contaminating non-phosphorylated retinoids, as revealed by t.l.c. (Fig. 3, II). These t.l.c. separations, however, indicated the presence of small amounts of labelled Ret-P-Man, representing 0.24–0.33% of the total radioactive incorporation; which was not discernible by DEAE-Sephacel chromatography.

The anhydroretinol content as determined by alumina chromatography (Table 2) of the non-phosphorylated retinoid fraction was 9.5%, whereas that of the fraction eluting with 0–10 mm-ammonium acetate on DEAE-Sephacel was 0.5%

Table 3. *Incorporation of radioactivity from β -[15,15'-³H]carotene into the retinoids of Brugia pahangi in vitro*

Approx. 0.2g wet wt. of *B. pahangi* (both sexes) was incubated in the presence of 125 μ Ci (4625 kBq) of β -[15,15'-³H]carotene under conditions described in the Materials and methods section.

Total incorporation

96720 c.p.m. (0.08% incorporation)

Alumina chromatography

Eluting with n-hexane, 770 c.p.m.

Eluting with 2% acetone in n-hexane, 20270 c.p.m.

Eluting with 10% acetone in n-hexane, 40880 c.p.m.

Silicic acid chromatography

Eluting with chloroform/methanol (8:1, v/v),
50480 c.p.m.

Eluting with chloroform/methanol (1:1, v/v),
12280 c.p.m. (V)

DEAE-Sephacel chromatography of V

Eluting with 0–10 mm-ammonium acetate,
7740 c.p.m. (VI)

Eluting with 50 mm-ammonium acetate, and just after
carrier Ret-P, 245 c.p.m. (VII)

Silica-gel 60F-254 t.l.c. of VI and VII

VI: co-chromatographing with Ret-P-[¹⁴C]Man
(R_F 0.37), 210 c.p.m.

VII: co-chromatographing with Ret-P (R_F 0.34),
202 c.p.m.

Fig. 3. T.l.c. distribution of radioactivity in various retinoid fractions extracted from *Brugia pahangi* after *in vitro* incubation with $[11,12(n)-^3H]$ vitamin A alcohol

Separated by Merck silica gel 60F-254 t.l.c. (left) and Whatman KC₁₈F r.p.t.l.c. (right). Sample I (fraction I described in Table 2, the non-phosphorylated retinoid fraction, eluted with chloroform/methanol (8:1, v/v) on silicic acid; sample II (fraction III described in Table 2), putative Ret-*P*-Man fraction eluted with 0–10 mM-ammonium acetate on DEAE-Sephacel; sample III (fraction IV described in Table 2), putative Ret-*P* peak, eluted with 50 mM-ammonium acetate on DEAE-Sephacel; sample IV, approx. 2500 c.p.m. of standard biosynthetic Ret-*P*- $[^{14}C]$ Man. The position of authentic *all-trans*-retinoids are as follows: A, Rep-*P*; B, retinoic acid; C, retinol; D, retinal; E, retinyl acetate; F, anhydroretinol; G, retinyl palmitate; O, origin; SF, solvent front.

Uptake and incorporation of β -[15,15'- 3H]carotene

Adult *B. pahangi* took up and incorporated only 0.08% of the available exogenous label during the 24 h incubation *in vitro* (Table 3). Alumina chromatography demonstrated that less than 1% of the incorporated radioactivity was either unchanged β -carotene or anhydroretinol; of the remaining incorporated radioactivity, 21% was accounted for as retinyl esters and 42% as the free alcohol (retinol). Silicic acid chromatography confirmed that greater than 50% of the incorporated radioactivity was in the non-phosphorylated retinoid fraction, with nearly 13% of the incorporated radioactivity eluting as phosphorylated retinoids. DEAE-Sephacel chromatography (Fig. 5) demonstrated that at least 245 c.p.m. above background (0.25% of the total incorporation) was eluted with 50 mM-ammonium acetate just after Ret-*P* and may again represent the unknown material described by Sasak *et al.* (1979). However, 202 c.p.m. of this fraction appeared to correspond to Ret-*P*, as judged by silica gel 60F-254 t.l.c. of this fraction (Fig. 6, I). As for the $[^3H]$ retinol experiment, DEAE-Sephacel chromato-

graphy did not yield a clearly defined Ret-*P*-Man peak. Once again t.l.c. separation of this fraction indicated the presence of a small amount (210 c.p.m. above the background value) of labelled Ret-*P*-Man, representing 0.22% of the total incorporation (Fig. 6, II).

Discussion

The present study with *B. pahangi* microsomal vesicles represents the first demonstration in an invertebrate of the transfer of mannose from GDP-mannose to exogenous Ret-*P*. The cofactor requirement and conditions for this transfer *in vitro* by *B. pahangi* utilizing the filter assay procedure of Shidoji & De Luca (1981) were not too dissimilar from the results reported by these authors in their studies with rat liver microsomes. The synthesis of Ret-*P*-Man and Dol-*P*-Man from endogenous lipid acceptors present in rat liver microsomes likewise was similar to that recently reported by De Luca *et al.* (1982). However, in this circumstance we have been unable to demonstrate comparable endogenous Ret-*P*-Man or Dol-*P*-Man synthesis in *B. pahangi*

Fig. 4. DEAE-Sephacel chromatography of a mixture of non-radioactive Ret-P and the phosphorylated retinoids extracted from *B. pahangi* after incubation in vitro with [11,12(n)- ^3H]vitamin A alcohol (fraction II described in Table 2)

The column was eluted with 25 ml of 99% methanol, followed by 100 ml of 10 mM-ammonium acetate in 99% methanol and finally by 100 ml of 50 mM-ammonium acetate in 99% methanol. Each fraction (5 ml each) was monitored for ^3H content (●). Fractions 25–45 were monitored for u.v. absorption at 325 nm (○).

Fig. 5. DEAE-Sephacel chromatography of a mixture of non-radioactive Ret-P and the phosphorylated retinoids extracted from *Brugia pahangi* after incubation in vitro with β -[15,15'- ^3H]carotene (fraction V, described in Table 3)

The column was eluted with 25 ml of 99% methanol, followed by 100 ml of 50 mM-ammonium acetate in 99% methanol and finally by 100 ml of 50 mM-ammonium acetate in 99% methanol. Each fraction (5 ml each) was monitored for ^3H content (●). Fractions 25–45 were monitored for u.v. absorption at 325 nm (○).

microsomes. The identity of the main endogenous lipid acceptor in *B. pahangi* microsomes functioning in the absence of exogenous Ret-P remains unknown,

Fig. 6. T.l.c. distribution of radioactivity in the retinoid fractions extracted from *Brugia pahangi*, after incubation in vitro with β -[15,15'- ^3H]carotene, and separated on Merck silica gel 60F-254

Sample I (fraction VII described in Table 3), putative Ret-P fraction, eluted with 50 mM-ammonium acetate just after Ret-P on DEAE-Sephacel; sample II (fraction VI described in Table 3), putative Ret-P-Man peak, eluted with 0–10 mM-ammonium acetate on DEAE-Sephacel. A, Ret-P; B, Ret-P-Man; O, origin; SF, solvent front.

although it may be a short-chain polyprenol since these molecules were shown to be biosynthesized from mevalonate by *B. pahangi* and *D. immitis* (Comley & Jaffe, 1981). The high levels of endogenous mannosyl transfer in the *B. pahangi* microsomal preparation may be in part due to the presence of these unknown lipid components within the microsomal membranes. Our apparent failure to demonstrate Ret-P reserves in *B. pahangi* microsomes should not, however, be taken to indicate that filariae *in vivo* lack this capacity, particularly as our *B. pahangi* microsomes were prepared from whole worm tissues and less than half the amount of whole-worm microsomal protein was available in the parasite assay compared with that derived specifically from rat liver.

The failure of exogenous dolichol phosphate or undecaprenyl phosphate to markedly promote mannosyl transfer to the microsomes of rat liver or *B. pahangi* is probably due to the poor solubility of these lipid acceptors in the aqueous environment of the BSA-based assay, which did not contain any detergent (De Luca, 1982). Dol-P-Man and an unidentified mannosylated lipid were, however, biosynthesized from endogenous acceptor lipids in rat liver and *B. pahangi* microsomes respectively; presumably these reserves were already present within the isolated membranes.

These studies *in vitro* allow us to conclude that *B. pahangi* microsomal membranes can biosynthesize Ret-*P*-Man when provided with exogenous Ret-*P*. Our results do not, however, tell us whether *B. pahangi in vivo* utilizes this pathway, or if sufficient concentrations of Ret-*P* will be achieved within the nematode's microsomal membranes so as to promote this transfer. Furthermore, the circumstances and conditions under which Ret-*P*-mediated mannosyl-transfer is favoured in *B. pahangi* relative to those transferases utilizing other lipid acceptors (such as dolichol phosphate or the unknown polyprenyl phosphate) remains to be determined.

Our findings that *B. pahangi* took up and incorporated significant amounts of retinol supports the hypothesis that Ret-*P* may function in filarial glycoprotein synthesis, since under our incubation conditions *in vitro* intact worms were able to phosphorylate retinol, and limited amounts of Ret-*P*-Man accumulated. Interestingly, similar percentages (0.25–0.5%) of the total radioactive incorporation derived from retinol or from β -carotene were tentatively identified as Ret-*P* (or Ret-*P*-like material in the case of β -carotene) and Ret-*P*-Man, even though greater than 50 times more retinol was incorporated than β -carotene during comparable incubations.

It seems most probable that the high degree of lipid solubility of these retinoids permits their uptake trans-cuticularly despite their relatively large molecular size, particularly as recent fluorescent-microscopic studies on the trematode *Schistosoma mansoni* have shown that free retinol can penetrate the surfaces of adults and schistosomulae of these parasites, and that it then disperses throughout the internal membrane systems of these organisms (Kusel *et al.*, 1982). By analogy with vertebrate studies (Goodman, 1981), we might then expect the intracellular transport of retinoids to be mediated by cellular retinoid-binding protein(s) for delivery to the target organelle(s). This prompted us to investigate whether filarial parasites possess cellular retinoid-binding protein(s) and our preliminary results indicate that both *D. immitis* and *B. pahangi* do possess proteins with binding affinities for retinol and retinoic acid (B. P. Sani & J. C. W. Comley, unpublished work).

Our preliminary indication that retinol may be metabolized by *B. pahangi* to retinyl acetate in addition to retinyl palmitate is unusual since, at least in mammalian systems, only the long-chain fatty acid esters are normally found (Ross, 1982). The apparent dehydration of nearly 10% of the incorporated labelled retinol to anhydroretinol suggests that this parasite may metabolize retinol in a way similar to spontaneously transformed mouse fibroblasts (3T12 cells) (De Luca *et al.*, 1979b).

In addition to the postulated involvement of Ret-*P*

in filarial glycoprotein synthesis, retinoids may have other biochemical functions in helminths. One possibility is that vitamin A influences the host-parasite interaction by virtue of its stimulatory role on antibody synthesis (Leutskaya & Prokhorova, 1979). Such a finding might explain why vitamin A deficiencies are observed in acute helminthiasis (Mahalanabis *et al.*, 1976), as there would be increased demand for vitamin A by the host owing to increased antibody formation directed against the parasite infection. This immunological role for vitamin A would appear to be supported by the work of Storey (1982), who found that vitamin A-deficient cotton rats were more susceptible to infection by the filarial worm *Litomosoides carinii*.

None of these observations, however, explain why various intestinal helminths (Leutskaya, 1961, 1971, 1974; Juhasz & Babos, 1969; Czczuga, 1971) apparently take up, accumulate and metabolize β -carotene and retinol, results that we believe indicate a biochemical function of retinoids in helminths. Interestingly, in support of the glycoprotein synthesis function of retinol, a previous investigation (Leutskaya, 1971) with *Ascaris suum* implied a connection between the accumulation of vitamin A in the pseudocoelomic fluid and the intensive protein synthesis of the reproductive organs of this parasite.

The recent finding of Storey (1982) that the development of microfilariae is retarded in the uteri of female *L. carinii* recovered from partially vitamin A-deficient hosts suggests that the processes of uptake and transport of vitamin A in filariae may represent a chemotherapeutic target, since compounds capable of interfering at these sites might be expected to cause inhibition of all vitamin A-mediated processes within the parasite. The presence of cellular retinoid-binding proteins in filariae may be important in this respect, since the preventative effects of some exogenous retinoids on cancer have been correlated with their high degree of affinity for these binding proteins (Muto *et al.*, 1981).

This investigation was supported by funds from the Filariasis Component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We gratefully acknowledge the expert technical advice given by Dr. Luigi M. De Luca, and his constructive criticisms of the manuscript.

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