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Synthesis of retinoids by human retinal epithelium and transfer to rod outer segments

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The synthesis and release of 11-*cis*-retinoids by primary cultures of human retinal pigment epithelium (RPE) and the transfer of these retinoids to co-incubated human rod outer segments (ROS) were studied. Monolayers of 2–3-week-old cultured RPE incorporate tritiated all-*trans*-retinol, esterify it to the corresponding retinyl palmitate, form 11-*cis*-retinol and 11-*cis*-retinaldehyde and release retinaldehyde into the culture medium. The ratio of 11-*cis* to all-*trans* isomers of retinol, retinyl palmitate and retinaldehyde formed in the cells along with retinaldehyde released and incorporated into the ROS progressively increases, indicating a progressive increase in the concentration of 11-*cis* isomer from the time it is formed in RPE cells until its transfer to ROS. Incorporation of 11-*cis*-retinaldehyde into the ROS is directly related to the amount of albumin present in the media, suggesting the transfer of retinoids from RPE to photoreceptor to be a protein-mediated process. Events leading to isomerization, esterification, oxidation and release of retinoids by human RPE and incorporation of retinoids into ROS can therefore be examined *in vitro*.

INTRODUCTION

The isomerization of all-*trans*- to 11-*cis*-retinoid is the critical reaction in the vitamin A cycle of vision. The interaction of the hindered 11-*cis*-retinaldehyde with a specific protein, opsin, is responsible for enabling the photoreceptor to respond to light. All-*trans*-retinol taken up from serum by retinal pigment epithelium (RPE) is enzymically converted (Krinsky, 1958) into retinyl esters in microsomes (microsomal fractions) (Andrews & Futterman, 1964; Berman *et al.*, 1980) by a non-fatty-acyl-CoA-mediated process (Saari & Bredberg, 1987*a*, 1988) via lecithin (phosphatidylcholine):retinol acyltransferase (Saari & Bredberg, 1989). We have shown that cultured human RPE, devoid of any neural retina, not only takes up supplied ^{3}H-labelled all-*trans*-retinol and esterifies it to corresponding palmitate, but can also form 11-*cis*-retinol and 11-*cis*-retinyl palmitate (Das *et al.*, 1986, 1987, 1988). These results indicated the presence of an isomerization system in these cultured cells. This isomerization is thought to be due to an enzyme, shown to be present in frog and bovine retinal epithelium (Bernstein *et al.*, 1987*a*; Fulton & Rando, 1987), that could convert all-*trans*-retinol into 11-*cis*-retinoid, but was unable to isomerize all-*trans*-retinaldehyde (Bernstein *et al.*, 1987*b*). Recent reports, however, suggest that all-*trans*-retinyl palmitate formed in RPE is processed to 11-*cis*-retinol (Deigner *et al.*, 1989), which is then oxidized to its corresponding aldehyde by a stereospecific dehydrogenase (Lion *et al.*, 1975).

In the present paper we show that, in addition to 11-*cis*-retinyl palmitate, 11-*cis*-retinol and 11-*cis*-retinaldehyde are also formed from all-*trans*-retinol by human RPE *in vitro*. Like retinol (Das & Gouras, 1988), retinaldehyde is also released from these cells in the presence of a protein in the medium capable of binding retinoid. In these experiments, serum albumin, as a binding protein for 11-*cis*-retinaldehyde, facilitates its release from RPE and its incorporation into co-incubated rod outer segments (ROS), which show selectivity for this isomer. The failure to detect transfer of any retinaldehyde from RPE to ROS in the absence of albumin suggests that the presence of a protein capable of binding retinoids is essential for the movement of 11-*cis*-retinaldehyde from RPE to the photoreceptors *in vitro*. Our results indicate that major steps of the vitamin A cycle of vision, namely, isomerization of all-*trans*-retinol to 11-*cis*-retinol, its oxidation to the corresponding retinaldehyde and its transfer to ROS can be carried out by human RPE *in vitro*.

EXPERIMENTAL

Materials

Human donor eyes from subjects ranging in age from 20 to 77 years old were received through the New York Eye Bank for Sight Restoration, the National Diabetes Research Interchange and Retinitis Pigmentosa Donor Eye Program. ^{3}H-labelled 11,12-all-*trans*-retinol was from New England Nuclear Research Products, Boston, MA, U.S.A. Betafluor was supplied by National Diagnostics, Manville, NJ, U.S.A. Hanks balanced salt solution and other supplies for tissue culture were from GIBCO, Grand Island, NY, U.S.A. Palmitoyl chloride was from Sigma Chemical Co., St. Louis, MO, U.S.A. Neutral Al_2O_3 (activity grade) was from ICN Nutritional Biochemicals, Cleveland, OH, U.S.A. Sources of other chemicals and solvents were as described elsewhere (Das & Gouras, 1988). ^{3}H-labelled 11-*cis*-retinaldehyde was a gift from Dr. James R. Mertz, who isomerized it from ^{3}H-labelled all-*trans*-retinol by first oxidizing it with activated MnO_2 (Fisher Scientific) and then irradiating it with white light for 15 min. The resultant isomer mixture of retinaldehyde was separated and purified by h.p.l.c. as described under 'Methods' below.

Methods

Human RPE cells were grown as primary cultures by using the method described previously (Flood *et al.*, 1980). The '*in vitro*' age of the cultures used was between 2 and 3 weeks unless otherwise mentioned. ^{3}H-labelled all-*trans*-retinol in tracer amounts, and bound to BSA, was used for supplementation of the cultured cells. Standard precautions for handling retinoids to minimize degradation and removal of serum-containing media from the cultures 2 h before supplementation, for maximum uptake of retinol, were followed as described elsewhere (Das & Gouras, 1988). To study the rate of formation of different

Abbreviations used: RPE, retinal pigment epithelium; ROS, rod outer segments; IRBP, interphotoreceptor retinol-binding protein; CRALBP, cellular retinaldehyde-binding protein.

metabolites of retinol by RPE cells in primary culture, all-*trans*-retinol was supplemented for different time points over a maximum period of 24 h. Supplementation of retinol, homogenization of the cells and determination of protein content of the cells were done as described by Das & Gouras (1988).

Extraction of retinoids from the cells, media and ROS was done as described by Zimmerman (1974) with minor modifications. Initially 2 ml of acetone was added to retinoid-containing tissue homogenate or media and, after vortex-mixing for 1 min, left at 20 °C for 5 min. After addition of 4 ml of n-hexane, the mixture was vortex-mixed for 2 min. This process was repeated twice. The top hexane/acetone layer was removed after centrifugation at 1040 *g* for 5 min. Extracts were pooled and evaporated under N_2 and re-suspended in 250 μl of n-hexane containing butylated hydroxytoluene (25 μg/ml) and stored at −20 °C before being analysed by h.p.l.c. After this extraction procedure, 96% of the total radioactivity due to retinoids, including retinaldehyde from photoreceptors, was recovered.

In order to determine the authenticity of 11-*cis*-retinol formed by human RPE after supplementation of ^{3}H-labelled all-*trans*-retinol, retinoid that was co-eluted from h.p.l.c. with authentic 11-*cis*-retinol was collected and evaporated to dryness under N_2. The residue was redissolved in 10 μl of anhydrous methylene chloride containing 55 μg of dissolved palmitoyl chloride. The same amount of palmitoyl chloride dissolved in 100 μl of methylene chloride was added twice more with an interval of 10 min, together with 50 μl of 2.5% (v/v) of pyridine in methylene chloride. The reaction was carried out at 20 °C for 30 min and stopped by the addition of 1 ml of water. Reaction products were extracted with acetone and hexane as described above and washed with dilute acid (0.2 mM-HCl) to remove residual pyridine. The extract was dried over anhydrous Na_2SO_4 and evaporated under N_2. The residue was redissolved in 100 μl of n-hexane and applied to a column (0.5 cm × 5.0 cm) containing 1.5 g of Al_2O_3 [+5% (v/w) water] to remove the palmitic acid formed. Retinyl palmitate was eluted with 1% (v/v) diethyl ether in hexane and was analysed by h.p.l.c.

Human ROS were isolated from eight to ten pairs of frozen (−70 °C) neural retinas at a time, as described by Kwok-Keung Fung & Stryer (1980).

To study the transfer of 11-*cis*-retinaldehyde formed in the cultured RPE to co-incubated ROS, RPE cells were supplemented with ^{3}H-labelled all-*trans*-retinol bound to albumin as described above. After 5 h the medium containing radioactive retinol was removed and the cells were washed twice with 2 ml of Hanks balanced salt solution. Washed cells were then supplied with 2.2 ml of minimal essential medium and 0.5% glucose containing 0, 0.5, 1.0, 3.0 and 5.0 mg of BSA/ml and equal amounts (containing 450 μg of total protein) of isolated ROS suspended in it. RPE cells containing ROS were co-incubated at 37 °C for 18 h, at which time the reaction was stopped by putting the cultures on ice. Media containing suspended ROS were removed from the cells and centrifuged at 1040 *g* for 5 min in a standard Dynac centrifuge with angle heads (Clay Adams, Parsippany, NJ, U.S.A.). The supernatant medium was removed, and pelleted ROS were washed twice with a total of 800 μl of 10 mM-Tris/HCl, pH 7.0, vortex-mixed for 1 min and re-pelleted by centrifuging at 1040 *g* for 5 min. The ROS pellet was homogenized with 1 ml of Tris buffer in a Dounce glass/glass tissue grinder, and retinoids were extracted as described above.

H.p.l.c.-purified ^{3}H-labelled 11-*cis*-retinaldehyde (50000 c.p.m./assay) in 10 μl of ethanol was added to 200 μl of 10 mM-Tris/HCl, pH 7.2, containing 0, 1.0, 3.0 and 5.0 mg of BSA/ml, together with equal amounts (450 μg of total protein) of isolated ROS suspended in 200 μl of Tris buffer in amber-coloured tubes at 0 °C. In order to show the incorporation of 11-*cis*-retinaldehyde into the ROS as a function of albumin concentration, the reaction mixture was flushed with O_2-free argon and incubated at 37 °C for 30 min. After stopping the reaction by putting the mixture on ice, the tubes were centrifuged at 1040 *g* for 5 min. The pellets were washed twice with a total of 800 μl of Tris buffer after removal of the supernatant. The washed pellets were then extracted for retinoids as described above, and retinoids were analysed by h.p.l.c. as described below.

Das & Gouras (1988) described the procedure for h.p.l.c. separation of retinyl palmitate and retinol isomers with a satisfactory resolution of all four isomers tested. The same procedure was used in the present study to separate retinol, retinyl palmitate and, in addition, retinaldehyde isomers. By using the same columns as described for retinol and retinyl palmitates, retinaldehydes were eluted with 1% dioxan/n-hexane at a flow rate of 0.6 ml/min. The eluate from the h.p.l.c. column was collected in 7 ml-capacity polyethylene scintillation vials (Fisher Scientific, Fair Lawn, NJ, U.S.A.) by using a Gilson FC 203 programmable fraction collector (Gilson Medical Electronics, Middletown, WI, U.S.A.) at 0.5 min intervals. A 4 ml portion of Betafluor was then added to each vial and radioactivity was counted in an LKB 1217 Rackbeta liquid-scintillation counter. In all experiments (except in Figs. 2 and 4) radioactivity eluted with authentic retinoids was normalized for protein after correction for background radioactivity and was expressed as c.p.m./mg of protein present in RPE cells.

RESULTS

H.p.l.c. profiles of separation of the retinoid isomers studied in these experiments are shown in Fig. 1. The separation attained by using the different solvent systems described in the Experimental section permitted us to identify unequivocally the 9-, 11-, 13-*cis*- and all-*trans*-retinol, -retinaldehyde and -retinyl palmitate.

The time-dependent appearance of radiolabelled all-*trans*- and 11-*cis*-retinol, -retinaldehyde and -retinyl palmitate in cultured RPE after supplementation with ^{3}H-labelled all-*trans*-retinol and subsequent appearance of retinaldehyde in the medium is shown in Table 1. The amounts of all retinoids increase with time in the first 5 h of incubation. After this time the amounts of both 11-*cis*- and all-*trans*-retinol and -retinaldehyde reach a plateau. The amounts of retinyl palmitate isomers decrease after 5 h. In the case of retinol and retinyl palmitate, the amount of all-*trans* isomer is greater than the 11-*cis* form at all times. However, for retinaldehyde the amounts of 11-*cis* isomer formed and released into the media are greater than for the all-*trans* isomer after 3 h of incubation, an observation that reflects the higher cellular levels of the 11-*cis*-aldehyde. In all experiments, different amounts of 9-*cis* and 13-*cis* isomers were formed (not shown in Table 1), but amounts were always less than those of the 11-*cis* isomer. We have also noted that prolonged storage of the samples at −20 °C increased the formation of 9-*cis* and 13-*cis* isomers.

In order to confirm the identity of the radiolabelled retinoid formed from all-*trans*-retinol after incubation with cultured RPE, the fraction co-eluted with authentic 11-*cis*-retinol was collected and chemically esterified with palmitoyl chloride. The h.p.l.c. profile of the product of esterification (Fig. 2) shows that the radioactivity was almost exclusively found in the fraction corresponding to 11-*cis*-retinyl palmitate. Only a small amount of radioactivity was found in the fraction corresponding to 9-*cis*-isomer, and no radioactivity was found in the all-*trans*- or 13-*cis*-isomer fractions.

The amounts of retinaldehyde incorporated in the ROS co-incubated with RPE cells pre-supplemented with ^{3}H-labelled all-*trans*-retinol is shown in Fig. 3. These results also show a relationship between the amounts of retinaldehyde taken up by

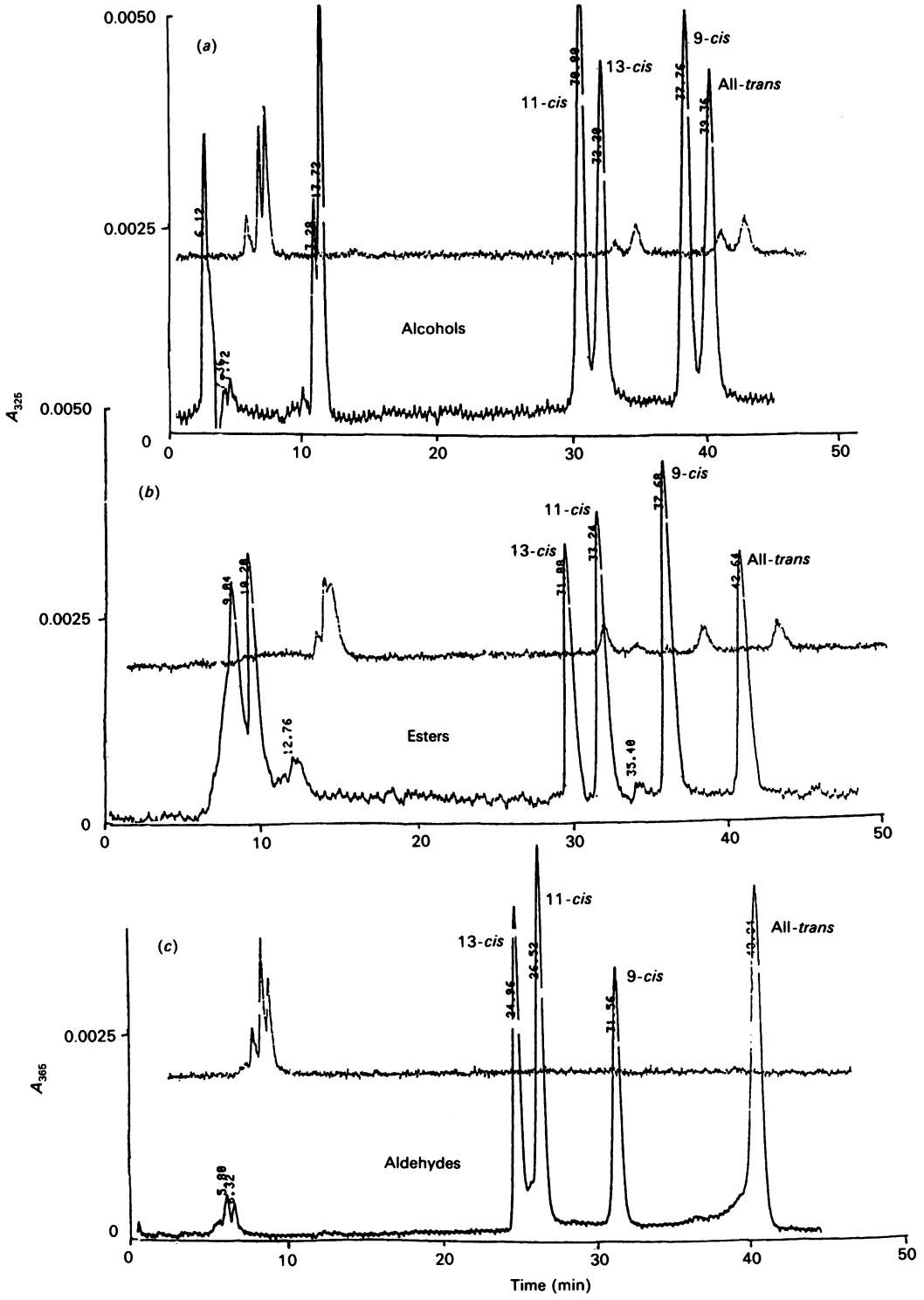

Fig. 1. H.p.l.c. profiles of three *cis* and all-*trans* isomers of retinol, retinyl palmitate and retinaldehyde

(*a*) H.p.l.c. chromatogram of the three *cis* isomers of retinol and all-*trans*-retinol. The Resolve (Waters; 5 μm particle size; spherical silica) columns, two in series, had dimensions 3.9 mm × 15 cm; the mobile phase was n-hexane/diethyl ether/dioxan (473:2:25, by vol.) at a flow rate of 0.6 nl/min. (*b*) Chromatogram of the three *cis* isomers of retinyl palmitate and all-*trans*-retinyl palmitate. The column, which was the same as that used in the retinol system, was eluted with a mobile phase of n-hexane/diethyl ether (249:1, by vol.), at a flow rate of 0.4 ml/min. (*c*) Chromatogram of the three *cis* isomers of retinaldehyde and all-*trans*-retinaldehyde. The column was the same as that mentioned above, the mobile phase being n-hexane/dioxan (99:1, by vol.) and the flow rate 0.6 ml/min. The trace going across the middle of the u.v. absorption peaks represents the output of the fluorescence detector set at 338 nm excitation and 460 nm emission. The fluorescence peaks are plotted about ~1.3 cm after the u.v. absorption peaks to avoid superimposition.

Table 1. Retinoids formed and released by RPE cells

In each experiment, 2–3-week-old primary cultures of RPE cells from human donors, of age ranging from 20 to 77 years, were depleted of fetal-bovine serum from their nutrient media for 2 h before supplementation of ^{3}H-labelled 11,12-all-*trans*-retinol as described in the Experimental section. After incubation at 37 °C over a period of 24 h, retinoids were extracted from the cells and medium, and different metabolites were analysed by h.p.l.c. as described in the legend to Fig. 1. Values are means ± S.D. for six observations.

	10^{-3} × Radioactivity (c.p.m./mg of RPE-cell protein)							
	Cells						Medium	
	Retinol		Retinyl palmitate		Retinaldehyde		Retinaldehyde	
Incubation time (h)	All-*trans*	11-*cis*	All-*trans*	11-*cis*	All-*trans*	11-*cis*	All-*trans*	11-*cis*
2	59±40	29±14	33±17	22±10	22±3	20±5	25±4	20±5
3	69±42	35±16	61±21	36±15	27±6	27±6	28±7	27±7
4	106±74	41±17	101±40	51±20	32±4	40±4	36±12	39±10
5	118±79	48±15	131±50	59±19	34±5	45±6	47±11	62±20
24	119±83	56±40	85±46	48±28	33±6	45±6	43±10	62±20

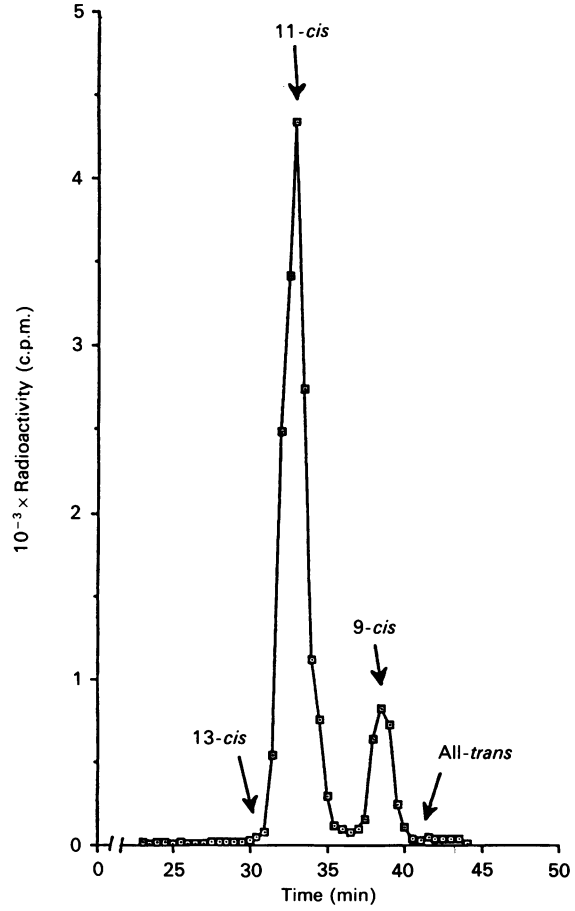

Fig. 2. H.p.l.c. elution profile of radioactivity due to the product of chemical esterification of the fraction co-eluted with authentic 11-*cis*-retinol on h.p.l.c.

The reaction was carried out and the product extracted with acetone and hexane as described in the Experimental section and was analysed by h.p.l.c. using the ester solvent system described in the legend to Fig. 1. The abscissa shows the h.p.l.c. retention time, and the ordinate shows the radioactivity (c.p.m.) due to the product of chemical esterification. The arrows indicate the retention times of authentic retinyl palmitate standards.

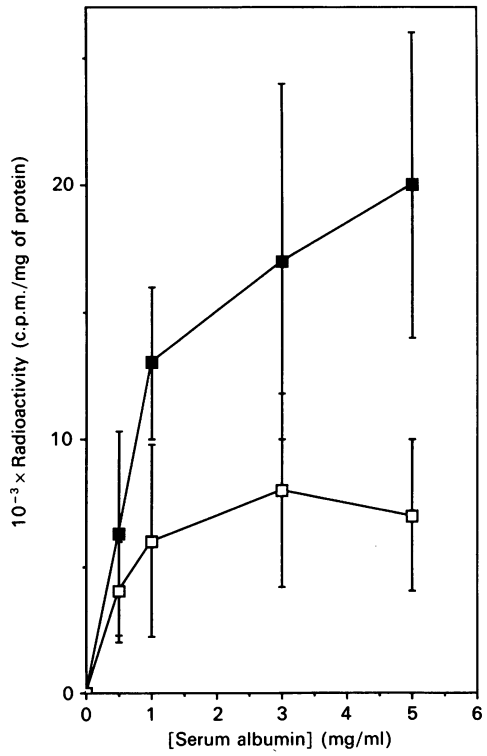

Fig. 3. Uptake of 11-*cis*- (■) and all-*trans*- (□) retinaldehyde by human ROS after they had been co-incubated with human RPE cells pre-supplemented with ^{3}H-labelled all-*trans*-retinol as described in the Experimental section

The number of replicate experiments was four, and the error bars in this Figure indicate the standard deviation from the mean.

ROS and the amount of serum albumin present in the medium. If there is no albumin in the medium, no retinaldehyde is found in the ROS. As the concentration of albumin in the media is increased, there is an increase in the amount of retinaldehyde incorporated by the ROS. The ROS incorporate relatively more 11-*cis*- than all-*trans*-retinaldehyde. Isomers of retinol were not detected in the ROS in any of the four experiments.

Table 2 illustrates the ratio of 11-*cis* to all-*trans* isomer for retinol, retinyl palmitate and retinaldehyde found in the cells and

Table 2. Ratio of 11-*cis* to all-*trans* isomer of retinol, retinyl palmitate and retinaldehyde formed in cultured RPE cells and retinaldehyde released into the media after incubation for 5 h with ^{3}H-labelled all-*trans* retinol

The ratio was calculated from the mean (±S.D.) values from six replicate experiments. To study the retinaldehyde incorporation into ROS, purified ROS were co-incubated for 18 h with the radiolabelled RPE cells as described in the Experimental section. The ratio was calculated from the mean values obtained in four replicate experiments.

Retinoid	10^{-3} × Radioactivity (c.p.m./mg of protein)		Ratio (A/B)
	11-*cis* (A)	All-*trans* (B)	
Retinol in cells	48±15	118±79	0.41
Retinyl palmitate in cells	59±19	131±50	0.45
Retinaldehyde in cells	45±6	34±6	1.32
Retinaldehyde in media	62±20	47±11	1.32
Retinaldehyde in ROS	20±6	7±3	2.9

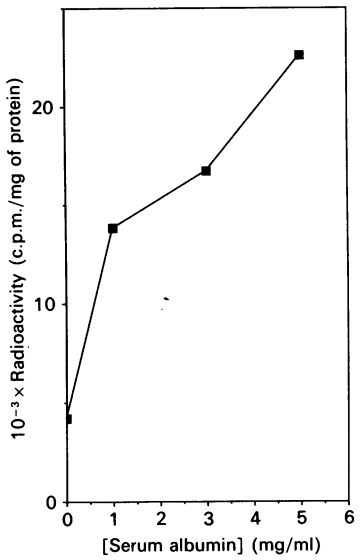

Fig. 4. Incorporation of ^{3}H-labelled 11-*cis*-retinaldehyde into the isolated human ROS in the absence of RPE cells

H.p.l.c. purified ^{3}H-labelled 11-*cis*-retinaldehyde was incubated with isolated ROS at 37 °C for 30 min as described in the Experimental section. After the incubation the ROS were pelleted, washed and homogenized, and the retinoids were extracted and analysed by h.p.l.c. as described in the legend to Fig. 1. The ordinate represents the radioactivity (c.p.m./mg) of protein in ROS) due to 11-*cis*-retinaldehyde incorporation.

the medium after the cells were supplemented with radiolabelled all-*trans*-retinol and in the ROS after being co-incubated with the radiolabelled-retinol-supplemented cells. The ratio is higher for retinaldehyde than for the other two retinoid forms. The ratio for the retinaldehydes is higher in the ROS than in the cell or in the medium.

In order to determine whether the uptake of 11-*cis*-retinaldehyde by the ROS is due to some influence of the RPE cells in conjunction with albumin, we incubated ROS in the presence of different concentrations of albumin and in the absence of cultured epithelial cells. Fig. 4 shows that the incorporation of 11-*cis*-retinaldehyde by ROS is dependent upon the amount of albumin present in the medium, even in the absence of epithelial cells.

DISCUSSION

The results indicate that cultured human retinal epithelium can satisfy all of the major requirements of the vitamin A cycle of vision. These cells incorporate all-*trans*-retinol from the media, isomerize it to 11-*cis*-retinol, oxidize 11-*cis*-retinol to the corresponding retinaldehyde and release this molecule into the media, where it is incorporated by ROS. This incorporation of retinaldehyde by ROS is proportional to the amount of albumin present in the media. Though there is incorporation of some 11-*cis*-retinaldehyde in the absence of albumin, increasing concentrations of albumin lead to increased amounts of incorporation. We have previously shown that albumin and interphotoreceptor retinol-binding protein (IRBP) both facilitate the release of retinoids from cultured RPE, but haemoglobin does not (Das & Gouras, 1988). We have assumed that it is the retinoid-binding capacity of albumin and IRBP that is responsible for this effect. The facilitation of 11-*cis*-retinaldehyde uptake by albumin may also be related to its retinoid-binding capacity and its possible interaction with the lipid bilayer of the outer segments. IRBP being the only protein capable of binding retinoids in the subretinal space (Adler & Evans, 1985; Bridges & Adler, 1985; Chader, 1989), possibly mediates the transfer of 11-*cis*-retinaldehyde from RPE to ROS *in vivo*. The role of this retinoid-binding protein is not entirely understood. It has been suggested that it plays a role in the transport of retinoids between the RPE and the ROS (Chader, 1989). Experiments on the transfer of all-*trans*-retinol between liposomes and ROS indicate that this retinoid can pass through an aqueous phase between these two organelles without any need for IRBP (Ho *et al.*, 1989). Our results, however, suggest that a protein capable of binding retinoids plays a significant role in the uptake of 11-*cis*-retinaldehyde by ROS *in vitro*.

Our study also shows that isomerization of all-*trans*-retinol to 11-*cis*-retinoids occurs in cultured human RPE and that 11-*cis*-retinol can be oxidized to the corresponding aldehyde form. Both of these reactions are thought to be due to enzymes present in RPE: an isomerase (Bernstein *et al.*, 1987*a*,*b*) and an 11-*cis*-retinaldehyde-specific dehydrogenase (Lion *et al.*, 1975). Presumably both of these enzymes are present in these cultured RPE cells. The latter enzyme may be responsible for the fact that the ratio of 11-*cis* to all-*trans* isomer is much higher for the aldehyde than for the alcohol or ester form of this molecule in the cultured cells. There is evidence that cellular retinaldehyde-binding protein (CRALBP), which binds 11-*cis*-retinaldehyde, facilitates the oxidation of 11-*cis*-retinol (Mertz *et al.*, 1987), and therefore this protein may also be present in these cultured cells.

If CRALBP is the main storage site for the cellular stores of 11-*cis*-retinaldehyde we detect, it is not easy to understand how this aldehyde molecule is then released into the media. CRALBP has a high affinity for 11-*cis*-retinaldehyde, so that it is unlikely to release this molecule to either bleached or unbleached photoreceptor membranes (Saari & Bredberg, 1987*b*). Therefore it should not be expected to release it to the surface membrane of the epithelial cell. The exact mechanism of the release of 11-*cis*-retinaldehyde from RPE cells needs further investigation.

There are reports that cultured RPE cells lose their ability to esterify retinoids with time in culture (Bridges *et al.*, 1986). Recently Saari & Bredberg (1989) have demonstrated

lecithin:retinol acyltransferase activity, a novel enzyme activity in bovine RPE, that is responsible for esterification of retinol, and low levels of this activity may be rate-limiting in cultured cells. Edwards & Adler (1989) failed to observe stimulation of esterification by addition of palmitoyl-CoA, but have observed that the presence of defined components in the medium such as insulin and retinal extracts can enhance retinyl ester synthesis.

Our results demonstrate that esterification occurs in these cultured cells. At the earliest incubation time with all-*trans*-retinol, esterification exceeds the rate of isomerization. It has recently been suggested that the enzymic isomerization of all-*trans*-retinol to 11-*cis*-retinol is coupled to the hydrolysis of all-*trans*-retinyl palmitate, which provides the driving force for isomerization (Deigner *et al.*, 1989). Our results are in accord with this hypothesis, because both the esterification and the hydrolysis of all-*trans*-retinyl palmitate and the isomerization reaction occur concomitantly in these cultured cells.

The kinetics of the formation of the retinoid 11-*cis* isomers by these cultures of human RPE cells follow a predictable time course under the conditions we have defined. It is possible that some human retinal degenerations may be the result of defects in the metabolism of these 11-*cis* isomers, for example, at either the isomerization or the oxidation stage. Therefore an examination of isomer-formation kinetics in cultured human RPE cells obtained from donors with certain hereditary forms of retinal degeneration could cast some light on the nature of such defects.

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