Biochimica et Biophysica Acta 901 (1987) 255–264
Elsevier

BBA 73607

Studies of the spontaneous transfer of retinol from the retinol : retinol-binding protein complex to unilamellar liposomes

Göran Fex and Gunvor Johannesson

Department of Clinical Chemistry, Malmö General Hospital, S-214 65 Malmö (Sweden)

(Received 12 December 1986)
(Revised manuscript received 10 April 1987)

Key words: Retinol transfer; Unilamellar liposome; Liposome; Retinol-binding protein; (Human protein)

The transfer of retinol from its complex with the retinol-binding protein to cell surfaces was studied using unilamellar liposomes as a cell surface model. The transfer of retinol to liposomes at 37 °C was rapid and reached an apparent equilibrium within 60 min. The amount of retinol transferred to the liposomes at equilibrium was directly proportional to the starting concentration of retinol : retinol-binding protein over a wide range of retinol : retinol-binding protein concentrations and also directly proportional to the concentration of liposomal phospholipid in the system, when the concentration of retinol : retinol-binding protein was held constant. The transfer increased slightly with temperature. Transfer was increased by a factor of 1.8 at pH 4.5 compared to pH around 7. Prealbumin in amounts sufficient to complex all retinol : retinol-binding protein, decreased retinol transfer to liposomes indicating that prealbumin increases the affinity of retinol-binding protein for retinol. Addition of apo retinol-binding protein to the system decreased the transfer of retinol to liposomes considerably probably through competition with the liposomes for retinol. In similarly designed experiments delipidated bovine serum albumin competed much less with liposomes for retinol. The results show that spontaneous transfer of retinol from the retinol : retinol-binding protein complex to liposomal membranes occurs in vitro and suggests that a similar transfer may occur in vivo from retinol : retinol-binding protein to cell surface membranes.

Introduction

Retinol (vitamin A alcohol) is a slightly water-soluble, light- and oxidation-sensitive and potentially toxic substance. It is transported in plasma tightly bound to a specific transport protein, the retinol-binding protein. The retinol : retinol-binding complex, is, in turn, bound to another plasma protein, prealbumin [1]. The stoichiometry in the retinol : retinol-binding protein: prealbumin complex is 1 : 1 : 1 (molar ratio). The uptake of retinol from the retinol : retinol-binding protein complex into the cell is assumed to occur at a cell surface receptor for retinol-binding protein. A cell surface receptor for retinol-binding protein has been demonstrated in bovine pigment epithelium [2] in rat testis [3] and in monkey small intestine [4]. Whether there are retinol-binding protein receptors in other tissues as well, is unknown. The receptor has so far not been characterised.

There are, however, also reports which indicate that retinol might be taken up by cells by mechanisms not involving a receptor for retinol-binding protein [5–7]. The mechanism for this uptake has not been clarified.

In the present paper we have used unilamellar

Correspondence: G. Fex, Department of Clinical Chemistry, Malmö General Hospital, S-214 65 Malmö, Sweden.

liposomes as a model to study the transfer of retinol from the retinol : retinol-binding complex to the cell surface. Using this model we have investigated the time course of the transfer and how this transfer is affected by variation in the concentration of the retinol : retinol-binding protein complex and the liposome concentration, by variations in temperature and pH and by prealbumin, apo retinol-binding protein and delipidated bovine serum albumin.

Materials and Methods

Egg yolk phosphatidylcholine (PC) dimyristoylphosphatidylcholine (DMPC), cholesterol, α-tocopherol, octyl-β-glucoside and bovine serum albumin were from Sigma Chemicals (St Louis, Mo, U.S.A.). Sephadex G-100, Sepharose CL-4B, CNBr-Sepharose 4B, DEAE-Sepharose CL-4B and prepacked Sephadex G-25 columns (PD-10 columns) were from Pharmacia Fine Chemicals (Uppsala, Sweden), Spectrapor 3 dialysis tubing was from Spectrum Industries Inc. (Los Angeles, CA, USA), Bio-Beads SM-2 from Bio-Rad (Richmond, CA, U.S.A.) and Lumagel® from Lumac/3M bv (Schaesberg, The Netherlands). [^{14}C]Triolein (specific radioactivity 111.8 mCi/mmol) and [^{3}H]retinol (specific radioactivity 29.0 Ci/mmol) were from New England Nuclear (Dreieich, F.R.G.). All other reagents were of analytical grade unless otherwise specified.

Proteins. Human retinol-binding was purified from serum and urine by affinity chromatography on human prealbumin linked to Sepharose 4B as previously described [8]. The purified retinol-binding protein was about 95 percent pure as judged from polyacrylamide gradient gel electrophoresis in the presence of dodecylsulphate. Retinol-free retinol-binding protein was prepared according to Goodman and Raz [9] and purified on prealbumin-Sepharose use [8]. The resulting apo retinol-binding protein preparation contained 1.63 mg retinol-binding protein/ml of which less then 4 mole percent contained retinol. Retinol-binding protein with high retinol content was prepared by first incubating purified retinol-binding protein with an excess retinol (in ethanol) for 60 min in the dark under N_2 at 20 °C and then purifying the resulting retinol : retinol-binding protein complex on prealbumin-Sepharose 4B. This preparation contained 2.21 mg retinol-binding protein/ml of which 82 mole percent was holo retinol-binding protein. Purified serum retinol-binding protein contained 0.47 mg/ml and contained 87 mole percent 'endogenous' retinol.

The concentrations of holo and apo retinol-binding protein in the preparations was determined using $\epsilon_{330} = 46\,000\ M^{-1} \cdot cm^{-1}$ [10] and $\epsilon_{280} = 46\,760\ M^{-1} \cdot cm^{-1}$ for holo retinol-binding protein and $\epsilon_{280} = 40\,400\ M^{-1} \cdot cm^{-1}$ for apo retinol-binding protein [11].

Prealbumin was isolated from plasma by thiol-disulphide interchange chromatography followed by affinity chromatography on retinol-binding protein linked to Sepharose CL-4B [12]. The product was better than 95 percent pure according to polyacrylamide gradient gel electrophoresis in the presence of dodecylsulphate. The concentration of prealbumin in solutions was determined using $\epsilon_{280} = 76\,140\ M^{-1} \cdot cm^{-1}$ [13].

Bovine serum albumin was delipidated as described [14]. The bovine serum albumin concentration was determined using the figure 6.7 for the absorbance of a 10 g/l bovine serum albumin solution at 280 nm [14].

[^{3}H]Retinol-labelled retinol : retinol-binding protein complex. Retinol-binding protein (10–20 nmol in 200 μl 0.02 mol/l Tris-HCl (pH 7.4) containing 0.2 mmol/l sodium EDTA) was incubated with 5 μl [^{3}H]retinol in absolute ethanol for 1 h at 20 °C in the dark under nitrogen. The [^{3}H]retinol : retinol-binding protein complex was then purified by ion exchange chromatography on a 0.7 × 1 cm DEAE-Sepharose CL-4B equilibrated in the same Tris buffer (start buffer). The column was washed with 8–10 volumes of start buffer and then eluted with 0.3 mol/l NaCl in start buffer [15]. The resulting [^{3}H]retinol-labelled retinol : retinol-binding protein complex was diluted with start buffer to give a final concentration of 0.05 mol/l NaCl before used in the incubations (see below). The characteristics of a typical preparation is shown in Fig. 1.

[^{3}H]Retinol-labelled retinol : albumin complex. [^{3}H]Retinol and unlabelled retinol were complexed to delipidated bovine serum albumin by mixing 100 μl delipidated bovine serum albumin (100 μmol/l in 0.02 mol/l Tris-HCl buffer (pH

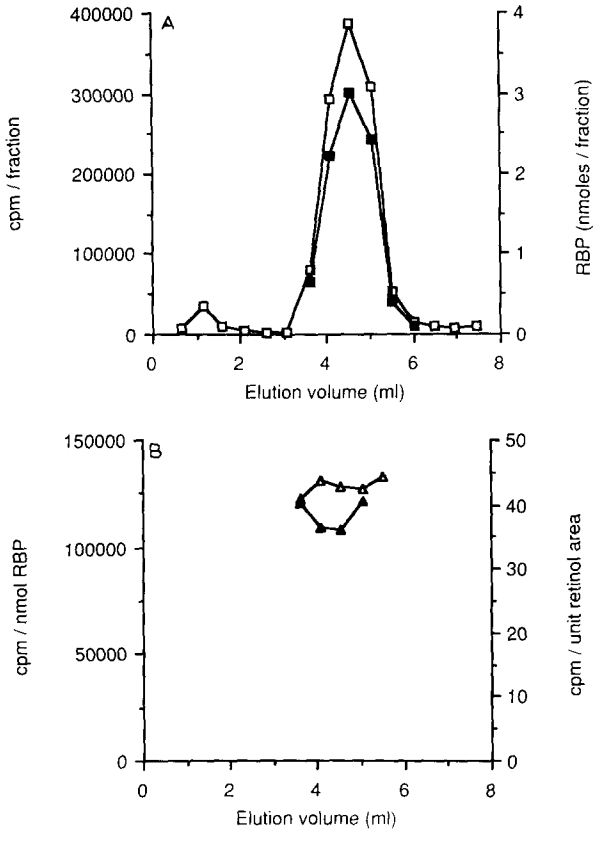

Fig. 1. Characteristics of the [^{3}H]retinol-labelled retinol : retinol-binding protein complex used in the transfer experiments. [^{3}H]Retinol was incubated with retinol-binding protein under N_2 in the dark at room temperature. The [^{3}H]retinol-labelled retinol : retinol-binding protein complex was then purified on small DEAE-Sepharose CL-4B columns from which it was eluted with 0.3 mol/l NaCl in start buffer. Retinol-binding protein was determined by radial immunodiffusion (recovery of retinol-binding protein $\geq$ 95 percent and of ^{3}H $\geq$ 70 percent and more than 91 percent of the ^{3}H-radioactivity coeluted with the retinol-binding protein peak). Retinol was isolated by HPLC and the specific radioactivity of retinol was expressed as cpm ^{3}H/unit surface area under the retinol peak in the HPLC chromatogram. (A) Retinol-binding protein concentration in nmoles (□) and ^{3}H-radioactivity in cpm per fraction (■). (B) Specific radioactivity of the retinol : retinol-binding protein complex expressed as cpm/nmol retinol-binding protein (△) and cpm/unit retinol area (▲).

7.4) containing 0.2 mmol/l sodium EDTA) with [^{3}H]retinol and 10.7 nmoles unlabelled retinol (in a total volume of 15 μl absolute ethanol). The retinol concentration in ethanol was determined using $\epsilon_{330} = 46000\ M^{-1} \cdot cm^{-1}$ [11]. The mixture was incubated for 30 min in the dark under N_2 at 20 °C and then applied on a 0.7 × 1 cm DEAE-Sepharose CL-4B column equilibrated in the above buffer. The column was washed with start buffer and the retinol-BSA complex eluted with 0.3 mol/l NaCl in start buffer. The recovery of ^{3}H was $\geq$ 80 percent. Of the recovered radioactivity > 83 percent was eluted with 0.3 mol/l NaCl. Assuming 100 percent recovery of bovine serum albumin it was calculated that the bovine serum albumin contained $\geq$ 0.71 mole of retinol/mole bovine serum albumin (BSA).

^{125}I-labelling of retinol-binding protein. ^{125}I-labelling of retinol-binding protein was done with the chloramine T method [16]. Free ^{125}I was removed by gel filtration on small prepacked Sephadex G-25 columns.

Preparation of liposomes. Liposomes were prepared essentially according to Mimms et al. [17]. [^{14}C]Triolein was used as a non-exchangeable marker and α-tocopherol was included as an antioxidant. In short, 5.3 μmol (4 mg) PC in chloroform/methanol (9 : 1, v/v), 4.7 μmol (1.8 mg) cholesterol, 0.053 μmol (23 μg) α-tocopherol and 10 μl (about 20 μCi) [^{14}C]triolein were taken to dryness with nitrogen in a glass tube. Octyl-β-glucoside, 0.15 mmol (44 mg) and 240 ml 0.02 mol/l Tris-HCl buffer (pH 7.4) containing 0.2 mmol/l sodium EDTA and 0.05 mol/l NaCl was then added and the tube gently shaken to dissolve the detergent and the lipids. The mixture was then dialyzed overnight against 2 litre of the above Tris-HCl buffer with 2 g/l Bio-Beads SM2 added to the dialysis fluid to trap the octyl-β-glucoside [18]. After dialysis the liposomes were in some cases gel filtered on a Sepharose CL-4B column (not shown) to check that they had the appropriate size. On this column more than 80 percent of the [^{14}C]triolein radioactivity eluted with or close to the void volume together with 70 percent of the phospholipid phosphorus. The phospholipid : cholesterol molar ratio of the liposomes in the peak was 1.126 : 1 which was close to the theoretical value (1.128 : 1). In some experiments liposomes were prepared using the above protocol but in buffers with pH ranging from 4.6 to 7.7. There was no gross difference in size between the liposomes prepared at pH 7 and pH 5 as judged from gel filtration experiments on Sephacryl S-1000. Liposomes made from di-

myristoylphosphatidylcholine with 1 mole percent α-tocopherol and [^{14}C]triolein as a non-exchangeable marker were also prepared according to the above protocol.

Electron microscopy. Suspensions of the egg lecithin : cholesterol liposomes prepared as described above were applied on carbon-coated copper grids and stained with a 2 percent (w/v) aqueous solution of uranyl acetate. The grids were viewed in a Philips EM 301 electron microscope. The resulting liposomes (not shown) looked spherical and had a diameter of 40 ± 4 nm (mean ± S.D.).

Determination of retinol transfer. Various concentrations of the [^{3}H]retinol-labelled retinol : retinol-binding protein complex or [^{3}H]retinol-labelled retinol : bovine serum albumin complex and liposomes were incubated in 0.02 mol/l Tris-HCl buffer (pH 7.4) containing 0.2 mmol/l sodium EDTA and 0.05 mol/l NaCl (or other buffers, see legend to the respective figures) for various times and at various temperatures in a total volume of 1 ml. Zero-time incubations were performed by mixing [^{3}H]retinol-labelled retinol : retinol-binding protein or bovine serum albumin complexes with liposomes on ice and immediately separating them again. The other time points were obtained by taking samples from the incubation mixture at the indicated times. Separation of liposomes and retinol : retinol-binding protein complex was achieved essentially as described by McLean and Phillips [19] by chromatography of 50 µl of the incubation mixtures on 0.7 × 0.5 cm columns (column volume 0.2 ml) of DEAE-Sepharose CL-4B equilibrated in 0.02 mol/l Tris-HCl buffer (pH 7.4) containing 0.2 mmol/l sodium EDTA and 0.05 mol/l NaCl. The columns were eluted (at 20 °C) with 1.5 ml of ice-cold equilibration buffer directly into scintillation vials. Under these conditions the liposomes do not bind to the column and 70–90 percent of the ^{14}C radioactivity is recovered within 1 ml of eluting buffer. Retinol-binding protein is retained on the column. The whole separation procedure took 2–3 minutes. Scintillator fluid, 10 ml, was added to the eluate and the ^{3}H and ^{14}C radioactivity of the mixture determined using standard settings for simultaneous determination of ^{3}H and ^{14}C in a Packard model 3255 Liquid Scintillation Spectrometer. The transfer of retinol from the retinol : retinol-binding protein complex to the liposomes was then calculated as

$A^{-1} \cdot B \cdot C$ = retinol transferred to liposomes (nmol)

where $A = {}^{3}\mathrm{H}/{}^{14}\mathrm{C}$ ratio of the whole unfractionated incubation mixture; $B = {}^{3}\mathrm{H}/{}^{14}\mathrm{C}$ ratio in the eluate from the DEAE-Sepharose 4B (i.e. the liposomes, which do not bind to DEAE under the conditions used) and C = the retinol concentration of the mixture expressed in nmol/ml. From this result was subtracted the calculated amount of retinol eluted in blank experiments without liposomes (see below).

To ensure that the radioactivity transferred to the liposomes was in the form of retinol and not some degradation product and that the retinol radioactivity and retinol mass were transferred in parallel, liposomes were incubated with [^{3}H]retinol-labelled retinol : retinol-binding protein complex for 1 h, isolated by DEAE-Sepharose chromatography as described above and the lipids extracted. The extract was then subjected to HPLC, the retinol concentration determined using retinyl acetate as internal standard and the ^{3}H radioactivity of the collected eluate determined. Both serum retinol-binding protein with 0.87 mol 'endogenous' retinol/mol retinol-binding protein and retinol-binding protein isolated from urine and loaded in vitro with retinol (see above) was used for these experiments. The recovery of ^{3}H in the HPLC fractions was ≥ 80 percent of which > 90 percent coeluted with unlabelled retinol (not shown). The specific radioactivity of retinol in the incubated liposomes was very similar to that of the [^{3}H]retinol-labelled retinol : retinol-binding protein complex indicating parallel transfer of [^{3}H]retinol and unlabelled retinol regardless if the retinol-binding protein used had been loaded with retinol in vivo or in vitro.

Two kinds of blanks were run in parallel with most incubations: one in which liposomes were omitted, which measures how much ^{3}H radioactivity is eluted with starting buffer and one where ^{125}I-labelled retinol-binding protein was added to the complete system (liposomes and [^{3}H]retinol-labelled retinol : retinol-binding protein complex) to measure the amount of retinol : retinol-binding protein complex with elutes together with the lipo-

somes. The former blank was used to 'correct' the retinol transfer to liposomes (see above). Other methods. Retinol-binding protein and prealbumin in incubation mixtures were quantitated by radial immunodiffusion [20] or electroimmunoassay [21] using the purified proteins as calibration standards, Polyacrylamide gradient gel electrophoresis in the presence of dodecylsulphate was performed as described by Blobel and Dobberstein [22] using the buffer system of Maizel [23]. Retinol was determined by HPLC [24], cholesterol according to Webster [25] and lipid phosphorus according to Chen et al. [26]. Lipids were extracted according to Folch et al. [27].

Results

The transfer of retinol from the retinol : retinol-binding protein complex to liposomes as a function of time is shown in Fig. 2. To ensure that it was not the whole retinol : retinol-binding protein complex or that free retinol was eluted with start buffer, control experiments with ^{125}I-labelled retinol-binding protein and liposomes and with [^{3}H]retinol : retinol-binding protein complex without liposomes were performed. It is evident from Fig. 2 that the abovementioned mechanisms could not account for the observed transfer of retinol to liposomes. Transfer was rapid and an apparent equilibrium was reached within 60 min. For the sake of comparison a similar experiment was performed using retinol complexed with bovine serum albumin instead of retinol-binding protein (experiment not shown). Transfer of retinol from this complex to liposomes was more rapid than from its complex with retinol-binding protein and the process seemed to have reached an equilibrium already in the zero time incubation i.e. during the separation step. The point of equilibrium with bovine serum albumin as retinol donor was at about 50 percent retinol transferred to liposomes compared to 20–30 percent transfer with retinol-binding protein as retinol donor at comparable concentration of retinol and liposomes.

In Fig. 3 are shown the results obtained when a constant concentration of liposomes was exposed to an increasing concentration of retinol, in the form of the retinol : retinol-binding protein complex, for 60 min. The retinol concentrations used covered the physiological range of retinol con-

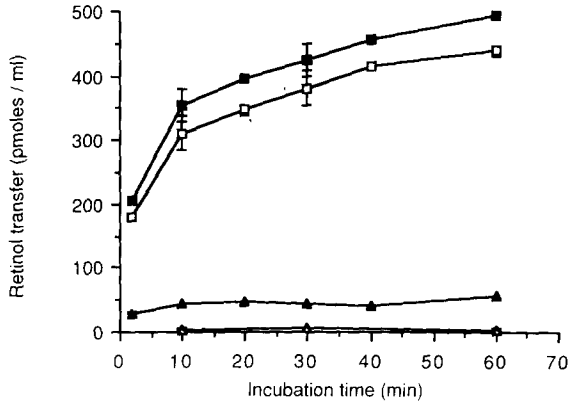

Fig. 2. Transfer of retinol from the retinol : retinol-binding protein complex to liposomes as a function of time. [^{3}H]Retinol-labelled retinol : retinol-binding protein (3 nmol retinol-binding protein/ml containing 0.82 mol retinol/mol retinol-binding protein) was incubated with (■) or without (▲) liposomes (0.65 μmol phospholipid/ml) at 37 °C for the indicated times. In some experiments (△), ^{125}I-labelled retinol-binding protein was added to the retinol : retinol-binding protein : liposome mixture. Retinol transfer corrected for blank transfer (incubation without liposomes) is shown also (□). Each point represents the mean of duplicate incubations with the range indicated.

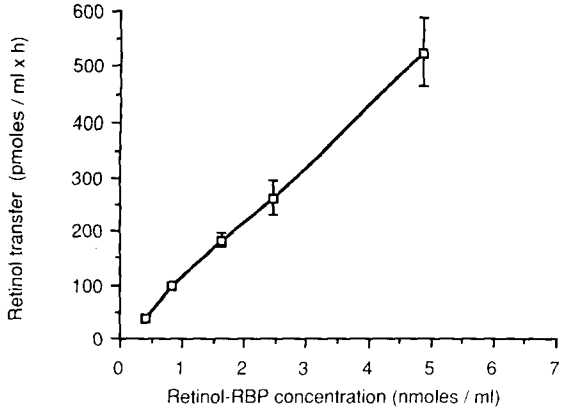

Fig. 3. Effect of the concentration of retinol : retinol-binding protein complex on the transfer of retinol to liposomes. Liposomes (0.65 μmol phospholipid/ml) were incubated for 1 h at 37 °C with varying concentration of the [^{3}H]retinol-labelled retinol : retinol-binding protein complex (retinol concentration of the complex was 0.82 mol/mol retinol-binding protein). Each time point is the mean of duplicate incubations with the range indicated. Results are corrected for blank transfer (incubation without liposomes).

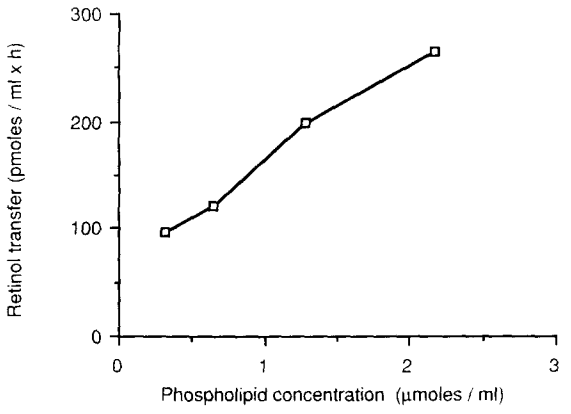

Fig. 4. Effect of different concentrations of liposomes on the transfer of retinol from the retinol : retinol-binding protein complex to liposomes. Different concentrations of liposomes were incubated for 1 h at 37 °C with 1 nmol [^{3}H]retinol-labelled retinol : retinol-binding protein complex (containing 0.82 mol retinol/mol retinol-binding protein). Each point is the mean of duplicate incubations with the range indicated. Results are corrected for blank transfer (incubation without liposomes).

centration in plasma. The transfer of retinol to liposomes looked almost linear over the range of concentrations tested (Fig. 3). Doubling of the retinol : retinol-binding protein concentration doubled the amount of retinol transferred to liposomes. At the highest concentration of retinol tested the molar ratio of liposomal lipid (phospholipid + cholesterol) to liposomal retinol after 60 min incubation was $\leq 1000 : 0.5$.

Variation of the concentration of liposomes keeping the concentration of retinol : retinol-binding protein complex, time and temperature constant (Fig. 4), showed that the transfer was directly proportional to liposome concentration. The transfer of retinol to liposomes/h increased with a factor of about three when liposome concentration was increased with a factor of factor of 6.8.

The transfer process showed some slight temperature-dependency in the 6–37 °C range (not shown). Transfer/h was lower with liposomes made from DMPC than with liposomes made

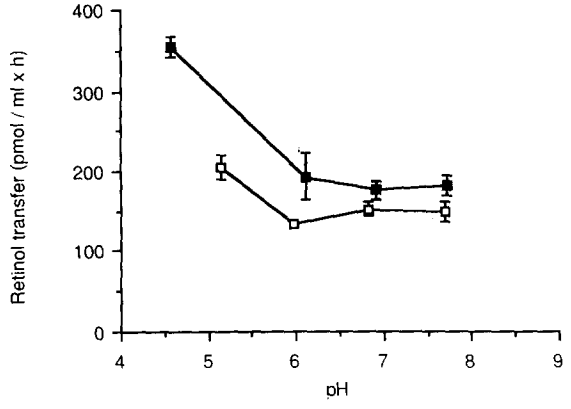

Fig. 5. Effect of pH on the transfer of retinol from the retinol : retinol-binding protein complex to liposomes. Liposomes (0.65 μmol phospholipid/ml) were incubated at 37 °C at pH 4.5–7.7 for 1 h with 1 nmol/ml [^{3}H]retinol-labelled retinol : retinol-binding protein complex (containing 0.82 nmol retinol/mol retinol-binding protein). Results are corrected for blank transfer (incubation without liposomes) and are given as the mean ± S.E. of three determinations for each pH. Two experiments using different buffers are displayed. In one experiment (□) 10 mmol/l sodium phosphate buffers with 0.05 mol/l NaCl were used for all pH values. In the other (■) 10 mmol/l citrate/phosphate buffers with 0.05 mol/l NaCl were used for the two lower pH values and 10 mmol/l sodium phosphate with 0.05 mol/l NaCl for the two higher pH values. Results were corrected for blank transfer (incubation without liposomes).

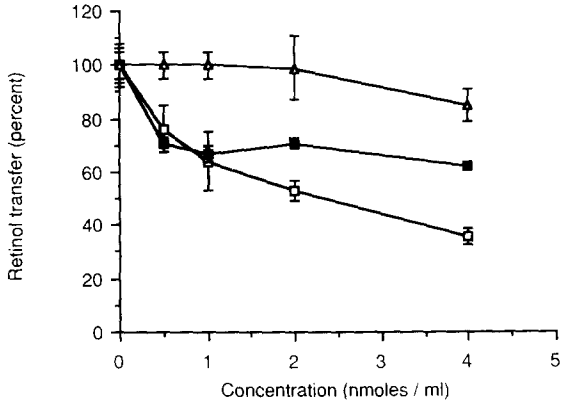

Fig. 6. Effect of prealbumin, delipidated retinol-binding protein and delipidated bovine serum albumin on the transfer of retinol from the retinol : retinol-binding protein complex to liposomes. Liposomes (0.65 μmol phospholipid/ml) and 1 nmol/ml [^{3}H]retinol-labelled retinol : retinol-binding protein complex (containing 0.82 mol retinol/mol retinol-binding protein) were incubated at 37 °C for 1 h in the absence/presence concentrations of prealbumin (■), delipidated retinol-binding protein (□) or delipidated bovine serum albumin (△). The fraction of retinol transferred from retinol : retinol-binding protein in the basal system (without any added prealbumin, apo retinol-binding protein or bovine serum albumin) was assigned the value of 100 percent. Each point is the mean ± S.E. of triplicate (duplicate and range for bovine serum albumin) incubations. Results are corrected for blank transfer (incubation without liposomes).

from egg PC at all temperatures. pH also seemed to influence transfer (Fig. 5). Transfer was enhanced at pH 4.5 relative to neutral pH by a factor of about 1.8. The effect of prealbumin, apo retinol-binding protein and delipidated bovine serum albumin on the transfer process is shown in fig. 6. When prealbumin was added in amounts sufficient to complex most of the retinol : retinol-binding protein comlex in a 1 : 1 (molar ratio) complex, transfer/h decreased with about 30 percent. Little further decrease in retinol transfer occurred when additional prealbumin was added. Addition of apo retinol-binding protein to the system strongly and progressively decreased the transfer while addition of the same molar amounts of delipidated bovine serum albumin had much less effect. The effect of prealbumin on the transfer was further investigated (Fig. 7) and the main effect of prealbumin seemed to be to decrease the equilibrium level of the system. The test system was not sensitive enough to detect also a probable decrease in retinol transfer rate.

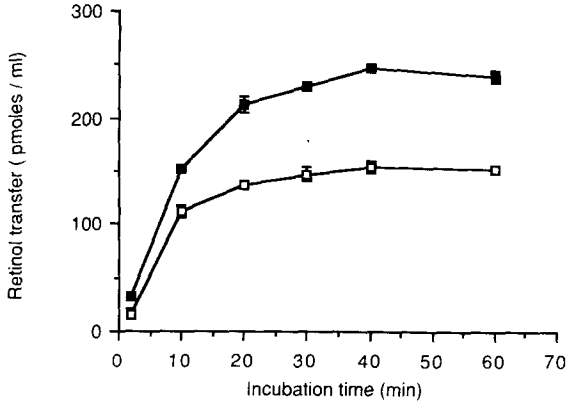

Fig. 7. Time course for the transfer of retinol from the retinol : retinol-binding protein complex to liposomes in the presence/absence of a two times molar excess of prealbumin. Liposomes (0.65 μmol phospholipid/ml) were incubated for various times with 1 nmol/ml [^{3}H]retinol-labelled retinol-binding protein (containing 0.82 nmol retinol/mol retinol-binding protein) in the presence (□) or absence (■) of 2 nmol/ml prealbumin. Each point is the mean and range of duplicate incubations. Results are corrected for blank transfer (incubation without liposomes).

Discussion

The cellular uptake of retinol from the retinol : retinol-binding protein complex has for long been assumed to occur in conjunction with the binding of the complex to a cell surface receptor for retinol-binding protein [2–4]. It is not known if endocytosis of the complex is involved similar to what has been described for transferrin [28] but it known that after delivery of retinol to the cell, retinol-binding protein is relased back into the blood and subsequently catabolized in the tubular cells of the kidney [1]. Precisely how retinol is detached from retinol-binding protein and transferred into the cell's interior is unknown. There are reports of retinol-binding membrane proteins [29] which might suggest that retinol uptake is similar to what has been suggested for fatty acids by Stremmel et al. [30,31]. These authors recently isolated a 40 kDa cell surface membrane protein from hepatocytes and enterocytes which specifically bound fatty acids and participated in their translocation into the cell. However, there is also the possibility that retinol can transfer non-specifically from the retinol : retinol-binding protein complex via the aqueous phase to the cell surface in a manner similar to fatty acids [32,33,35], cholesterol [19,36] and phospholipids [19].

The results obtained in this study show that retinol can transfer from its complex with retinol-binding protein to phospholipid bilayers with a halflife of probably less than 30 min. This is similar to the 40 min halflife for α-tocopherol transfer between liposomes and lipoproteins [34]. For fatty acid transfer from albumin to liposomes the halflife is less than 5 s [36] and cholesterol and phospholipid transfer between liposomes with halflives of 2–7 h [19,36] and 48 h, respectively [19]. The magnitude of the halflife for the transfer of retinol from retinol : retinol-binding protein to liposomes was similar to the in vivo halflife of 30–50 min for retinol in complex with retinol-binding protein in rats [38].

The time course of the transfer (Fig. 2) was similar to the retinol uptake curve which Rask et al. [39] obtained with isolated keratinocytes from cornea incubated with retinol complexed to retinol-binding protein. These authors interpreted the uptake of retinol into the cells as mediated by a

retinol-binding protein receptor. However, from a purely kinetic point of view it might as well have been the result of a non-specific transfer from the retinol : retinol-binding protein complex to the cell surface membrane of the keratinocytes.

The amount of retinol transferred to liposomes per h was directly proportional to the amount of retinol in the system over a broad range of retinol concentrations other factors held constant. There was also a direct proportionality between transfer per h and the concentration of liposomal phospholipid at constant retinol : retinol-binding protein concentration. The system did not show any signs of saturation within the concentration ranges tested indicating that the equilibrium of the system was reached within 1 h at all tested concentrations. Using the data in Fig. 5, the equilibrium constant for the distribution of retinol between retinol-binding protein and liposomes was calculated according to Noy et al. [32] and was found to be $(2.88 \pm 0.28 \cdot 10^{-4}$ (mean $\pm$ S.E., $n = 8$) in favour of the retinol : retinol-binding protein complex. The corresponding figure for fatty acids in the albumin : fatty acid-liposome system [32] was $2.6 \cdot 10^{-3}$. The binding constants for retinol-binding protein and retinol and albumin and long-chain fatty acids are in the order of 10^7 M^{-1} [40,41] and 10^8 M^{-1} [42], respectively, i.e. the difference in equilibrium constants in the liposome system does not reflect the affinities of albumin and retinol-binding protein for their respective ligands. However, the affinity of liposomes for fatty acids depends on fatty acid structure [43] and it may be that the observed difference in equilibrium constants instead reflects differences in the affinity of the liposomes for retinol and fatty acids respectively.

Compared to neutral pH transfer was almost doubled at pH 4.5 but only slightly increased at pH 5.5 to which a hypothetical retinol : retinol-binding protein receptor complex would be exposed if it were endocytosed in a manner similar to the transferrin-transferrin receptor complex [28]. One might argue that the observed pH effect was due to differences in the properties of the acceptor liposomes which were prepared at different pH. However, the rate-limiting step in the process seems to be the desorption of ligand from the donor (i.e. in this case retinol-binding protein) while the properties of the acceptor seems to be less important [36]. Transfer was decreased but not abolished by the presence of a molar excess of prealbumin over retinol-binding protein (Fig. 6). Addition of more prealbumin did not seem to further reduce transfer. Prealbumin does not prevent the release of retinol from the retinol : retinol-binding protein complex by blocking the binding site for retinol on retinol-binding protein [44,45] but the complexing with prealbumin probably increases the affinity of retinol-binding protein for retinol. This would be in accord with the early findings of Peterson and Rask [46], and Goodman and Raz [8]. This is also demonstrated in Fig. 7, where it can be seen that the point of equilibrium of the system is changed by prealbumin. Retinol transfer rate is probably also affected but this could not be demonstrated with the present experimental model.

This effect of prealbumin may have physiological importance. In the blood the retinol : retinol-binding protein complex is exposed to the phospholipids of the surface membranes of blood cells and the phospholipid surface of lipoproteins which together amount to approx. 3 μmol phospholipid/ml blood. In addition there is the large endothelial cell surface. Since phospholipid surfaces can extract retinol from retinol-binding protein (Fig. 4) a considerable fraction of the retinol could be transferred to the abovementioned membranes in the circulation. In the circulation, however, most of the retinol : retinol-binding protein is complexed with prealbumin which would decrease this non-specific transfer to membranes. Prealbumin may thus have a role in the keeping of retinol bound to the retinol-binding protein in the circulation. Due to its small size, it is reasonable to believe, that retinol-binding protein would penetrate the tissues more efficiently than its three times larger complex with prealbumin. The concentration of free retinol : retinol-binding protein complex would, therefore, be relatively higher in the interstitium than in plasma which would favor retinol release in the interstitium. Apo retinol-binding protein had profound effect on the transfer (Fig. 6) probably due to competition with the liposomal surface for retinol. Apo retinol-binding protein can also extract retinol from liposomal surfaces (Fex, G., unpublished) demonstrating that retinol can trans-

fer reversibly between retinol-binding protein and membranes. In uremia, the plasma concentration of retinol is higher than normal. The concentration of retinol-binding protein is still higher and its molar concentrations outnumbers that of prealbumin [47–49]. Because of the increased retinol concentration it has been argued that uremic patients might be at risk for retinol intoxication [47]. However, in uremia only the skin seemed to contain higher levels than normal of vitamin A [48] while the vitamin A content was decreased in the liver and unchanged in a number of other tissues [49]. The fact that retinol transfer from the retinol : retinol-binding protein complex to liposomes is decreased in the presence of high concentration of apo retinol-binding protein (Fig. 6) suggest that a high total concentration of retinol in the blood may be necessary in order to ensure sufficient retinol uptake into the tissues if the concentration of apo retinol-binding protein in the blood is high.

The present results thus show that retinol can be transferred from the retinol : retinol-binding protein complex to phospholipid membranes in vitro and we propose that a similar process may be operative also in vivo, i.e. that cells can take up retinol from the retinol : retinol-binding protein complex by a non-specific transfer process. The postulated receptor for retinol-binding protein [2–4] may, thus, not be necessary for cellular uptake of retinol from the retinol : retinol-binding protein complex. The role of the receptor may, instead, be related to targeting of the retinol flux to specific cells in order to ensure these cells access to retinol in excess over what is spontaneously delivered the non-specific way.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (03X-03364), the Albert Påhlsson Foundation, the Magnus Bergwall Foundation, the Malmö General Hospital Cancer Foundation, the Malmö General Hospital foundation for Medical Research, the John and Augusta Persson Foundation, the Crafoord Foundation and the Medical Faculty, University of Lund.

References

1 Rask, L., Anundi, H., Böhme, J., Eriksson, U., Fredriksson, Å., Nilsson, S.F., Ronne, H., Vahlquist, A. and Peterson, P.A. (1980) Scand, J. Clin. Lab.. Invest. 40, Suppl. 154, 45–61
2 Heller, J. (1975) J. Biol. Chem. 250, 3613–3619
3 Krishna Bhat, M. and Cama, H.R. (1979) Biochim. Biophys. Acta 587, 273–281
4 Rask, L. and Peterson, P. (1976) J. Biol. Chem. 251, 6360–6366
5 Yoshikami, S. and Nöll, G.N. (1978) Science 200, 1393–1394
6 Smith, J.E., Borek, C. and Goodman, DeW. S. (1978) Cell 15, 865–873
7 Takahashi, Y.I., Smith, J.E. and Goodman, DeW. S. (1977) Am. J. Physiol. 2, E263–E272
8 Vahlquist, A., Nilsson, S.F. and Peterson, P.A. (1971) Eur. J. Biochem. 20, 160–168
9 Goodman, DeW. S. and Raz, A. (1972) J. Lipid Res. 13, 338–347
10 Horwitz, J. and Heller, J. (1973) J. Biol. Chem. 248, 6317–6324
11 Heller, J. and Horwitz, J. (1973) J. Biol. Chem. 248, 6308–6316
12 Fex, G., Laurell, C.-B. and Thulin, E. (1977) Eur. J. Biochem. 75, 181–186
13 Raz, A. and Goodman, DeW. S. (1969) J. Biol. Chem. 244, 3230–3237
14 Chen, R.F. (1967) J. Biol. Chem. 242, 173–181
15 Saari, J.C., Bredberg, L. and Futterman, S. (1980) Invest. Ophthalmol. Vis. Sci. 19, 1301–1308
16 Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) Biochem. J. 89, 114–123
17 Mimms, L., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds J.A. (1981) Biochemistry 20, 833–840
18 Phillipot, J., Mutaftschiev, S. and Liautard, J.P. (1983) Biochim. Biophys. Acta 734, 137–143
19 McLean, L.R. and Phillips, M.C. (1981) Biochemistry 20, 2893–2900
20 Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235–254
21 Laurell, C.-B. (1972) Scand. J. Clin. Lab. Invest. 29, Suppl. 124, 7–19
22 Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835
23 Maizel, J.V. (1969) in Fundamental Techniques in Virology (Habel, K. and Salzman, N.P., eds.), pp. 334–362, Academic Press, New York
24 Fex, G. and Felding, P. (1984) Eur. J. Clin. Invest. 14, 146–149
25 Webster, D. (1962) Clin. Chim. Acta 7, 277–284
26 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
27 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
28 Dautry-Varsat, A., Ciechanover, A. and Lodish, H, (1983) Proc. Natl. Acad. Sci. USA 80, 2258–2262
29 Maraini, G., Ottonello, S., Gozzoli, F. and Merli, A. (1977) Nature 265, 68–69

30 Stremmel, W., Strohmeyer, G., Borchard, F., Kochwa, S. and Berk, P.D. (1985) Proc. Natl. Acad. Sci. USA 82, 4–8
31 Stremmel, W., Strohmeyer, G. and Berk, P.D. (1986) Proc. Natl. Acad. Sci. USA. 83, 3584–3588
32 Noy, N., Donnelly, T.M. and Zakim, D. (1986) Biochemistry 25, 2013–2021
33 Pownall, H.J., Hickson, D.D. and Smith, L.C. (1983) J. Am. Chem. Soc. 105, 2440–2445
34 Massey, J.B. (1984) Biochim. Biophys. Acta 793, 387–392
35 Doody, M.C., Pownall, H.J., Kao, Y.J. and Smith, L.C. (1980) Biochemistry 19, 108–116
36 Fugler, L., Clejan, S. and Bittman, R. (1985) J. Biol. Chem. 260, 4098–4102
37 Vahlquist, A., Peterson, P.A. Wibell, L. (1973) Eur. Clin. Invest. 3, 352–362
38 Lewis, K.C., Green, M.H. and Underwood, B. (1981) J. Nutr. 111, 1135–1144
39 Rask, L., Geijer, C., Bill, A. and Peterson, P.A. (1980) Exp. Eye. Res. 31, 201–211
40 Cogan, U., Kopelman, M., Mokady, S. and Shinitzki, M. (1976) Eur. J. Biochem. 65, 71–78
41 Fex, G., Albertsson, P.-Å. and Hansson, B. (1979) Eur. J. Biochem. 99, 353–360
42 Spector A.A. (1975) J. Lipid Res. 16, 165–179
43 Daniels, C., Noy, N. and Zakim, D. (1985) Biochemistry 24, 3286–3292
44 Newcomer, M.E., Jones, T.A. Åqvist, J., Sundelin, J., Eriksson, U., Rask, L. and Peterson, P.A. (1984) EMBO J. 3, 1451–1454
45 Sandblom, P., Åqvist, J. Jones, T.A., Newcomer, M.E., Van Gunsteren, W.F. and Tapia, O. (1986) Biochem. Biophys. Res. Commun. 139, 564–570
46 Peterson, P.A. and Rask, L. (1971) J. Biol. Chem, 246, 7544–7550
47 Farrington, K., Miller, P., Varghese, Z., Baillod, R.A. and Moorhead, J.F. (1981) Brit. Med. J. 282, 1999–2002
48 Vahlquist, A., Berne, B. and Berne, C. (1982) Eur. J. Clin Invest. 12, 63–67
49 Stein, G., Schöne, S., Geinitz, D., Abendroth, K., Kokot, F., Fünfstück, R., Sperschneider, H. and Keil, E. (1986) Clin. Nephrol. 25, 87–93