Terminal differentiation of the keratinocytes (cornification) has been linked to a restricted supply of retinol. Retinol is distributed to target cells by the retinol-binding protein (RBP), which circulates in the plasma in complex with transthyretin (TTR). In this study we have addressed the question of retinol delivery to the epidermis via RBP.

Retinol radiobinding assays, affinity chromatography with TTR coupled to Sepharose beads, polyacrylamide gel electrophoresis, and immunoblotting techniques were used to show that epidermal extracts contain retinol binding sites with no affinity for TTR. Immunoreactive RBP was detected in the epidermal extracts. The RBP in the epidermis was in the apoform (without retinol) in contrast to serum where the majority of RBP is in the holoform (with retinol). Epidermal RBP was converted in vitro into the holoform only after addition of 20 times more retinol, which was needed to reconstitute holoforms of RBP in dermal extracts, human buccal mucosal extracts, and delipidized normal serum or purified delipidized RBP. Moreover, several immunoreactive RBP bands (possibly degradation products) were identified in the epidermal but not in dermal extracts. Retinol-binding protein from nonkeratinizing human oral mucosa showed different immunoblotting patterns when compared to epidermal RBP.

These results suggest that degradation of RBP within the epidermis may result in a decreased retinol supply to the keratinocytes, and may lead to the cornification of the epidermis.

**MATERIALS AND METHODS**

The [11,12-3H]all-trans-retinoic acid (30 Ci/mmol) used was a gift from F. Hoffmann-La Roche (Basel, Switzerland). [15-3H]All-trans-retinol (14.3 Ci/mmol) and Biofluor were purchased from...
NEN (F.R.G.). All-trans-retinoic acid, all-trans-retinol, and antiproteases were purchased from Sigma (St. Louis, Missouri). Purity of retinoids was checked by high-performance liquid chromatography (HPLC) [13]. Solutions of retinoids were stored at −2°C in absolute ethanol containing 0.05% butylated hydroxytoluene. Nitrocellulose sheets were obtained from Bio-Rad (Richmond, California), the densitometer, TLC Scanner 76510 from Camag (Muttenz, Switzerland), and the Polytron PT7 homogenizer from Kinematica (Lucerne, Switzerland). Complete and incomplete Freund's adjuvants were obtained from Difco (Detroit, Michigan). Rabbit antigoat IgG Fab fragments were purchased from Cappel (Cochraneville, Pennsylvania). All other chemicals were of analytical grade.

**Tissue Samples** Normal human skin samples were obtained from healthy donors by cosmetic surgery from several body sites. Epidermis was separated from dermis by heat (52°C, 1 min) in saline [14]. In some experiments the epidermis was obtained by using a keratome set at 0.18 mm. This procedure resulted in samples with very little dermal contamination as checked by histology. Samples were stored at −70°C.

Normal noninflamed human oral mucosa was obtained from surgical excision during extractions of wisdom teeth. The tissue was washed several times in saline and compressed between pieces of gauze in order to eliminate blood. Histologic assessment of the samples indicated little contamination by dermis and absence of keratinization of the epithelium. Samples were stored at −70°C and processed in a manner similar to skin tissue.

**Tissue Supernatants** Lyophilized aliquots of tissue samples representing about 15 mg of epidermis, 30 mg of dermis, or 15 mg of mucosa were reconstituted at full speed for 30 s, 3 times in 800 μl of extraction buffer (50 mM Tris, 25 mM NaCl, 2.5 mM EDTA, pH 7.5) using a tissue homogenizer [15]. Tissue supernatants were obtained after centrifugation at 100,000 g for 60 min at 4°C and were immediately used for assays or frozen at −20°C. Protein contents were determined using bovine serum albumin (BSA) as standard [16]. To the samples analyzed by polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting a mixture of antiproteases (0.025 mM N-p-tosyl-L-lysine chloromethyl ketone, 0.025 mM 1-tosylamido-2-phenylethyl chloromethyl ketone, 0.01 mM aprotinin, 0.02 mM leupeptin) was added to the extraction buffer.

**Serum Delipidation** Normal human serum and 2 vol of methylene ketone were vortexed 3 times for 4 s (maximum speed) at 0°C. Organic solvent layer was separated by centrifugation for 3 min at 10,000 g and discarded. This procedure was repeated. The sample was then flushed with N2 to evaporate the solvent and the volume was readjusted with water to initial serum volume. Identical procedure was utilized to delipidize pure RBP solution. This procedure did not impair the binding properties of RBP.

**Competitive Radiobinding Assays** Vitamin A binding proteins were quantitated by incubating the samples with [11,12,13,14,15,16,17]H]all-trans-retinoic acid 50 nM or [15,16,17]H]all-trans-retinol 50 nM [17]. Alkaline solutions of retinoids containing 0.5 mg/ml of butylated hydroxytoluene were depleted in microtubes and were evaporated by a stream of N2. Cytoplasmic fractions (about 300 μg of protein) in a final volume of 100 μl were added and incubated at 4°C for 16 h. For binding and competition studies, an excess of 200-fold of unlabeled ligand was added in the same manner as for radioactive compounds. Samples were then subjected either to gel filtration on a Sephadex G75 column (0.8 × 15 cm) [16] or to charcoal-dextran technique [15].

**Affinity Chromatography** Affinity chromatography on a column of human TTR coupled to Sepharose 4B was performed according to Volhquist et al. [18]. Ten milligrams of purified human TTR [2] was covalently coupled with 2 ml of swollen CNBr-activated Sepharose 4B. The coupling efficiency was found to be 97%. A column (0.5 ml) was prepared in a 2-ml syringe and equilibrated with extraction buffer containing 0.1 M NaCl. A small column with a high capacity of binding RBP was chosen to avoid retinol dissociation in the gel. This column was used several times without appreciable loss of binding capacity.

Binding capacity was checked with pure holo-RBP and was found to be more than 0.6 mg. Holo-RBP was eluted with distilled water adjusted to pH 8 with diluted ammonia solution. Tritiated retinol-binding peaks eluted from Sephadex G75 column were collected and applied to the TTR-Sepharose column, washed, and eluted. Fractions of 0.3 ml were collected and their radioactivity assessed in a liquid scintillation counter using 8 ml Bio-fluor per fraction.

**Protein Transfer and Immunoblotting** Proteins separated by PAGE were electroblottransfered onto a nitrocellulose filter in 0.25 mM sodium phosphate solution and further processed according to Towbin et al [19]. To reduce nonspecific binding of the immunoglobulins (IgG), the filters were incubated for 1 h in 5 mM sodium phosphate, 0.13 mM NaCl, pH 7.2 (PBS) containing 3% BSA (blocking solution) prior to a 2-h incubation at room temperature in washing solution (PBS, 0.5% BSA, 0.2% Tween 20) containing suitable amounts of purified anti-RBP IgG or antisera (see below). The filters were then washed 3 times in washing buffer followed by incubation of horseshard peroxidase-labeled rabbit antigoat IgG Fab fragments, appropriately diluted in washing solution. This incubation, which lasted 1 h at room temperature, was terminated by 3 washings of the filter in washing solution. Bound enzyme was visualized by incubating the filter in 100 mM Tris/HCl buffer (pH 7.4), containing 0.5 mg/ml of diaminobenzidine and 0.03% H2O2. The percentage of apo- to holo-RBP was estimated by optical densitometry.

**Goat Monospecific Immunoglobulin Against Human RBP** A 2-ml solution of RBP (0.4 mg/ml) in PBS was emulsified with an equal volume of complete Freund's adjuvant and injected s.c. in multiple spots on the back of a goat. The procedure was repeated 3 times at weekly intervals with incomplete Freund's adjuvant. The serum from the bleeding 7 days after the last injection was used for further purification. Monospecific goat antihuman RBP antibodies were purified by passage of the immunosorbed immunoglobulin adsorbed through a column containing highly purified human RBP coupled to Sepharose 4B. After extensive washing with PBS, specific IgGs were eluted with 3 M KSCN and then dialyzed against PBS and concentrated. The specificity of both the monospecific IgGs and immunosorbed antiserum were verified by radial immunodiffusion and immunoblotting techniques with whole serum and pure RBP.

**Metabolism Studies** In order to ensure that retinol or retinoic acid was not metabolized under our binding assay conditions, supernatant tissue fractions were incubated with 1 μM of either [14]retinol or [14]retinoic acid at 4°C for 18 h. The cytosol was then acidified to pH 4.0 with diluted HCl before it was extracted with 2 × 1 ml diethyl ether, followed by drying. The organic phase of the residue was reconstituted in 50 μl of ethanol and subjected to HPLC on a monoceric C18 reversed-phase column (TSK, MCH 5, 25 × 4 mm). A two-solvent system was used: solvent A, 100% methanol; solvent B, 10 mM ammonium acetate.
in water. An isocratic elution with a mixture of 80% solvent A and 20% solvent B for 12 min at 1 ml/min was followed by a linear gradient to 94% of solvent A for 12 min. Fractions of 0.5 ml were collected, and their radioactivity assessed in a liquid scintillation counter using 5 ml Biofluor per fraction.

**Retinol-RBP Dissociation Studies** The possible dissociation of retinol from its RBP during protein extraction procedure was considered. First, serum was homogenized in conditions similar to those utilized for tissues. Second, serum diluted 1/10 or 1/50 in 800 µl of extraction buffer was incubated with the pellet resulting from proteins extracted from dermis, epidermis, or whole skin. Third, serum diluted at 1/10 or 1/50 with an epidermal protein extract was incubated in the same conditions as for radiobinding assays. In all the cases cited above, supernatants were analyzed by immunoblotting technique.

**RESULTS**

**Epidermal Extracts Contain Retinol-Binding Sites With No Affinity to TTR** Table I shows the 

![Figure 1. Gel filtration analysis on Sephadex G75 column of human epidermis. Supernatant (0.3 mg protein) was incubated with 50 nM [3H]retinol (open circles), in the presence of an excess of 200-fold retinoic acid (solid circles) or in the presence of an excess of 200-fold retinol (solid triangles).](image)

![Figure 2. Human TTR coupled to Sepharose affinity chromatography. Tritiated retinol activities after Sephadex G75 were deposited on the column and washed with extraction buffer (A). Elution was performed with dissociation solution (B).](image)

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![Table I. Cellular Retinoic Acid-Binding Protein (CRABP) and Cellular Retinol-Binding Protein (CRBP) Binding Activities and Specificities in Normal Human Epidermis](image)

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Delipidation of serum readily dissociated retinol from the holo-RBP, giving one sharp band of apo-RBP. Total reconstitution was obtained after addition of 10 μM retinol. This value is about 50 times higher than the $K_d$ of retinol for pure RBP [25].

Electrophoresis dissociates the complex RBP-TTR from the blood totally, so that immunoreactive bands showed free RBP forms. This was confirmed by the same electrophoretic mobility observed for RBP in the serum and for pure RBP.

**Retinol-Binding Protein Species in Dermal, Epidermal, and Mucosal Extracts:** Dermal and epidermal extracts were subjected to the PAGE-immunoblotting technique under the same conditions as for serum (Fig 5). Dermal extracts showed only a major band of apo-RBP but when incubated with 10 μM retinol they showed total reconstitution.

Epidermal extracts (from both heat-separated and keratinized samples) showed very different immunoblotting patterns (Fig 5).

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### Table II. Estimation of Retinol-Binding Protein (RBP) Species and Holo-RBP Reconstitution

<table>
<thead>
<tr>
<th></th>
<th>RBP (%)</th>
<th>Holo-RBP % Reconstitution</th>
<th>RBP Degraded Forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>95</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>Epidermis</td>
<td></td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>Mucosa</td>
<td></td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

*Estimated from total RBP (apo- + holoforms).

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**Figure 3.** Polyacrylamide gel electrophoresis of normal human serum and purified RBP. Slab gel PAGE (7.5%) with 5 μl normal human serum (lane 1), 20 μg of freshly purified human RBP (lane 2), 8 μg of excessive handling of purified RBP (lane 3), and 8 μg of delipidized pure RBP (lane 4). Pure RBP, 10 μg on SDS-PAGE 5–20% slab gel (lane 5).

**Figure 4.** Immunoblotting of normal human serum (NHS) using goat antihuman RBP serum. Samples (5 μl) were subjected to PAGE and electrophoretically transferred to a nitrocellulose filter. Lanes 1 and 2, NHS and delipidized NHS; lanes 3–6, delipidized NHS incubated with 1, 2.5, 5, and 10 μM retinol, respectively; lane 7, 0.4 μg of purified RBP. Antibodies bound to the filter were detected with peroxidase-conjugated IgG, and visualized with diaminobenzidine colorization, as described in Materials and Methods.

**Figure 5.** Immunoblotting of RBP species in epidermis, dermis, and mucosa. Tissue extracts were subjected to PAGE and electrophoretically transferred to nitrocellulose. Dermal extracts (250 μg protein/lane) were incubated without (lane 1) or with 10 μM retinol (lane 2). Epidermal extracts (300 μg protein/lane) were incubated without (lane 3), with 10 μM (lane 4) and with 200 μM retinol (lane 5). Mucosal extracts (250 μg/lane) were incubated without (lane 6) or with 10 μM retinol (lane 7). Six distinct immunoreactive bands with slow or fast electrophoretic mobilities were detectable. Proteolytic activity during homogenization was minimized as antiproteases were present in the extraction medium. One of these bands corresponded to apo-RBP. In order to estimate the affinity of retinol for apo-RBP, different concentrations of retinol were incubated with epidermal extracts. About 80% reconstitution was obtained with 200 μM, i.e., 20 times more retinol than needed for serum.

On the other hand, oral mucosal extracts showed a pattern of RBP species different from those present in the epidermis and dermis (Fig 5). No significant fast-, but various slow-migrating immunoreactive bands were detected. In contrast to that in epidermis, apo-RBP could be partially reconstituted into holo-RBP when mucosal extracts were incubated with 10 μM retinol. (Table II shows the estimation of RBP species and holo-RBP reconstitution in these tissues.)

**Retinol RBP Dissociation Control Experiments:** To determine possible dissociation of retinol from RBP during extraction of tissue proteins, we performed 3 control experiments: (a) Minimal retinol release from RBP was observed by the PAGE-immunoblotting technique, when serum was subjected to the procedure similar to tissue homogenization. (b) Incubation of normal diluted serum with the pellet resulting from protein extraction of dermis and epidermis produced a negligible amount of apo-RBP. These results show that retinol can be only partially dissociated from its RBP by dermal or epidermal components. (c) Incubation of normal human serum with epidermal supernatants did not result in the appearance of immunoreactive RBP bands as compared to the
in epidermis since the TTR affinity binding technique cannot discern qualitative differences in RBP species. For instance, a modified epidermal RBP that possesses no affinity for TTR could still bind to retinol.

### The RBP Species of Human Epidermis

The concept that RBP is responsible for the delivery of retinol from liver to the extrahepatic action sites of vitamin A is generally accepted (see review [8]). Although no strong evidence has been reported that RBP leaves the blood and diffuses throughout dermis to bring retinol to epidermis, the detection of RBP in dermis and epidermis makes this concept acceptable, even if the synthesis of mRNA for RBP in tissues other than liver has been reported [30].

Qualitative analysis of RBP species in tissues, including sera, is of great importance for two reasons. First, these studies are necessary for understanding the metabolism of RBP in normal and pathologic tissues. Second, such information could be related to the putative epidermal RBP membrane receptors in order to better understand the mechanisms involved in retinol delivery and regulation [4,7,8]. To address these questions, we used an immunoblotting technique, which is the most sensitive method for detection of such immunoreactive compounds at present. Moreover this technique allows for the identification of a binding protein with (holo) or without (apo) its ligand. This is the first time that such RBP molecules can be visualized and their binding properties studied when expressed in very low amounts as found in tissues.

This technique directly showed that normal human serum contains the 2 forms apo- (5%) and holo-RBP (95%). This is in agreement with current knowledge indirectly derived from measurement of either vitamin A and total RBP [31,32] or holo-RBP in disc gel rod and total RBP [33].

Our results clearly demonstrate the presence of RBP, mainly as apo-RBP, in dermis, epidermis, and oral mucosa. Qualitatively RBP and its immunoreactive metabolic products are expressed very differently in these tissues: Surprisingly, dermal extracts showed only apo-RBP, when one could expect to find also the holoform because the dermis contains an important microvascularization and it could serve as a transit tissue toward the epidermis. Epidermal and oral mucosa extracts showed, besides apo-RBP, several immunoreactive bands probably due to partial hydrolysis of RBP. These bands remained unchanged when extraction buffer contained a cocktail of proteases excluding a possible degradation during protein extraction.

Sharp differences were seen when comparing the electrophoretic patterns of epidermal and oral mucosa extracts: high mobility bands were absent in oral mucosa. These results suggest that degradation of RBP is very low if not absent in the dermis whereas it is high, although different, in the epidermis and oral mucosa. The fact that no holo-RBP was found in these tissues and especially in the dermis could be explained by retinol dissociation during tissue homogenization, but reassociation would have taken place after protein extraction. Inactivation of endogenous retinol, by esterification for example, requires further studies.

Most importantly, apo-RBP from normal human serum and dermis could be almost totally reconstituted into the holoform with retinol, whereas this did not happen with the apo-RBP from the epidermis. It is likely that RBP does not diffuse throughout the epidermis in its intact holoform, but is rather metabolically inactivated (loss of affinity) and partially hydrolyzed. The absence of intact holo-RBP in epidermis might be explained by the inactivation of the binding protein in the lower layers of the epidermis. Since our study was nondynamic in design we cannot exclude the possibility that a certain amount of RBP may leave the epidermis as unmodified apo-RBP; we think that this is unlikely considering the immunoblotting patterns of the dermis.

In oral mucosa, apo-RBP appears to have only partially lost its affinity so that retinol might still be supplied to the upper layers of the epithelium. This suggestion is strengthened by the fact that the metabolism of RBP in oral mucosa is different from

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*Figure 6. Metabolism of [3H]retinol in vitro. Epidermal supernatant (open circles), or extraction buffer (solid circles) were incubated with [3H]retinol extracted with diethylether. The extracted radioactivity was chromatographed on reversed-phase HPLC. Details in text. The experiment was repeated 3 times. (†)R = retinol, (3H)RA = retinoic acid.*
the epidermis as seen in the patterns obtained by immunoblotting technique.

From studies on intestinal mucosal epithelial cells from the monkey [4], pigment epithelial cells of bovine retina [34], and rat testis [35], it has been suggested that only holo-RBP could interact with the cell membrane receptor for RBP. As far as the epidermis is concerned, our results suggest that this might occur in the lowest layers of the epithelium (basal cells?), where RBP might give up retinol and itself becomes structurally and chemically modified, with subsequent loss of its affinity for retinol; thus it cannot interact any longer with its receptor. If smaller retinol availability is involved in the terminal differentiation of the epidermis, it might be related to the lack of a functionally active form of RBP diffusing throughout the epidermis.

We thank Prof. F. Chytiril for helpful discussions and suggestions. We acknowledge the expert technical assistance of Mrs. Denise Grand. We thank Prof. G. Fiore-Dono and Dr. J. Samson for mucosal sampling, and Dr. Y. Meroit for histologic evaluation of samples, as well as Ms. Christine Wang and Mrs. Sylvie Deschamps for typing the manuscript.

REFERENCES