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THE EXTRACTION AND PARTIAL PURIFICATION OF THE DEOXYCHOLATE-SOLUBLE MATRIX PROTEIN FROM HUMAN PLANTAR HORNY LAYER

Tadashi Tezuka

From the Department of Dermatology, School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan

Abstract. The electron-dense, amorphous matrix protein in human horny cells was extracted with deoxycholate. The deoxycholate-soluble material was partially purified by differential centrifugation and molecular sieve chromatography. Approximately 44 % of the total protein was extracted into the 270 000 *g* supernatant fraction. Macroaggregates with histochemical characteristics identical with those of *in situ* keratohyalin granules were formed upon dialysing the 270 000 *g* supernatant fraction against redistilled water. Antibody was raised to the purified material of the initial peak from the Sepharose 6B column. The resulting antiserum yielded a single precipitin line when tested against the immunizing antigen. Electrophoresis in the presence of sodium dodecylsulfate showed this antigenic material to be heterogeneous. It was immunologically homogeneous when this antigenic material was examined by immuno-electrophoresis. Since the antibody was localized on the keratohyalin granules of human plantar skin by indirect immunofluorescence, the partially purified, deoxycholate-soluble material from human plantar horny layers is considered to originate from keratohyalin granules. Amino acid analysis revealed a high content of glycine, glutamic acid and aspartic acid, and a low content of histidine and leucine.

Key words: Matrix protein; Horny layer: Keratohyalin granule origin; Pauly's stain

In order to elucidate the nature of the abnormality in keratohyalin granules in disorders of keratinization, such as ichthyosis, it would be of value to examine such granules biochemically. Since keratohyalin granules cannot be isolated in sufficient quantities for biochemical examination from the skin of patients suffering from these disorders, little is known about them.

As the horny layers of skin contain material of keratohyalin origin, the following studies were undertaken. This material is thought by Mercer (13) and Brody (1) to exist in two forms. Each of these workers has proposed a concept of keratinization. Mercer considers that keratohyalin is added to tonofibrils and actually becomes involved in the morphological changes occurring in tonofibrils that result in the unstained filaments observed in cornified cells, whereas Brody believes that keratohyalin remains as the interfilamentous matrix in cornified cells.

Radioautographic studies have shown that ^{3}H-labelled amino acids are incorporated in keratohyalin granules 1–6 hours after injection and are later detected in the cornified layer (4–6). In addition to these radioautographic observations, extraction of keratohyalin granules with 0.1 N NaOH from glutaraldehyde-fixed epidermis, also extracts the electron-dense background substances in the horny cells, revealing the fibrous structure clearly (7).

When newborn rat epidermis was extracted with sodium deoxycholate, keratohyalin granules were extracted, the electron-dense material disappeared, and the fibrous structure was revealed in the lowermost layers of stratum corneum under electron-microscopy (16).

These observations suggest that keratohyalin granules, actively synthesized in granular cells, enter the horny layer and both they, and the electron-dense material in the horny cell, can be removed with 0.1 N NaOH or sodium deoxycholate.

EXPERIMENTAL PROCEDURES

Materials. Human plantar skin was obtained from 3 patients who had been operated on for osteosarcoma. Male albino rabbits weighing 3 kg were used to prepare antibodies against the Sepharose 6B peak. Sodium deoxycholate was purchased from Difco Laboratories, Detroit, Mich.; Sephadex G-200 and Sepharose 6B from Pharmacia Fine Chemicals Inc., Fair Lawn, N.J.; goat antisera raised against rabbit γ-globulin and labelled with fluorescein isothiocyanate from Behringwerke AG, Marburg, Lahn, BRD; glass homogenizers were

purchased from Kontes Glass Company, Vineland, N.J. All other chemicals were of reagent grade or analytic grade, obtained from various commercial sources.

Preparation and extraction of tissue

Human plantar skin was excoriated immediately after surgery and cut into thin strips (approx. 7 × 1 cm) after removal of subcutaneous tissue. The strips were placed on a stretching apparatus under moderate tension and were sliced carefully with a razor blade to obtain sheets consisting solely of horny layers. Horny layers were minced and homogenized in either 50 mM Tris-HCl byffer, pH 8.6, containing 3% deoxycholate or redistilled water, and stirred for 48 hours at 4°C. Subsequent procedures were carried out at 4°C. The extraction procedure was repeated three times, changing the extracting solution every 48 hours. The homogenate was centrifuged at 15 000 *g* for 10 minutes (Tominaga refrigerated automatic centrifuge) and the resulting deoxycholate-supernatant fraction was recentrifuged at 270 000 *g* for 60 minutes (Beckman-Spinco ultracentrifuge Model L-2-65). The lipid layer was discarded and the high speed supernatant fraction was dialysed against 10 mM Tris-HCl (pH 8.8) for 24 hours at 4°C, concentrated by lyophilization and stored at −80°C until further use.

Microscopic studies

Samples of tissue used in the fractionation procedures and the aggregates obtained by dialysis of the 270 000 *g* supernatant fraction were fixed in buffered formalin for examination in the light microscope, and in 2.5% buffered glutaraldehyde and post-fixed with osmium tetroxide for electron microscopy. Prior to fixation, deoxycholate was removed from specimens by rinsing in 50 mM Tris-HCl buffer (pH 8.8). Tissues for electron microscopy were fixed for one hour, dehydrated, embedded in Epon and cut on a Porter Blum MT-2 ultratome (Ivan Sorvall Inc.). Thin sections were stained with 1% uranyl acetate, counterstained with lead and examined in a Hitachi 11 electron microscope.

Histochemical studies

One ml of the 270 000 *g* concentrated supernatant fraction was dialysed against 2 liters of redistilled water at 4°C for 72 hours, changing the redistilled water every 24 hours. Aggregates were harvested by centrifugation at 700 *g* for 10 minutes, and used for the histochemical examinations. The pellet and normal human plantar skin were sectioned in a cryostat at 6 μm and fixed with 95% ethanol for 5 minutes. They were stained with toluidine blue (10), diazotized sulfanilic acid (15), and Harris hematoxylin-eosin. Counter stains were not employed with any of the histochemical techniques.

Gel filtration studies

Both the 270 000 *g* supernatant fraction and the aggregates, which were collected by centrifugation after dialysing the 270 000 *g* supernatant fraction, were dissolved in 50 mM Tris-HCl (pH 8.8), made 3% with respect to deoxycholate, and were subjected to gel filtration on a column (2.5 × 34 cm) of Sephadex G-200. The profile was monitored at 280 nm during elution with 50 mM Tris-HCl (pH 8.8) containing 10 mM 2-mercaptoethanol and, of the several peaks described below, the first peak which eluted immediately following the void volume was further fractionated on a Sepharose 6B column (2.5 × 34 cm) using the same eluant or 50 mM Tris-HCl (pH 8.8) containing 0.5% deoxycholate. These studies were carried at 4°C.

Gel electrophoresis

Various fractions were subjected to disc gel electrophoresis (17) in the presence of sodium dodecylsulfate using the technique of Weber & Osborn (20).

Chemical analysis

Protein was determined by the method of Lowry et al. (11), and hexose, by the phenol-sulfuric acid method of Dubois et al. (2). Amino acid analyses were obtained following hydrolysis at 100°C *in vacuo* for 18 hours in the presence of 6.0 M redistilled HCl. Analyses were performed on a Beckman automatic amino acid analyser equipped with an automatic integrator. RNA was measured by the orcinol method (12).

Immunological procedures

The lyophilized eluates from the Sepharose 6B column were dissolved in 50 mM Tris-HCl (pH 8.8), containing 3% sodium deoxycholate and 10 mM 2-mercaptoethanol and dialysed against distilled water at 4°C. The resulting turbid solution was emulsified with an equal volume of Freund's complete adjuvant (3) and injected into all foot pads of rabbits (1 mg of protein in a 2.0 ml emulsion was injected into each animal). The animals were bled and boosted at appropriate intervals. The boosting was done by the intracutaneous injection of 1 mg of protein in Freund's incomplete adjuvant into the neck 3 weeks after the initial injection.

Sera were assayed for precipitating antibody content using the Ouchterlony double diffusion technique (14). 2% ion agar in a barbituric acid–sodium barbiturate mixture of 0.1 ionic strength (pH 8.6) was used.

Immunoelectrophoresis was carried out according to a method based upon Graber's technique (8). The buffer was a barbituric acid–sodium barbiturate mixture of 0.1 ionic strength and pH 8.6, and 2 ml of 2% agar at pH 8.6 was deposited with a pipette on a thoroughly cleaned and dried microscope slide. Electrophoresis was performed for 60 minutes at 40 volts. The antigen–antibody reaction was developed in a humid chamber at room temperature overnight. To remove non-reacted, diffusible protein, the slides were washed for 24 hours in physiological saline with several changes of the solution, and the washed slides were stained by a 20 minute dip in an amido black 10B solution (100 mg of dye to 100 ml of a 9:1 methanol–acetic acid solution). After immersion in the solution and rapid washing in water, the slides were air dried.

Indirect immunofluorescent studies were performed according to the technique of Kawamura (9) using fresh normal human plantar skin, frozen and cut in a cryostat and fixed in 95% ethanol for 3 minutes.

RESULTS AND DISCUSSION

Tissue source and separation techniques

The plantar skin consists of very thick stratum corneum, three layers of stratum granulosum, several layers of spinous cells, a single basal cell layer and

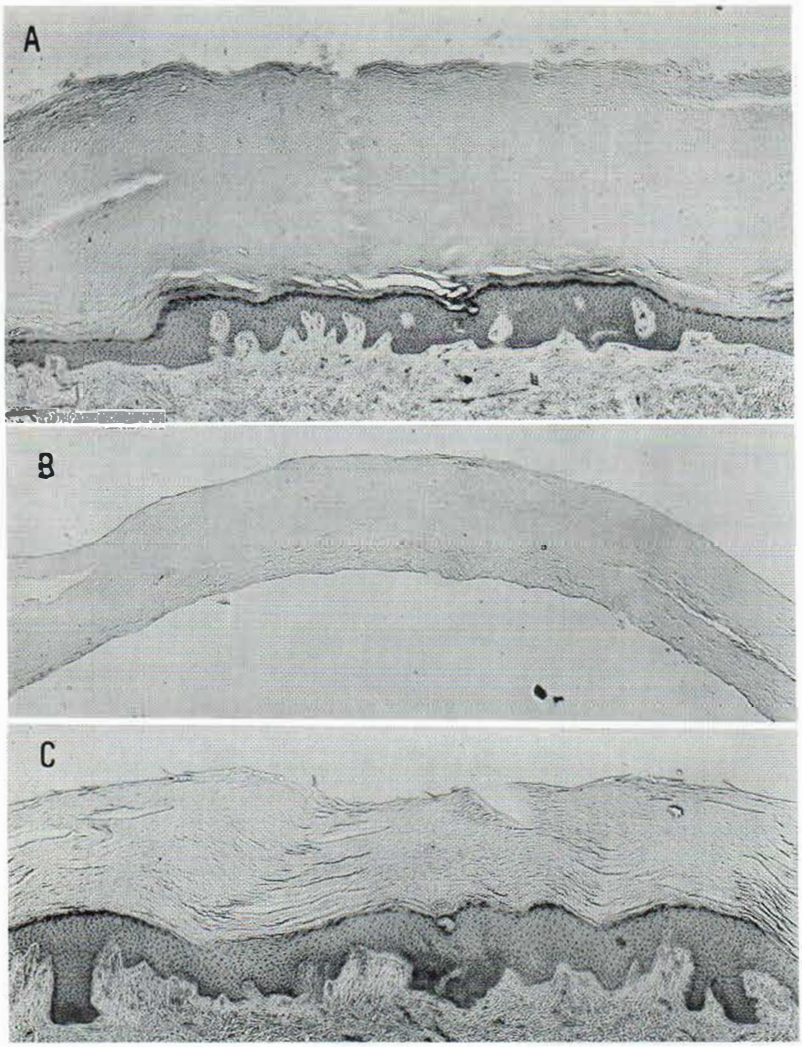

Fig. 1. Histologic sections of human plantar skin. Tissue from adult human sole skin was fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (A) tissue prior to the treatment; (B) horny layer separated from the skin with a razor blade under a moderate tension; (C) residual skin. × 50.

the dermis (Fig. 1A). Fig. 1B represents horny layers which have been separated by the stretch method. Fig. 1C shows that after the procedure, the residual skin has a thick horny layer. This separation technique was chosen in order to obtain a specimen consisting of horny cells without contamination by granular or spinous cell. Adult human plantar skin is a suitable material for obtaining horny layers as its horny layer is usually so thick (around 3 mm thick) that a material consisting only of horny cells can be obtained with ease.

Extraction of the deoxycholate-soluble material

From the separation of horny layer, following homogenization and incubation in deoxycholate as described above, the protein could be extracted. As

Table 1. *Extraction of the matrix protein with either 3% deoxycholate or redistilled water at 4°C*

The values given are averages from double experiments. Around 44.3% of the protein was extractable into the 270 000 *g* supernatant fraction with deoxycholate

	DOC-Tris[a] buffer		Redistilled water	
	μg of protein	%	μg of protein	%
270 000 *g* Supernatant fraction	72 580	44.3	32 800	19.4
270 000 *g* Pellet	45 000	27.5	1 300	0.8
15 000 *g* Pellet, 8 M alkaline, urea soluble	27 340	16.7	15 200	9.0
1 N NaOH soluble	18 750	11.5	120 000	70.9

[a] 50 mM Tris-HCl buffer, pH 8.6, containing 3% DOC.

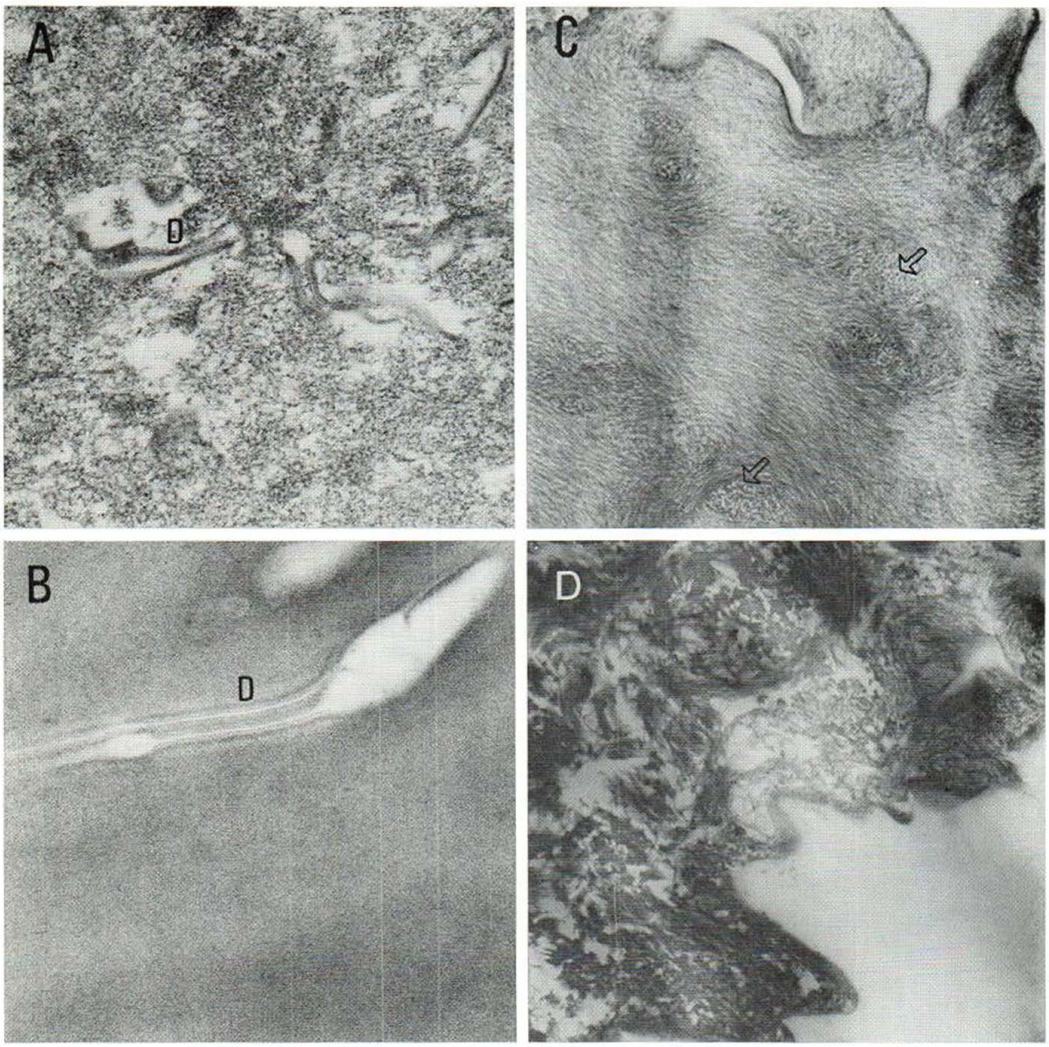

Fig. 2. Extraction of matrix protein with deoxycholate.(A) 270 000 *g* pellet; (B) control tissue following separation with a razor blade; (C, D) insoluble material which sedimented at 15 000 *g* following the three fold 48 hour extraction in deoxycholate (C) and in redistilled water (D). Arrow indicates an electron-dense tonofibril. *D:* desmosome. × 26 000 (A); × 23 000 (B); × 23 000 (C); × 23 000 (D).

is shown in Table I, around 44.3% of the total protein was released in the 270 000 *g* supernatant fraction following incubation with deoxycholate, but only 19.4% of the total protein was extracted in redistilled water and 80% of proteins remained unextracted. In this redistilled water-extractable fraction, only a few protein bands, which were of small molecular size, were found, as compared with the electrophoretic profile of the extract with DOC in disc gel electrophoresis in the presence of SDS (Fig. 3A, B).

The electron micrographs of sections from control tissue and from the 15 000 *g* pellet following three successive extractions for 48 hours are shown in Fig. 2B, C. The electron-dense background disappeared and many electron-dense fibrous structures (arrow) were revealed and remained unextracted (Fig. 2C). Fig. 2A demonstrates that the pellet fraction from such high-speed centrifugation contains only debris of fibrous materials and desmosome-like structures. No electron-dense amorphous material could be detected. The materials forming the elec-

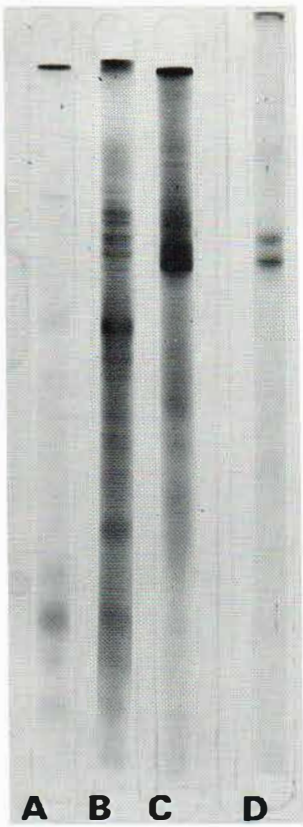

Fig. 3. Acrylamide gel electrophoretic profiles of the extracts from human plantar horny layers. The 270 000 *g* supernatant fractions, which were extracted with redistilled water (A), with deoxycholate (B), the aggregates obtained by dialysis (C), and the purified material obtained by molecular sieve chromatography (second peak in Fig. 8) (D) were analysed in the presence of sodium dodecylsulfate using acrylamide gel of 10% as described in Results and Discussion. (A) 160 μg, (B) 180 μg, (C) 150 μg, (D) 50 μg of protein.

tron-dense background of the horny cells (Fig. 2B) were removed to the 270 000 *g* supernatant fraction. Fig. 2D shows that the electron-dense material still remains and the fibrous structures are only partially revealed when redistilled water is used instead of Tris-deoxycholate buffer.

In addition to these biochemical, disc gel electrophoretic and electron-microscopic findings on the deoxycholate-soluble material, its histochemical characteristics were examined, in order to confirm that this extract was derived from the matrix proteins of the horny layer. The precipitate obtained by the dialysis against redistilled water for 72 hours (Fig. 4A-a) consisted of small, round, electron-dense particles and many, lined, fine particles (arrow) in the electron micrograph (Fig. 4B). These fine particles seem to aggregate and form electron-dense particles.

This precipitate was stained orange-yellow with Pauly's reagent, blue with toluidine blue, and basophilic with hematoxylin-eosin staining (Fig. 5B, D, F). The aggregates of deoxycholate-soluble material demonstrate the same tinctorial properties as those of both the horny layers and keratohyalin granules in vivo (Fig. 5A, C, E). These histochemical observations showed that these aggregates were a substance of keratohyalin granule origin and were contained in the horny layers, since the granular cell was not contained in the original material from which the deoxycholate-soluble material was extracted. Taking into consideration that the fibrous structures were not extractable with deoxycholate (see the electron micrograph (Fig. 2C)) and tonofilaments could not be extracted with DOC, when keratohyalin granules of newborn rat were extracted with DOC (16), the deoxycholate-soluble material was the non-fibrous, matrix protein of the horny cells.

Molecular sieve chromatographical studies

Both the 270 000 *g* supernatant and the aggregate fraction were subjected to column chromatography on Sephadex G-200 in order to purify the deoxycholate-soluble material from the horny layer. The eluate was 50 mM Tris-HCl buffer (pH 8.6), containing 10 mM 2-mercaptoethanol without deoxycholate. These materials yielded an initial large peak of 280 nm absorbing material at the void volume, followed by a plateau, a small second peak, and a final peak which has been identified as deoxycholate (Fig. 6). The large initial peak was dialysed, lyophilized and rechromatographed on Sepharose 6B either with or without sodium deoxycholate. Without DOC, the initial large peak at the void volume was followed by a smaller peak and a large final peak which again represents deoxycholate (Fig. 7). When the deoxycholate-soluble material was chromatographed on a column not saturated with deoxycholate, the material formed aggregates which were eluted at the void volume.

This procedure separates the material from soluble protein of smaller molecular weight. The substances of very large molecular weight had already been removed by the ultracentrifugation. To ensure that the deoxycholate-soluble material was aggregated and formed large molecules in the absence of sodium deoxycholate, the initial peak from the Sephadex G-200 column was subjected to Sepharose 6B column

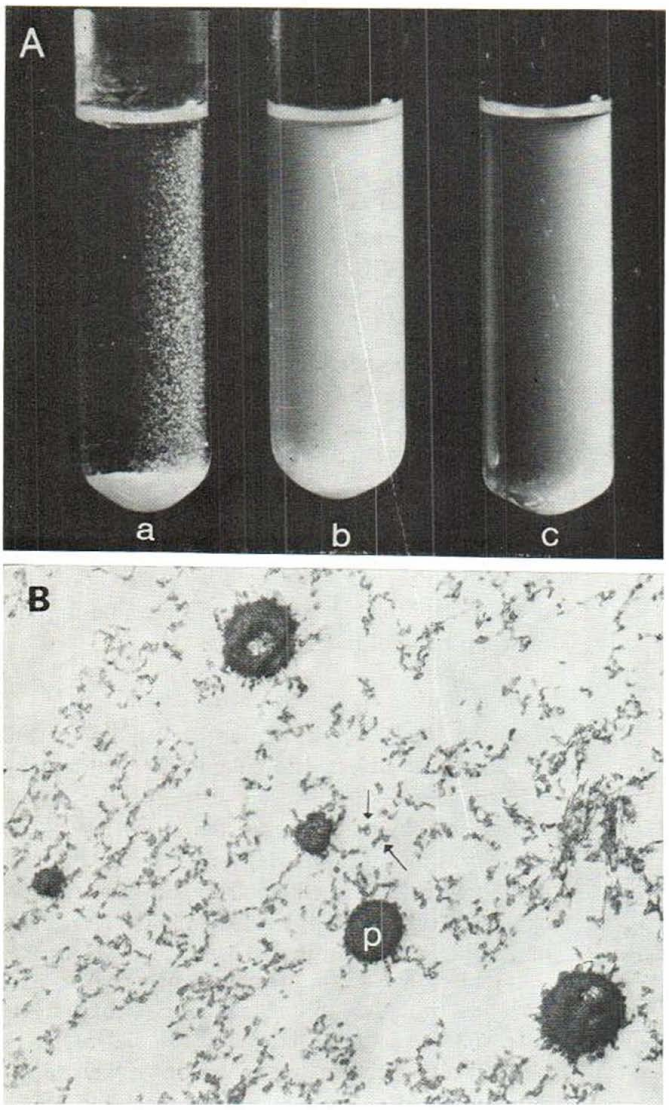

Fig. 4. (A) Aggregates were obtained by dialysing the 270 000 *g* supernatant fraction against redistilled water for 24 hours (*c*), 48 hours (*b*) and 72 hours (*a*). (B) Aggregates consist of round, electron-dense particles (*p*) and lined, fine particles.

chromatography in the presence of 0.5% deoxycholate, and three peaks were obtained (Fig. 8).

Electrophoretic studies

When 50 μg of the initial peak from the Sepharose 6B column (Fig. 7) was subjected to electrophoresis using a 5% acrylamide gel, two faster migrating bands were observed (Fig. 9A). When the material of the second peak from the Sepharose 6B column in the presence of 0.5% sodium deoxycholate was run at the same time, two bands were also seen migrating in both 5 and 10% gels (Figs. 9B and 3D).

Immunological studies

The material of the initial peak from the Sepharose 6B column was antigenic in rabbits, eliciting a precipitating antibody which formed a single, sharp line of recipitation in agar when assayed against the materials of the initial peak from the Sepharose 6B column and of the initial peak and the first half of the plateau region from the Sephadex G-200 column, which proved to be immunologically identical (Fig. 10A). A broad precipitin line also occurred, which was identical to the precipitin line occurring between the antigen-antibody described as above, when the material of the second peak on Sepharose 6B

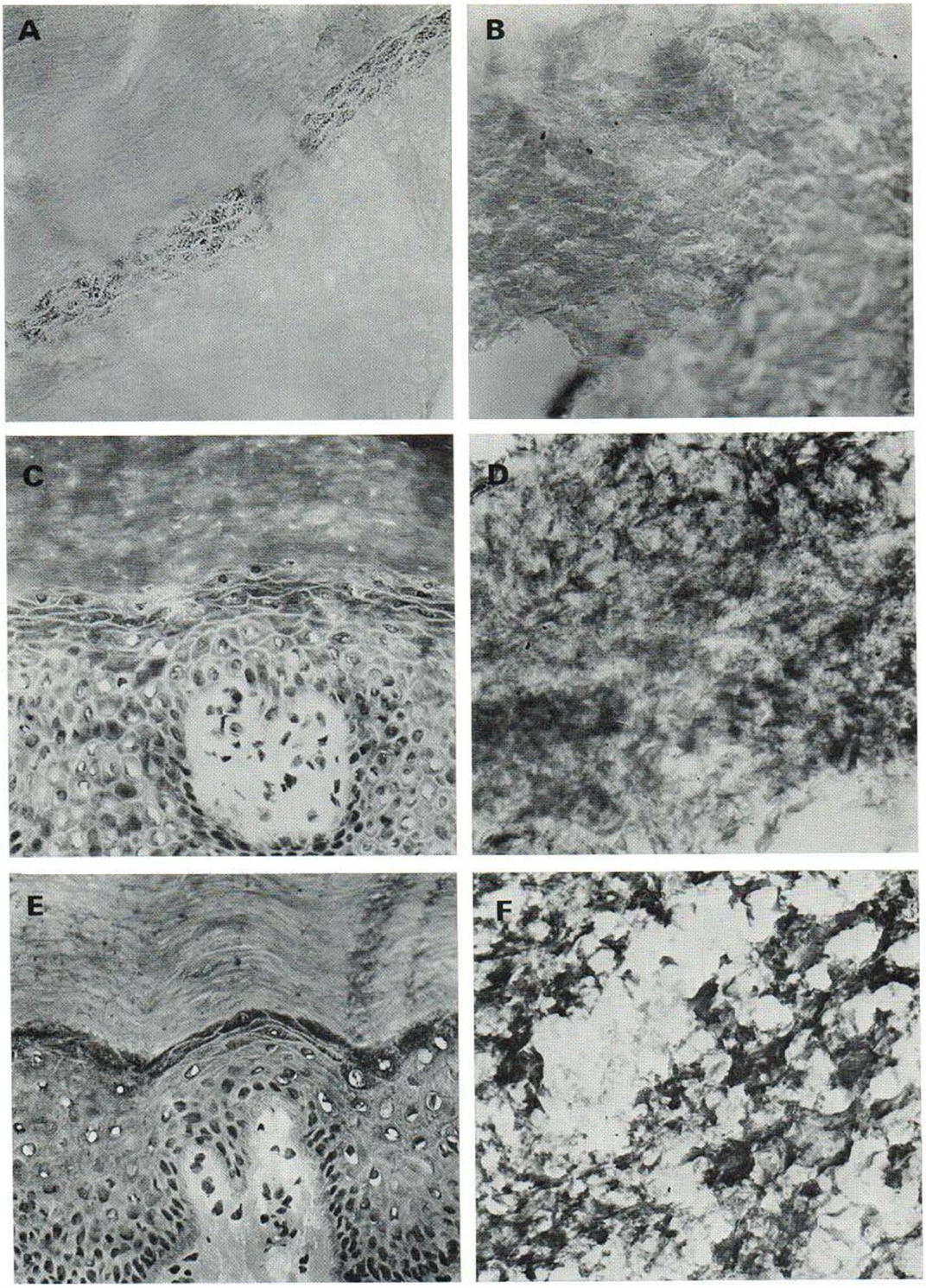

Fig. 5. Aggregates were stained with Pauly's reagent (B), toluidine-blue (D), and hematoxylin-eosin (F), showing the same tinctorial characteristics as keratohyalin granules *in vivo* (A, C, and E).

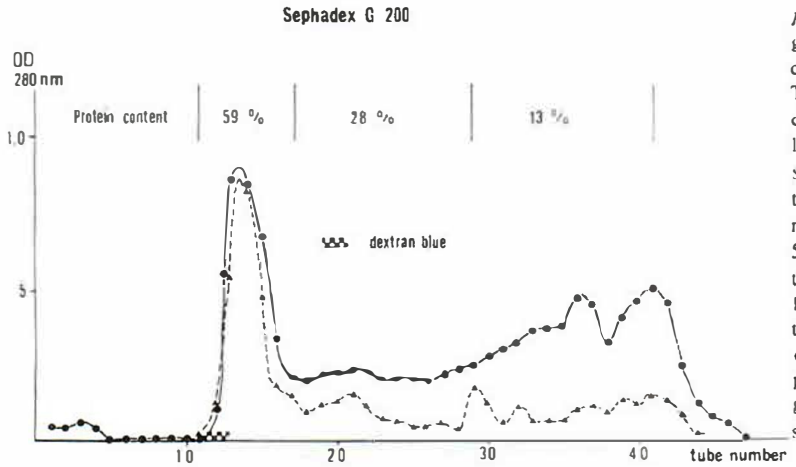

Fig. 6. Molecular sieve chromatography (Sephadex G-200) of the deoxycholate-soluble supernatant fraction. The soluble extract following deoxycholate extraction was dialysed, lyophilized and resolubilized as described in Results and Discussion. Elution from the Sephadex column was monitored at 280 nm and the eluate was 50 mM Tris-HCl buffer (pH 8.6), containing 10 mM 2-mercaptoethanol. Protein content was assayed according to the method of Lowry et al. (11). ●—●, the 270000 *g* concentrated supernatant fraction; ▲--▲, the aggregates obtained by dialysing the high-speed supernatant fraction.

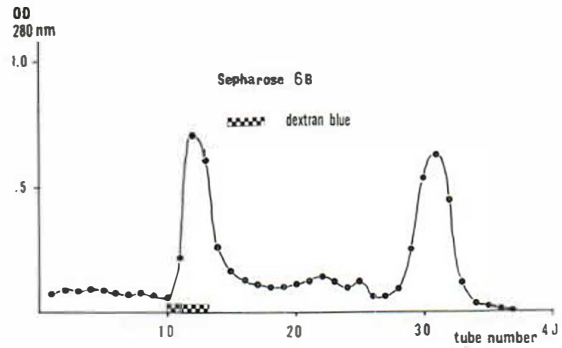

Fig. 7

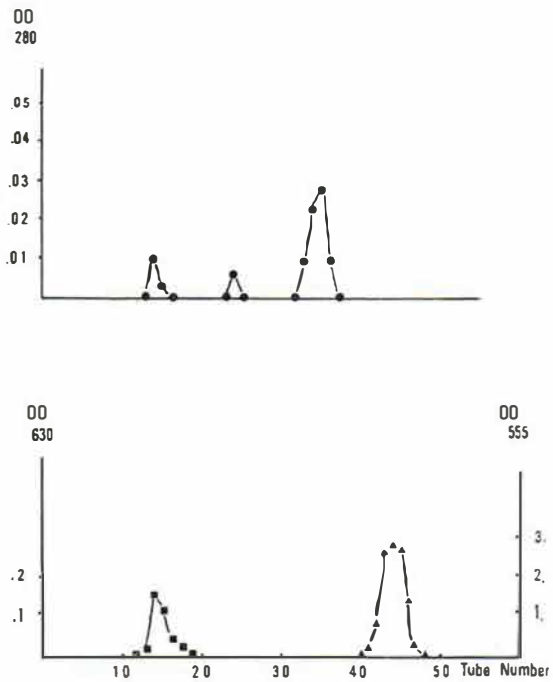

Fig. 8

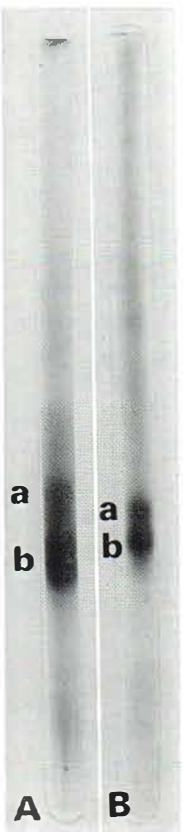

Fig. 9

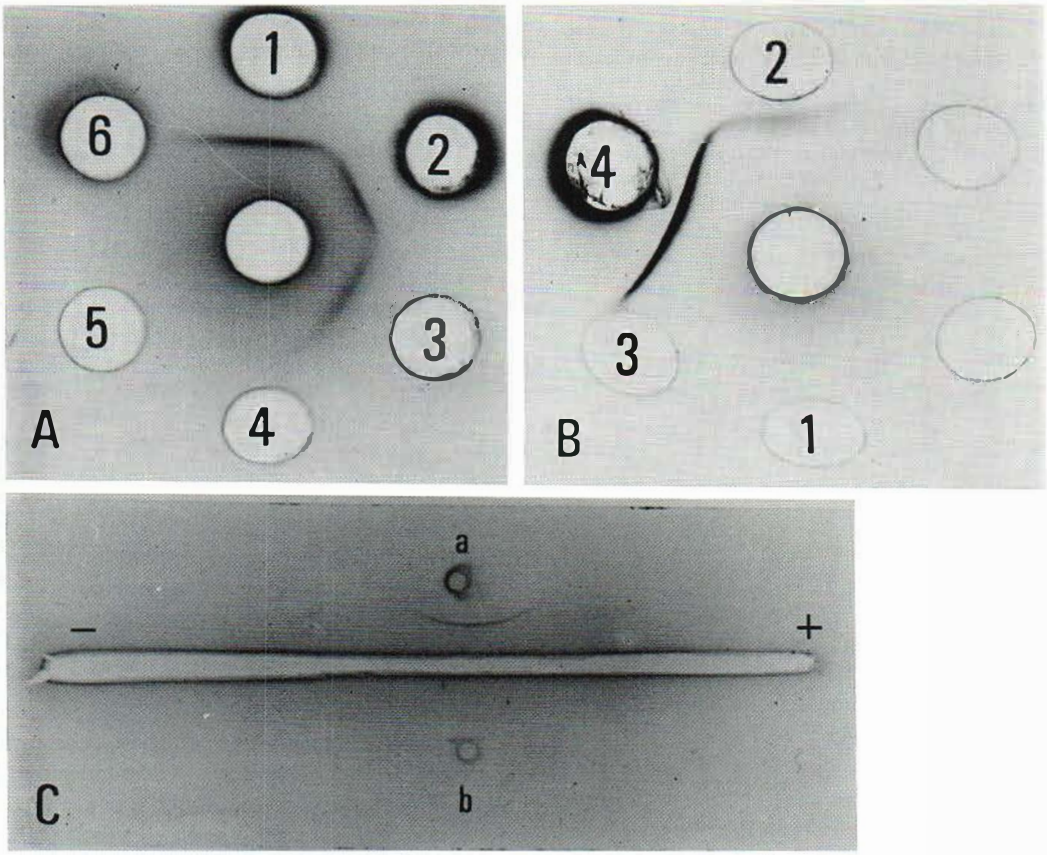

Fig. 10. (A) Analysis by double diffusion in agar of antibody produced to the human matrix protein extract. The center well contains rabbit antiserum raised to the protein of the initial peak from the Sepharose 6B column. The peripheral wells contain the fraction which was used for producing antibody (*1*), the initial peak from the Sephadex G-200 column (*2*), the first half (*3*) and second half (*4*) of the plateau region, the second peak from this same column (*5*), and human serum (*6*). (B) Analysis by double diffusion in agar of the protein of three peaks obtained from the column chromatography on Sepharose 6B in the presence of sodium deoxycholate. The center well contains rabbit antiserum raised to the protein of the initial peak from the Sepharose 6B column. The peripheral wells contain the material of the initial (*1*), second (*2*), and third (*3*) peaks from the Sepharose 6B column in the presence of 0.5% DOC. An identical precipitin line occurred between no. 2 well and the center well. (C) Immunoelectrophoresis was performed as described in Experimental Procedures. Material of the initial peak from the Sepharose 6B column in well (*a*) and human serum in well (*b*).

Fig. 7. Molecular sieve chromatography (Sepharose 6B) of the deoxycholate-soluble supernatant fraction. The dialysed, lyophilized, initial peak fraction from the Sephadex G-200 column was resolubilized with 3% deoxycholate and rechromatographed on Sepharose 6B as described in Results and Discussion. The eluate was the same as in Fig. 6. ——, Absorbance at 280 nm.

Fig. 8. Molecular sieve chromatography (Sepharose 6B) of the deoxycholate-soluble supernatant fraction. The dialysed, lyophilized and resolubilized initial peak fraction from the Sephadex G-200 column was rechromatographed on Sepharose 6B in the presence of 0.5% sodium deoxycholate. Optical density at 280 nm (●—●), at 630 nm (dextran blue) (■—■), and at 555 nm (phenol red) (▲—▲).

Fig. 9. Acrylamide gel electrophoretic patterns of the deoxycholate-soluble material. The material of the initial peak fraction from the Sepharose 6B column chromatography in the absence of DOC (A), and that of the second peak in the presence of 0.5% DOC (B) were analysed in the presence of sodium dodecylsulfate using acrylamide gels of 5%, as described in Results and Discussion.

Table II. *Amino acid analysis*

The initial peak from the Sepharose 6B column was subjected to amino acid analysis using the techniques described in the text. Results are expressed as residues/100 residues. Hydrolysis was performed for 18 hours
A, human plantar interfilamentous matrix; B, rat keratohyalin granules

	A	B		A	B
Lys	5.5	5.5	Ala	6.7	10.1
His	1.2	3.3	Val	3.2	4.6
Arg	5.9	8.2	Met	1.8	1.0
Asp	10.1	7.5	Ile	3.3	3.8
Thr	4.4	5.4	Leu	7.6	7.5
Ser	9.2	9.3	Tyr	4.1	2.4
Glu	15.2	14.5	Phe	2.7	2.9
Pro	([a])	3.8	Cys		1.6
Gly	19.1	10.6			

[a] Very small amount.

column chromatography in the presence of deoxycholate was used (Fig. 10B). The material, dissolved in 8 M alkaline urea, which was antigenic in rabbits, was considered to be immunologically homogeneous since a single precipitin line was seen when the immunoelectrophoresis was done (Fig. 10C), though heterogeneous on disc gel electrophoresis in the presence of sodium dodecylsulfate (Fig. 9A).

Chemical analysis

The purified material contained a small amount of hexose (14 μg per 100 μg of protein). The orcinol reaction was negative when an aliquot of eluate containing about 500 μg of protein was analysed for RNA.

In Table II are displayed the results of amino acid analyses performed on the material of the initial peak from the Sepharose 6B column without DOC, and of the keratohyalin fraction of newborn rat epidermis, for contrast. The most common amino acid residues in the material extracted with deoxycholate from human plantar horny layers are glycine and glutamic and aspartic acid. The leucine and histidine residues are very sparse and amino acid analysis of the material of the second peak from the Sepharose 6B column chromatography in the presence of DOC is under investigation in order to establish whether the further purified fraction might contain a larger histidine residue. Cystine content in this material is also under investigation. The reason why its histidine content was low, is that histidine-rich peptide was dissociated during the molecular sieve chromatography, since the material of the second peak from Sepharose 6B column in presence of DOC was negative with Pauly' stain, though the original macroaggregates stained positive (19). During the fractionation of the keratohyalin fraction obtained from the newborn rat epidermis, the histidine-rich fraction was dissociated and recovered in the slowly eluting fraction (18). A similar slowly eluting protein fraction was also found in the deoxycholate-soluble fraction from human plantar horny layers and its disc gel electrophoretic profile is similar to that of histidine-rich proteins of rat (19).

Immunofluorescent studies

The in vivo origin of the material extracted with deoxycholate from human plantar horny layers was established definitively by the immunofluorescent studies using the indirect technique detailed in Fig. 11. Specific fluorescence was apparent over keratohyalin granules, but no specific fluorescence was observed in control specimens. While a light area was observed on the horny layers (Fig. 11C), it does not represent fluorescence. As antigenic substance was extracted from the horny cells, it might be expected that diffuse fluorescence would occur on the horny layers, but no such observation was made. The reason why no fluorescence was observed on the horny layers is still unknown. However, since antibody was raised against the matrix protein of the horny layers, though not against keratohyalin granules, and as there was no contamination by granular cells in the material from which the antigenic substance had been extracted, it may well be that the inclusion of the material of keratohyalin origin into keratin fibers might cause the change of its tertiary structures, thus leading to a loss of the antigenecity of the keratohyalin material. The antigenicity could be regained by the isolation from keratin fibers with deoxycholate extraction. It is known that the matrix substance coats keratin fibers to yield the so-called "keratin pattern" in the electron micrographs. The material which was used to produce antibody, was treated at 4°C and never mixed with SDS. Thus, the possibility that this fluorescence could be a function of the fact that the isolated protein has been artifactually altered, can be excluded.

It can be concluded that the material which was extracted with deoxycholate and partially purified by the molecular sieve chromatography, is of keratohyalin granule origin.

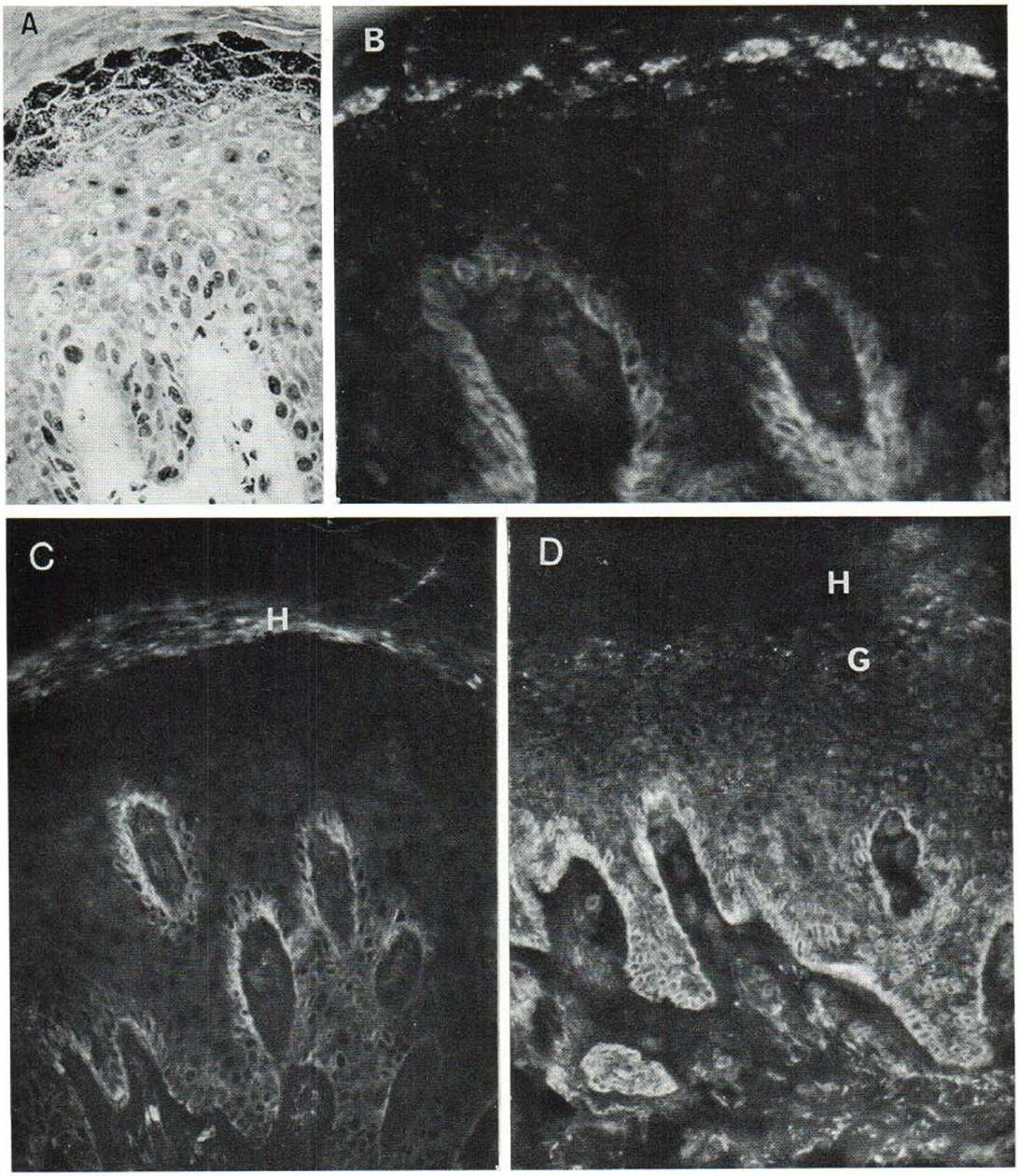

Fig. 11. Analyses by indirect immunofluorescence of antibody produced to the matrix protein extract. (A) Histologic section of human plantar skin stained with Harris hematoxylin-eosin. (B) The same tissue fixed in 95% ethanol and incubated with anti-matrix protein serum following the incubation with goat-anti-rabbit γ-globulin which was conjugated with fluorescein isothiocyanate, (C) with saline, and (D) with control rabbit serum. *G:* granular cell layer; *H:* horny cell layer. (A) × 300; (B) × 400; (C and D) × 300.

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T. Tezuka, M.D.
Department of Dermatology
School of Medicine
Tokyo Medical and Dental University
1-5-45, Yushima
Bunkyo-ku
Tokyo
Japan