STUDIES ON ISOLATED AGGREGATING OLIGORIBONUCLEOPROTEINS OF THE EPIDERMIS WITH HISTOCHEMICAL AND MORPHOLOGICAL CHARACTERISTICS OF KERATOHYALIN

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ABSTRACT

Histochemical and ultrastructural studies demonstrate that keratohyalin can be mobilized from fresh specimens of cattle hoof epidermis by 1.0 M potassium phosphate buffer (pH 7.0). Macroaggregates with histochemical characteristics identical to those of *in situ* keratohyalin granules (staining by Harris' hematoxylin, Congo red, diazotized sulfanilic acid, sodium alizarin sulfonate, toluidine blue, methyl green–pyronin, and acridine orange) and with similar morphological characteristics at the ultrastructural level are formed upon dialyzing the extracted keratohyalin against distilled water. Staining by basic dyes (toluidine blue, methyl green–pyronin, and acridine orange) is abolished by treating either *in situ* keratohyalin granules or isolated macroaggregates with ribonuclease. Electrophoresis of isolated macroaggregates on polyacrylamide gels in the presence of sodium decylsulfate results in the fractionation of a 13 member oligomeric series of ribonucleoproteins and two nonhomologous species of ribonucleoproteins. The oligomeric series can be purified by isolating "stacked" oligomers on low concentration (3%) polyacrylamide gels. Fractionated oligomers on polyacrylamide gels and aggregates formed from purified ribonucleoproteins demonstrate histochemical characteristics identical to those of *in situ* keratohyalin granules. Aggregates formed from denatured ribonucleoproteins are highly disordered and are markedly different from *in situ* keratohyalin granules or nondenatured isolated macroaggregates at the ultrastructural level, possibly due to irreversible denaturation of the oligomers by sodium decylsulfate.

INTRODUCTION

The stratum granulosum of the epidermis is a transitional area in which living epidermal cells undergo progressive and functional death, resulting in the formation of the outermost layer of skin, the stratum corneum. The unique morphological marker for which the stratum granulosum is named and which defines the boundaries of this transitional area is the keratohyalin granule (4, 10, 27, 37).

Previous studies have demonstrated that keratohyalin can be extracted from epidermis by potassium phosphate buffer (pH 7.0) as a function of molarity, indicating ranges of solubility of keratohyalin (45). If the extracted material is dialyzed against distilled water, it aggregates to form granules that are similar to keratohyalin granules in size, shape, histochemical staining, and ultrastructure (46). The morphological and physical-

chemical data in the present study suggest that the isolated material is an aggregating ribonucleoprotein.

MATERIALS AND METHODS

Isolation of Macroaggregates from 1.0 M Potassium Phosphate Buffer (pH 7.0) Extracts

Epidermis was obtained from the hairless area of skin on the posterior aspect of adult cream-colored cattle hooves at the time of slaughter and maintained at 4°C. Specimens were dissected as previously described, retaining only that epidermis located beneath the stratum corneum (48). 1 g (wet weight) of dissected tissue was minced with scissors, then extracted in 10.0 ml of 1.0 M potassium phosphate buffer (pH 7.0) for 15 min at 37°C with gentle stirring. The suspension was decanted and centrifuged twice at 75,000 *g* for 30 min at 4°C. The clear supernatant was dialyzed[1] against 32 volumes of distilled water for 24 hr at 4°C. The resultant turbid dialyzate contained macroaggregates which were collected on porous membranes (type HA Millipore filter, 0.45 μ pore size, 13 mm diameter, placed in a Swinnex filter holder [Millipore Corp., Bedford, Mass.]), using 1 ml portions of turbid dialyzate for each membrane preparation.

Preparation of Nonextracted and Extracted Tissue and Macroaggregates for Light Microscopy

Nonextracted and extracted tissue and porous membranes coated with macroaggregates were rinsed in distilled water for 2 min, fixed in 80% methanol or Carnoy's fluid (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 4 hr, embedded in paraffin, and sectioned at 6 μ. Porous membranes without a coating of aggregates were prepared simultaneously as controls. Sections of tissue (nonextracted and extracted) and porous membranes (noncoated and coated with macroaggregates) were stained with toluidine blue (18), Langeron's sodium alizarin sulfonate (23), diazotized sulfanilic acid (36), acridine orange (1), the Feulgen reaction (29), the periodic acid–Schiff reaction (29), and the dihydroxydinaphthyl disulfide method for sulfhydryl and disulfide groups (29). Other sections were stained with Harris' hematoxylin for 5 min, rinsed in distilled water for 2 min, and destained in tap water for 5 min. Additional sections were stained in a 1% aqueous solution of Congo red for 60 min, followed by destaining in 1% aqueous potassium iodide for 1 min and further destaining in 70% ethanol for 1 min.

For methyl green–pyronin staining, the following modification of Trevan and Sharrock's (44) method was employed. 1 g of pyronin Y and 3.0 g of methyl green were dissolved in a solution of 230 ml of 0.2 M disodium phosphate plus 170 ml of 0.1 M citric acid. The final pH was adjusted to 5.3 by the addition of 0.2 M disodium phosphate or 0.1 M citric acid. Sections were stained in the methyl green–pyronin solution for 30 sec and then rinsed in two changes of tap water (approximately 5 sec per each rinse). Sections were differentiated in 95% ethanol, dehydrated in 100% ethanol, cleared in xylene, and mounted.

Counterstains were not employed with any of the histochemical techniques. Specimens fixed in Carnoy's fluid were used for methyl green–pyronin, acridine orange, and Feulgen staining; all other staining was performed on methanol-fixed specimens. Sections stained with acridine orange were examined in ultraviolet light (440–530 mμ).

Preparation of Specimens for Ribonuclease Digestion

Sections of hoof epidermis, fixed in Carnoy's fluid or 80% methanol, were incubated in ribonuclease (salt free, 5 × recrystallized bovine pancreatic ribonuclease, obtained from Mann Research Labs. Inc., New York), 1.0 mg per ml in 0.005 M potassium phosphate buffer (pH 7.0) for 30 min at 37°C. Control sections were incubated in buffer without ribonuclease. After incubation, sections were rinsed with distilled water and stained with methyl green–pyronin or acridine orange using Carnoy's-fixed sections. Additional methanol-fixed sections were stained with toluidine blue, Harris' hematoxylin, Congo red, sodium alizarin sulfonate, and diazotized sulfanilic acid as described.

Porous membranes coated with macroaggregates were prepared as described. Whole unfixed membranes were incubated in ribonuclease or buffer as described above. After incubation, membranes were rinsed in distilled water, fixed in Carnoy's fluid or 80% methanol for 4 hr, embedded in paraffin, sectioned, and stained as described.

Preparation of Nonextracted and Extracted Tissue and Macroaggregates for Electron Microscopy

For electron microscopy, specimens of tissue (nonextracted and extracted) and porous membranes

[1] The dialysis tubing (1.7 inches wide, obtained from Union Carbide, New York) was boiled in 10% Na_2CO_3 until no yellow material appeared in the wash, then rinsed in distilled water to neutrality, stored in 0.01 M ethylenediaminetetraacetate (EDTA) at pH 7.0, then washed with distilled water prior to use.

(noncoated and coated with macroaggregates) were fixed in 6% glutaraldehyde buffered with 0.1 M phosphate buffer and embedded in Epon (46). Membrane preparations were cut into strips approximately 3 mm wide prior to fixation, and xylene was substituted for propylene oxide in the embedding technique (6). Thick sections were stained with methylene blue and Azur II, and thin sections were stained with uranyl acetate and lead citrate as previously described (46). Specimens were examined in a Siemens electron microscope at 80 kv.

Preparation of Crude Oligomers

Macroaggregates were prepared by dialyzing 1.0 M potassium phosphate buffer (pH 7.0) extracts of hoof epidermis against 32 volumes of distilled water as described above. All following procedures were performed at 4°C unless otherwise noted. Macroaggregates were harvested by centrifugation of the turbid dialyzate at 50,000 *g* for 30 min. The pellet was washed three times in distilled water with the aid of a glass rod, using 1.3 ml of distilled water per gram of initial minced epidermis, and collected by centrifugation at 50,000 *g* for 30 min. Washed pellets were solubilized in sodium decylsulfate (NaDS) (Eastman Organic Chemicals, Rochester, N.Y.), using 480 μl of 0.10 M NaDS per gram of initial minced epidermis. Solubilization was aided by homogenization for 1 min at 2000 rpm in a Potter-Elvejhem Teflon-glass homogenizer. Suspensions were incubated with stirring for 3 hr at 37°C, then dialyzed against 42 volumes of 0.02 M NaDS for 12 hr. Dialyzates were cleared by centrifugation at 75,000 *g* for 30 min. This cleared dialyzate was designated crude oligomers. Protein was determined by the method of Lowry (24) using crystalline bovine albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard, and ribonucleic acid was determined by the orcinol reaction (2) using adenosine monophosphate dihydrate as a standard (Mann Research Labs.). Spectra were determined on a Beckman DK-2A ratio recording spectrophotometer.

Purification of Crude Oligomers

300–500 μg of crude oligomers (in 0.02 M NaDS, 10% sucrose) were fractionated on 3% T/3% C[2] polyacrylamide gels as previously described, using system A (38, 47) containing 0.02 M NaDS in the upper buffer. Electrophoresis was conducted at 2 ma per tube at 25°C and terminated when the marking dye (bromphenol blue) approached gel bottom. Stacked oligomers were localized on one gel by precipitation with 7.5% acetic acid, and corresponding areas on nonacid treated gels were excised and extracted for 24 hr in 0.02 M NaDS, using 250 μl per gel slice. Extracts were decanted then centrifuged twice at 75,000 *g* for 30 min (to remove gel particles), then further cleared by passage through porous membranes (Millipore filter, type HA, 0.45 μ pore size, 13 mm diameter). Cleared filtrates were dialyzed against 1000 volumes of 0.02 M NaDS for 12 hr to remove marking dye and small molecular weight contaminants (glycine, Tris). This dialyzed filtrate was designated purified oligomers.

[2] % T, total gel concentration = % acrylamide + % bisacrylamide (w/v); % C, crosslinking = bisacrylamide/T × 100.

Electrophoresis of Crude and Purified Oligomers

100-μg samples of crude or purified oligomers (in 0.02 M NaDS, 10% sucrose) were fractionated on 9% T/3% C or 3% T/3% C polyacrylamide gels. Gels were stained overnight in 0.1% amido black in 7.5% acetic acid, then destained electrophoretically in 7.5% acetic acid.

Histochemical Staining of Fractionated Oligomers in Polyacrylamide Gels

Crude and purified oligomers (in 0.02 M NaDS, 10% sucrose) were fractionated on 3% T/3% C and 9% T/3% C polyacrylamide gels. Gels were removed from glass columns by reaming with water, placed in 7.5% acetic acid for 12 hr at 4°C, then rinsed in distilled water. Rinsed gels were placed in buffered formaldehyde (100 ml 40% formaldehyde, 20 g sodium acetate, 900 ml distilled water) for 12 hr, then rinsed again in distilled water. Fractionated oligomers on polyacrylamide gels were stained with (*a*) 100 ml of 0.002% solution of Congo red for 24 hr, and destained for 8 hr in distilled water; (*b*) 50.0 ml of a 0.002% solution of Harris' hematoxylin for 24 hr, and destained for 8 hr in distilled water; (*c*) 10.0 ml of a 1% solution of sodium alizarin sulfonate for 24 hr and destained for 24 hr in distilled water; (*d*) 10.0 ml of freshly prepared diazotized sulfanilic acid (36); (*e*) 10.0 ml of a 0.005% solution of toluidine blue for 3 hr then destained for 12 hr in distilled water (using nonacid or formaldehyde-fixed gels); (*f*) 10.0 ml of a solution of pyronin for 24 hr and destained in 200 ml of distilled water for 24 hr (prepared by mixing 1.0 g of pyronin in 230 ml of 0.2 M Na_2HPO_4 plus 170 ml 0.1 M citric acid and adjusting the pH to 5.3, then filtering through No. 5 Whatman filter paper); (*g*) 10.0 of a solution of methyl green for 24 hr and destained in 200 ml of distilled water for 24 hr (prepared as for pyronin but using 3.0 g of methyl green).

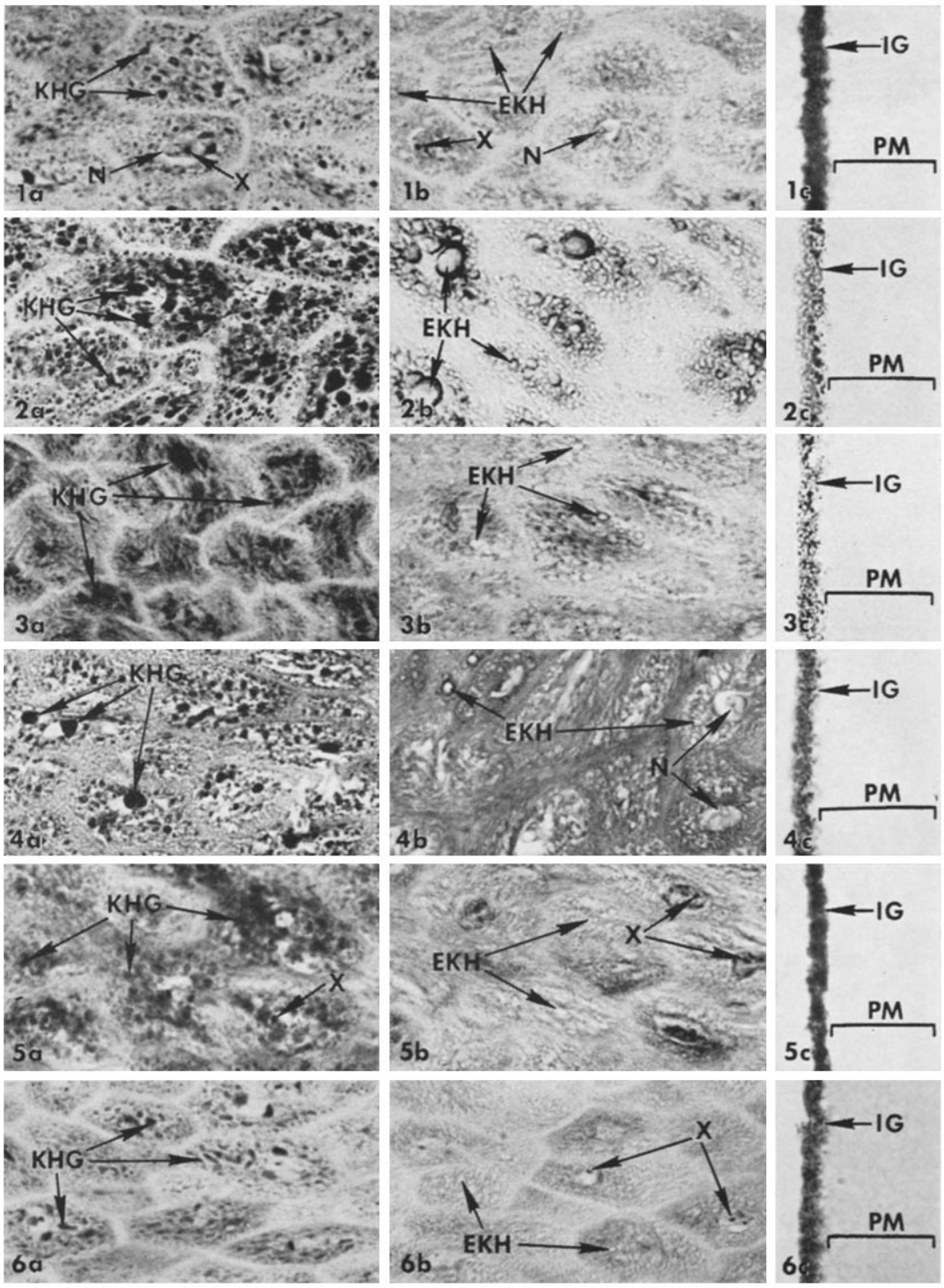
Preparation of Aggregates from Crude and Purified Oligomers

Crude and purified oligomers in 0.02 M NaDS were aggregated by dialysis against 1000 volumes of dis-

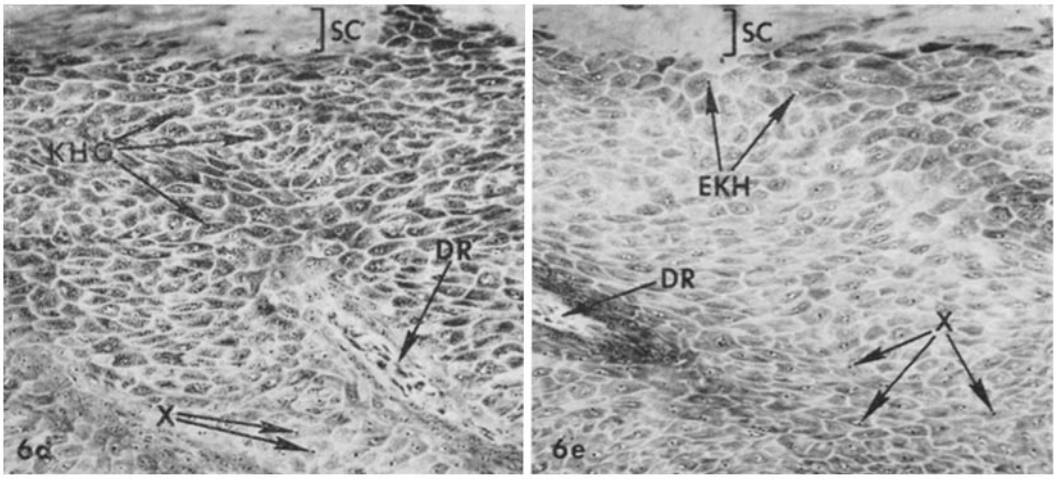
TABLE I

Histochemical Characteristics of In Situ Keratohyalin Granules, Isolated Macroaggregates, Sodium Decylsulfate–Denatured Aggregates, and Fractionated Oligomers on Polyacrylamide Gels

	In situ keratohyalin granules			Isolated macroaggregates		NaDS-denatured aggregates	Fractionated oligomers
(1) Stain	(2) Nonextracted tissue	(3) Extracted tissue	(4) RNAse-treated tissue	(5) Non-RNAse treated	(6) RNAse treated	(7)	(8)
Harris' hematoxylin	Deep blue	Minimal	Deep blue	Deep blue	Deep blue	Deep blue	Deep blue
Congo red	Orange-red	Minimal	Orange-red	Orange-red	Orange-red	Orange-red	Orange-red
Diazotized sulfanilic acid	Orange	Minimal	Orange	Orange	Orange	Orange	Orange
Sodium alizarin sulfonate	Red	Minimal	Red	Red	Red	Red	Red
Toluidine blue	Purple	Minimal	Negative	Purple	Negative	Purple	Purple
Methyl green–pyronin	Red	Minimal	Negative	Red	Negative	Red	Red
Acridine orange	Orange-red	Minimal	Negative	Orange-red	Negative	Orange-red	



FIGURES 1–6 Paraffin-embedded specimens of (Figs. 1 *a*–6 *a*) nonextracted tissue demonstrating numerous *in situ* keratohyalin granules; (Figs. 1 *b*–6 *b*) tissue extracted in 1.0 M potassium phosphate buffer (pH 7.0) demonstrating extraction of keratohyalin; (Figs. 1 *c*–6 *c*) isolated macroaggregates on surface of porous membranes. Figs. 1–6 are stained with Harris' hematoxylin, Congo red, diazotized sulfanilic acid, sodium alizarin sulfonate, toluidine blue, and methyl green–pyronin, respectively. *KHG*, keratohyalin granule; *N*, nucleus; *X*, nucleolus; *EKH*, area in which keratohyalin has been extracted; *IG*, isolated macroaggregates; *PM*, porous membrane. × 640.



FIGURES 6 *d* and 6 *e* Paraffin-embedded specimens of (*d*) nonextracted tissue; (*e*) tissue extracted in 1.0 M potassium phosphate buffer (pH 7.0). In nonextracted tissue, keratohyalin and nucleoli are stained, and a few cells reveal the presence of both keratohyalin granules and nucleoli. In extracted tissue, nucleoli are present, but the majority of keratohyalin has been extracted. The relative absence of nucleoli in the upper regions of the stratum granulosum is due to nuclear decay in this region. *KHG*, keratohyalin granule; *X*, nucleolus; *DR*, dermal rete; *SC*, stratum corneum; *EKH*, area in which keratohyalin has been extracted. Methyl green–pyronin. × 100.

tilled water for 24 hr. Aggregates were collected on porous membranes (type HA Millipore filter, 0.45 μ pore size, 13 mm diameter), then prepared for light and electron microscopy as described above.

RESULTS

Light Microscopy

Results are summarized in Table I.

In situ keratohyalin granules in nonextracted tissue and macroaggregates isolated from 1.0 M potassium phosphate buffer (pH 7.0) extracts were stained identically by Harris' hematoxylin, (Fig. 1 *a*, 1 *c*), Congo red (Fig. 2 *a*, 2 *c*), diazotized sulfanilic acid (Fig. 3 *a*, 3 *c*), sodium alizarin sulfonate (Fig. 4 *a*, 4 *c*), toluidine blue (Fig. 5 *a*, 5 *c*), methyl green–pyronin (Fig. 6 *a*, 6 *c*), and acridine orange. In extracted tissue varying amounts of residual keratohyalin were noted (Fig. 1 *b*–6 *b*), while nuclei and nucleoli continued to be stained (Figs. 1 *b*, 4 *b*, 5 *b*, 6 *b*, 6 *d*, 6 *e*). Epon-embedded specimens more clearly demonstrate the residual keratohyalin, usually located at the margins of extracted areas (Fig. 7 *b*), and that macroaggregates isolated from 1.0 M potassium phosphate buffer (pH 7.0) extracts are distinct particles which are similar to *in situ* keratohyalin granules in size and shape (Figs. 7 *a*, 7 *c*).

In situ keratohyalin granules and macroaggregates were not stained with the periodic acid–Schiff reaction, the Feulgen reaction, or by the dihydrodinaphthyl disulfide method.

Ribonuclease Studies

After ribonuclease digestion, *in situ* keratohyalin granules in nonextracted tissue and macroaggregates isolated from 1.0 M potassium phosphate buffer (pH 7.0) extracts were not stained with toluidine blue, methyl green–pyronin, or acridine orange, but continued to be stained with Harris' hematoxylin, Congo red, diazotized sulfanilic acid, and sodium alizarin sulfonate (Table I). Specimens incubated in buffer without ribonuclease were stained by all the above reagents.

Electron Microscopy

In situ keratohyalin granules vary in size (0.1–5.0μ) and shape (round to oval) and are composed of an electron-opaque substance which appears amorphous to finely granular. No limiting membrane surrounds the granule (Fig. 8). Certain granules are surrounded by numerous particles which range in size from 220 A to 290 A (Fig. 8). Those particles immediately adjacent to the margin of keratohyalin granules are difficult to

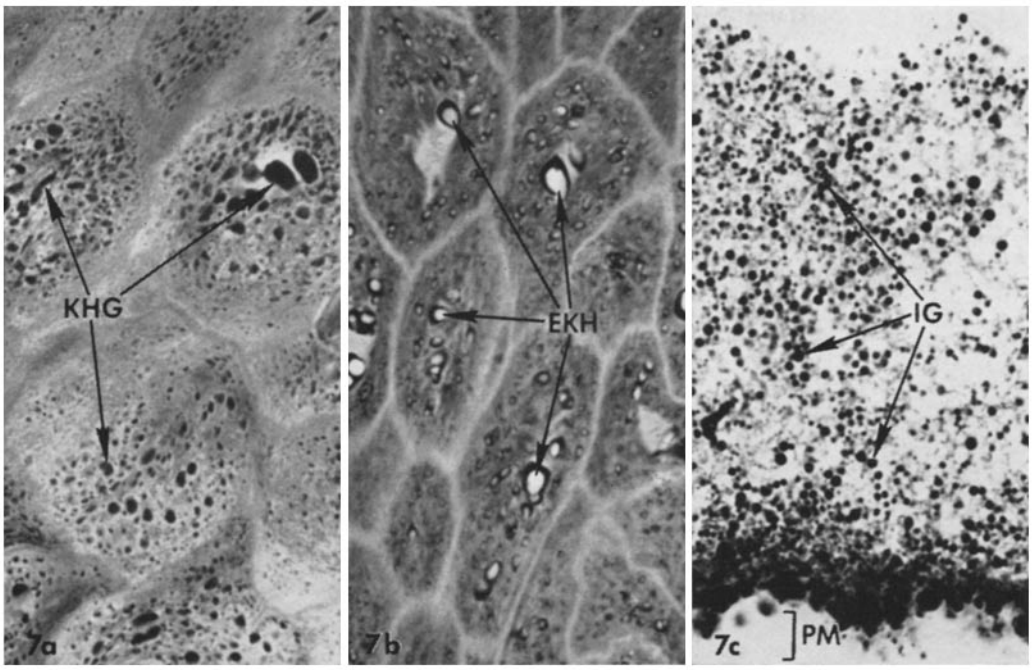


FIGURE 7 Epon-embedded specimens of (*a*) nonextracted tissue demonstrating numerous *in situ* keratohyalin granules (*KHG*); (*b*) tissue extracted in 1.0 M potassium phosphate buffer (pH 7.0) demonstrating extraction of keratohyalin (*EKH*) and residual rims of keratohyalin; (*c*) isolated macroaggregates (IG) on porous membranes (PM) demonstrating discrete nature of the macroaggregates and size and shape similar to *in situ* keratohyalin granules. Azur II and methylene blue. × 1000.

resolve, while those particles further removed from the margin are distinct (Fig. 8). Other keratohyalin granules are surrounded by tonofilaments and have only rare particles at their margins.

Keratohyalin in extracted tissue has undergone varying degrees of extraction, ranging from complete to minimal (Fig. 9–11). Where keratohyalin has been completely extracted a vacuole surrounded by tonofilaments is observed (Fig. 10), while sites which have been almost completely extracted are lined by a residual rim of keratohyalin (Fig. 9). Certain granules, which have been minimally extracted, reveal an area of complete extraction lined by densely staining keratohyalin. Further removed from the completely extracted area, partially extracted keratohyalin appears vacuolated and loosely packed (Fig. 11).

Macroaggregates isolated from 1.0 M potassium phosphate buffer (pH 7.0) extracts are similar to *in situ* keratohyalin granules in size and shape (Fig. 12). They are composed of an electron-opaque core which appears amorphous to finely granular (Fig. 13). This core is surrounded by a variable thickness of vacuolated, less densely stained material (Fig. 13). No limiting membrane is present.

Preparation of Crude and Purified Oligomers

Recovery of crude oligomer protein ranged from $6.88 \times 10^{-3}\%$ to $8.49 \times 10^{-3}\%$ of the starting wet weight of minced epidermis. Ribonucleic acid ranged from 16.1% to 18.0% of the crude oligomer protein. Recovery of purified oligomer protein ranged from 12.3% to 34.8% of the originally applied crude oligomers. Ribonucleic acid ranged from 6.9% to 10.7% of the purified oligomer protein. (mg orcinol RNA)/(mg Lowry protein) × 200%.[3]

Electrophoresis of Crude and Purified Oligomers

Fig. 14 demonstrates the fractionation of crude oligomers on a 9% T/3% C gel. Examination of

[3] A factor of 200% is used, since only purine-bound sugars react significantly (2).

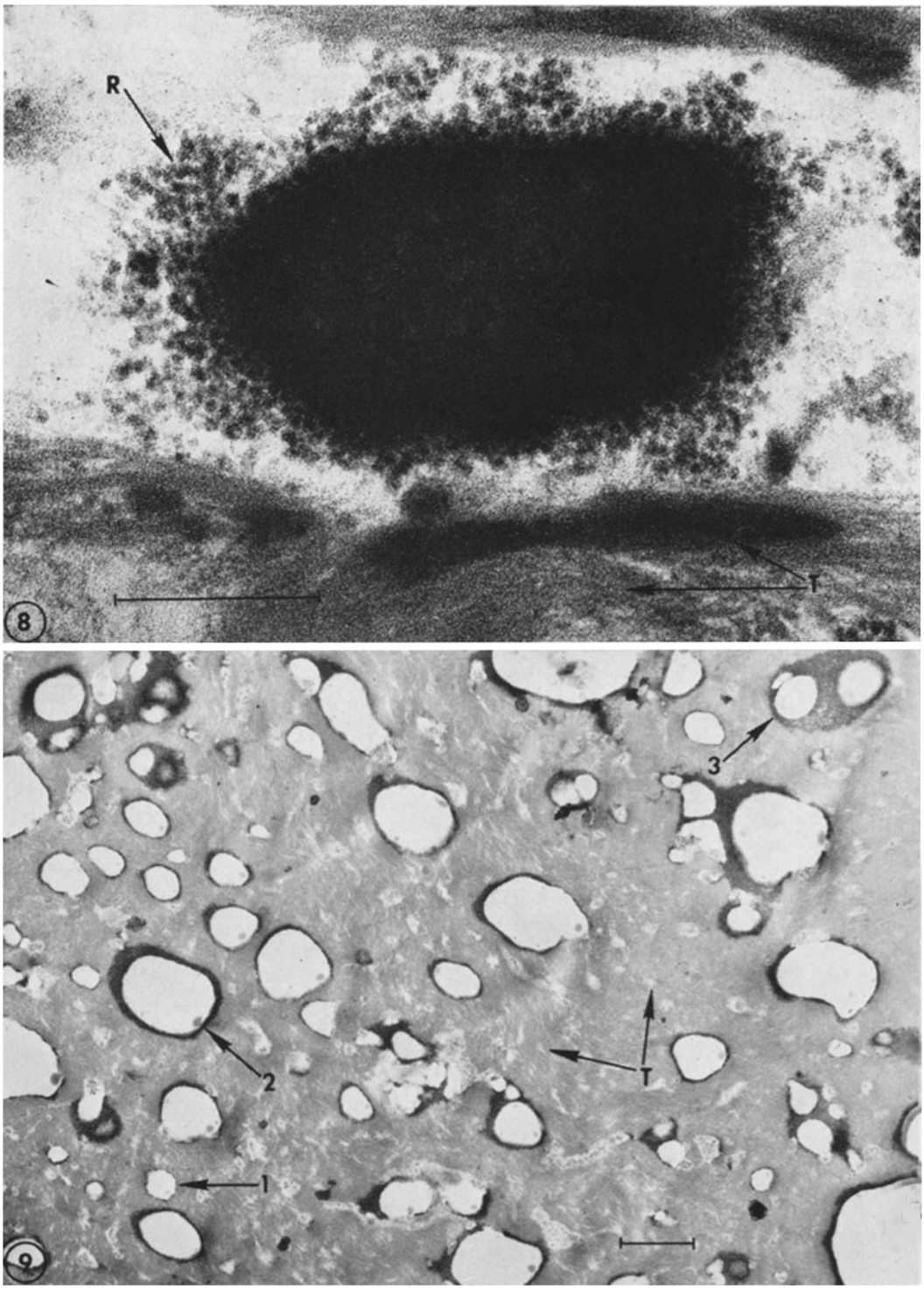


FIGURE 8 *In situ* keratohyalin granule in nonextracted tissue demonstrating small particles (220–290 A) surrounding certain granules and the amorphous nature of keratohyalin. Small particles are distinct at the extreme margin and indistinct at the edge of the keratohyalin granule. No limiting membrane is present. *R*, small particle; *T*, tonofilament. Uranyl acetate and lead citrate. Scale marker, 0.5 μ. × 46,000.

FIGURE 9 Epidermis extracted in 1.0 M potassium phosphate buffer (pH 7.0). Keratohyalin has undergone varying degrees of extraction ranging from complete to minimal. 1, area of complete extraction; 2, area demonstrating residual rim of keratohyalin; 3, area of minimal extraction; *T*, tonofilament. Uranyl acetate and lead citrate. Scale marker, 1 μ × 8000.

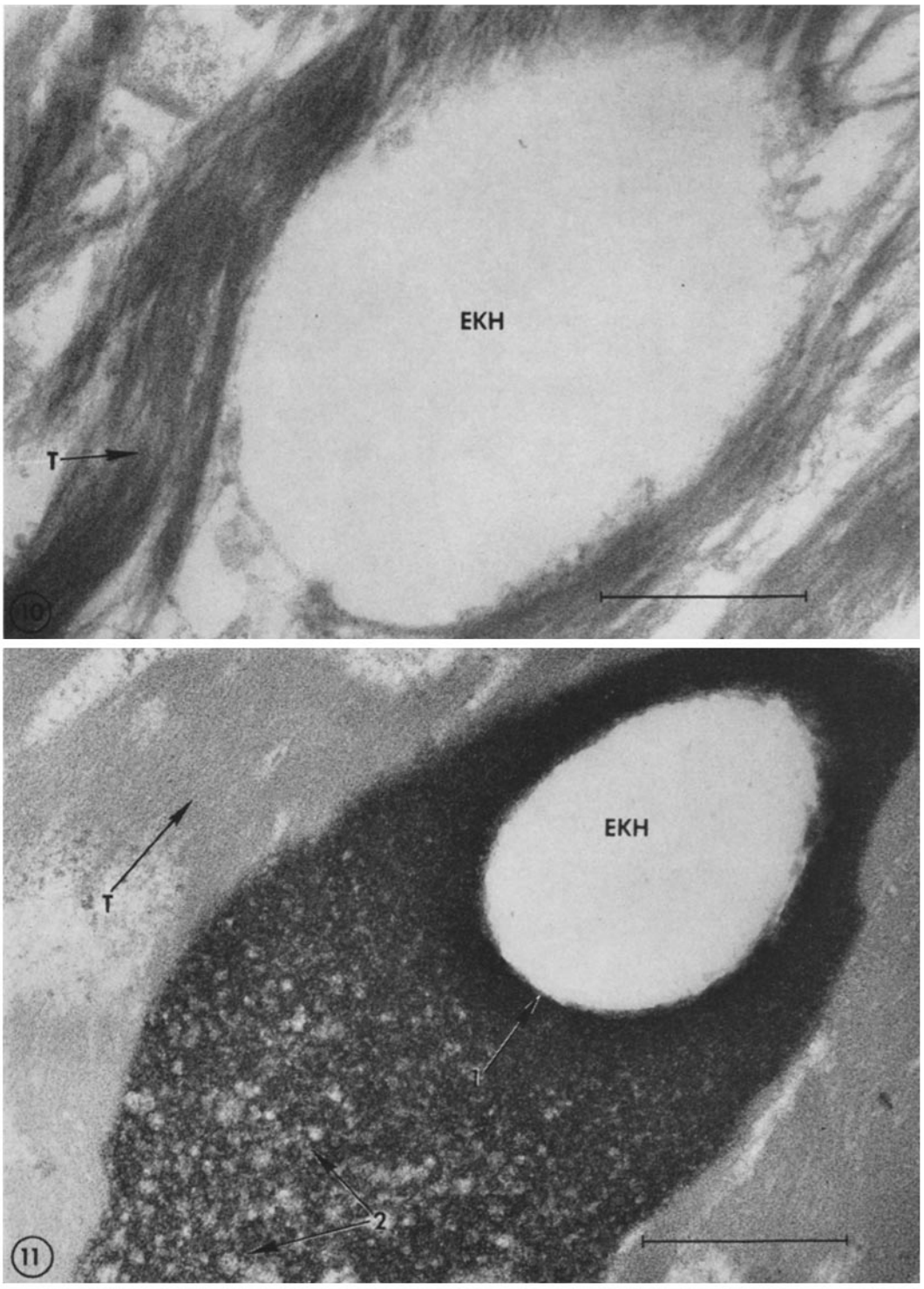
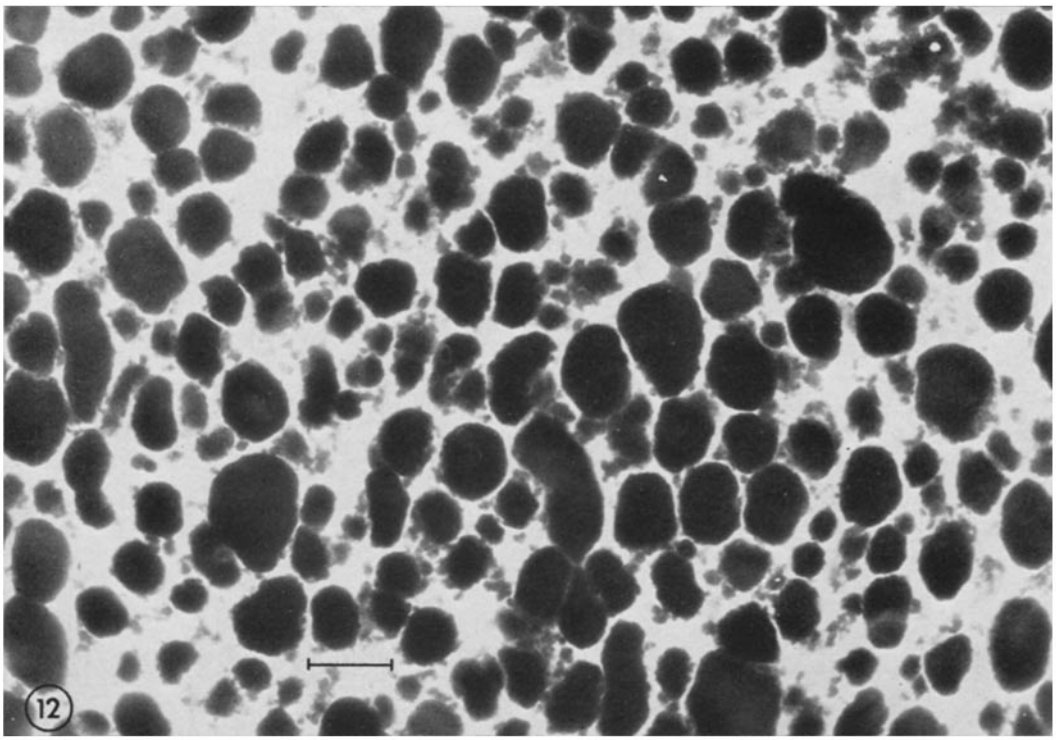


FIGURE 10 Area in which keratohyalin has been completely extracted demonstrating a vacuole (*EKH*) surrounded by tonofilaments (*T*). Uranyl acetate and lead citrate. Scale marker, 0.5 μ. × 46,000.

FIGURE 11 Minimally extracted keratohyalin granule demonstrating a small area of complete extraction (*EKH*) lined by densely staining keratohyalin (1). Further removed from the completely extracted area keratohyalin appears vacuolated and loosely packed (2). *T*, tonofilaments. Uranyl acetate and lead citrate. Scale marker, 0.5 μ. × 46,000.



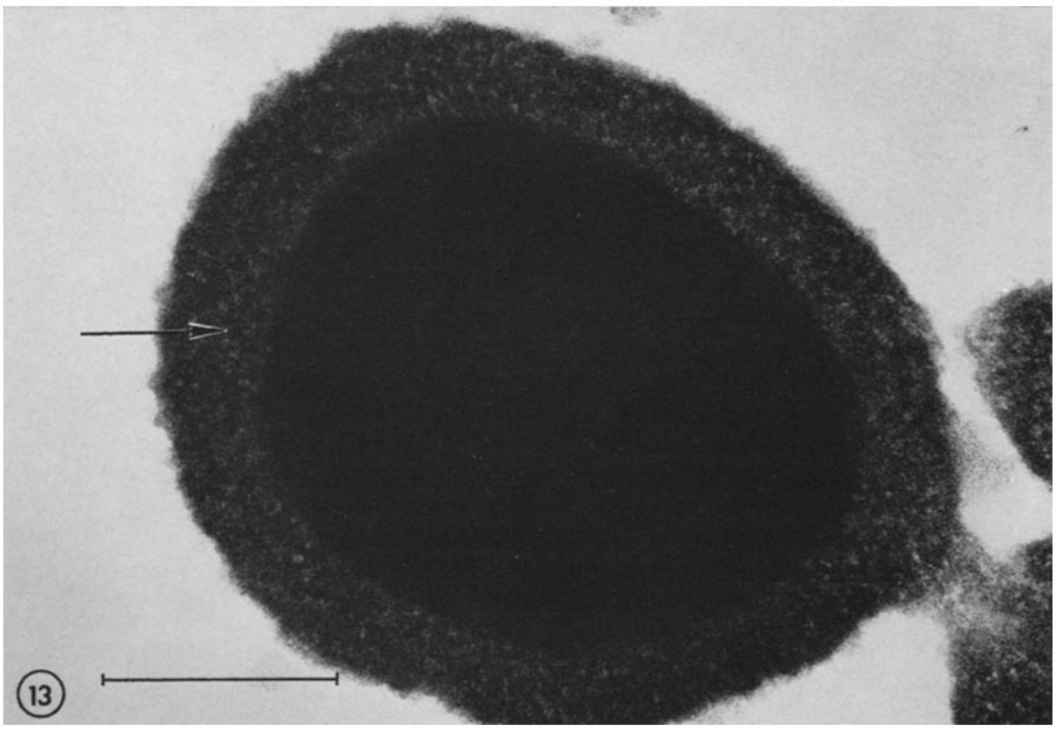


FIGURE 12 Isolated macroaggregates which are similar to *in situ* keratohyalin granules in size and shape and are discrete particles. Uranyl acetate and lead citrate. Scale marker, 1 μ. × 8000.

FIGURE 13 Isolated macroaggregate with a more prominent margin (arrow). The margin is similar in appearance to partially extracted keratohyalin as demonstrated in Fig. 11. Uranyl acetate and lead citrate. Scale marker, 0.5 μ. × 46,000.

the gel reveals the geometric distribution of the first 13 bands and the doublet pattern of certain bands. Also demonstrated are two diffuse bands which are not geometrically related to the oligomeric series (bands 14 and 15). Fig. 15 demonstrates the "stacking" of the oligomeric series and the separation of the two diffuse nonhomologous bands (Nos. 14 and 15) on 3% T/3% C polyacrylamide gels.

Fractionation of purified oligomers on 9% T/3% C polyacrylamide gels is demonstrated in Fig. 16. Examination of this gel reveals the presence of the oligomeric series and the absence of the nonhomologous bands (Nos. 14 and 15). Stacked oligomers and the absence of the nonhomologous bands (Nos. 14 and 15) are also demonstrated by the fractionation of purified oligomers on 3% T/3% C polyacrylamide gels (Fig. 17).

Fig. 18 demonstrates the spectrum of purified oligomers in 0.02 M NaDS.

Light and Electron Microscopy of Aggregates Prepared from Crude and Purified Oligomers

Aggregates formed from crude or purified oligomers were composed of nondistinct clumps which demonstrated histochemical characteristics identical to those of *in situ* keratohyalin granules and isolated nondenatured macroaggregates (Table I). Epon-embedded specimens were also composed of nondistinct clumps.

At the ultrastructural level, aggregates formed from crude or purified oligomers were composed of closely packed particles of varying size, shape, form, and degrees of opacity. Occasionally fine strands were noted but the over-all pattern was highly disorganized (Fig. 19).

Histochemical Staining of Fractionated Oligomers on Polyacrylamide Gels

On 9% T/3% C polyacrylamide gels faint but definite staining of the fractionated oligomers (including both members of each doublet) and the two nonogohomolus species (bands Nos. 14 and 15) was noted with Harris' hematoxylin, Congo red, sodium alizarin sulfonate, diazotized sulfanilic acid, toluidine blue, and pyronin (Table I and Fig. 20). Concentration on 3% T/3% C polyacrylamide gels increased the intensity of the staining of the oligomers and faint staining of the nonhomologous bands was also noted. Similar results were noted with purified oligomers, except that the nonhomologous species were not evident. Methyl green failed to permanently stain the oligomers or the nonhomologous bands, but transient blue staining was noted prior to destaining.

DISCUSSION

At present there is no absolute method for identifying keratohyalin. The identical staining characteristics of *in situ* keratohyalin granules and isolated macroaggregates, their susceptibility to ribonuclease digestion, and the appearance of extracted tissue are suggestive as to the origin of the isolated material. However, these data cannot permit the statement that keratohyalin has been isolated, and not until more definitive biochemical data are available on *in situ* keratohyalin granules can valid comparisons be made (46). The present studies serve only to compare the morphological and histochemical characteristics of *in situ* keratohyalin granules and isolated macroaggregates and to describe the physical-chemical properties of the isolated material. Because the origin of the isolated material has not been precisely documented, the nonspecific terms "oligomer" or "isolated macroaggregate" have been employed. The origin of the isolated material is under further study using immunological and radioautographical techniques.

The present studies suggest the presence of an aggregating ribonucleoprotein in the isolated material, and the purification of an oligomeric series of aggregating ribonucleoproteins from isolated macroaggregates has been demonstrated. The presence of ribonucleoprotein could explain much of the histochemical staining and metal binding characteristic of keratohyalin granules or isolated macroaggregates. Basic dyes such as toluidine blue, methyl green–pyronin or acridine orange could possibly complex with the phosphate residues of the nucleic acid or the nucleic acid itself (1, 18, 19, 33, 49). In the case of toluidine blue, such binding would result in the alcohol-stable β-metachromasia noted with keratohyalin granules (18, 19) or isolated macroaggregates. Similarly, metal cations might bind to such phosphate groups, explaining the laking seen with hematoxylin (formation of deeply colored chelate complexes with unoxidized hematoxylin after exposing substrate to cations) (22, 32), and the in vitro binding of zinc ions noted with dithizone (35). Chelation of sodium alizarin sulfonate might be due to alkali metals, such as calcium and magnesium, which are normally bound to ribonucleoproteins (30), presumably by binding to phosphate residues.

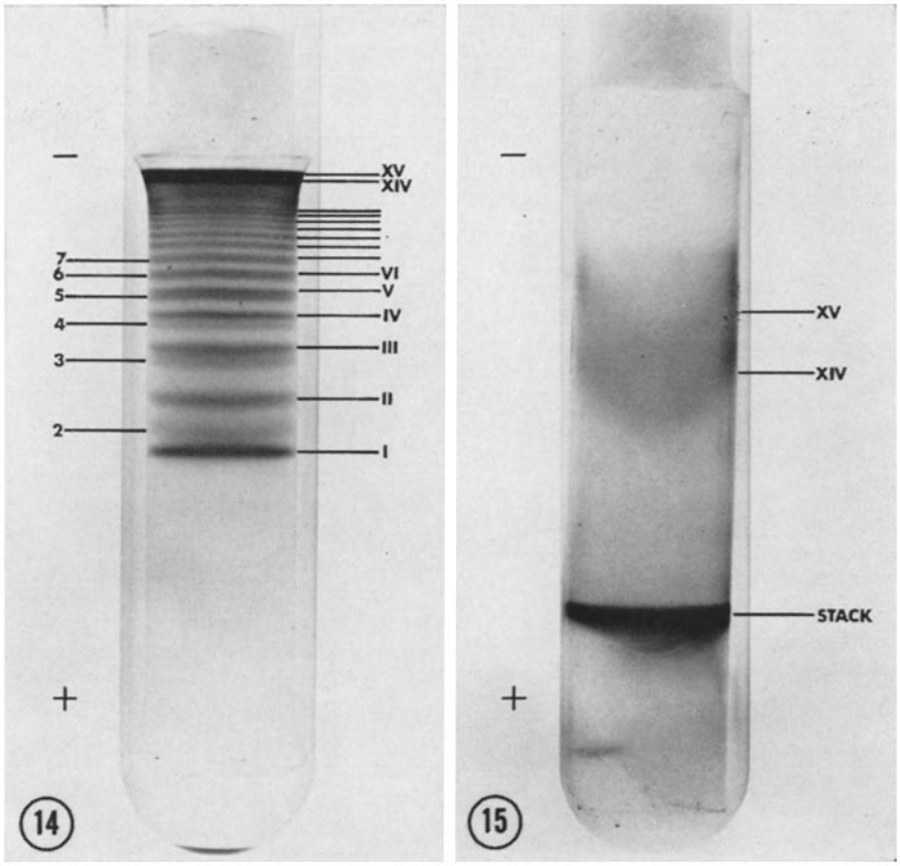


FIGURE 14 Fractionation of crude oligomers on a 9% *T*/3% C polyacrylamide gel stained with amido black. The first 13 bands are distributed in a geometric series and are composed of doublets in which the slower moving member of each doublet is more darkly stained (Roman numerals) than the faster moving member (Arabic numerals). The monomer of the faster moving series is not seen (Arabic No. 1) either due to loss during dialysis or failure for detection in the present gel system. Bands XIV and XV are not in series.

FIGURE 15 Fractionation of crude oligomers on a 3% T/3% C polyacrylamide gel stained with amido black. The entire series is stacked and separated from nonhomologous bands XIV and XV.

Staining of keratohyalin granules or isolated macroaggregates by acidic dyes (hematoxylin and Congo red) and diazotized sulfanilic acid (Pauly reaction) suggests the presence of a basic protein containing histidine. Preliminary amino acid analyses of isolated oligomers verifies the presence of significant amounts of histidine and arginine and small amounts of lysine (70, 120, and 40 residues per 1000 residues, respectively). Also present were significant amounts of serine, glycine, aspartic and glutamic acids (213, 139, 70, and 122 residues per 1000 residues, respectively) and small amounts of proline, cysteine, methionine, tyrosine, and phenylalanine (16, 8, 5, 22, and 10 residues per 1000 residues, respectively). Fukuyama, using the technique of radioautography, has demonstrated that serine, arginine, glycine, and histidine are concentrated into keratohyalin granules of the rat (12, 13), and the distribution agrees with the amino acid analyses of oligomers from cattle hoof epidermis. A "histidine-rich" protein has been isolated from rat and human epidermis, and this material also contains significant amounts of serine, arginine, glycine, and glutamic acid (3, 16, 50), and possibly urocanic acid (41). Matoltsy (25), using grid preparations as an assay system, has isolated keratohyalin granules from rat epidermis by solubilizing all nonkeratohyalin constituents. However, the particles isolated by Matoltsy have not been subjected to histochemical, biochemical

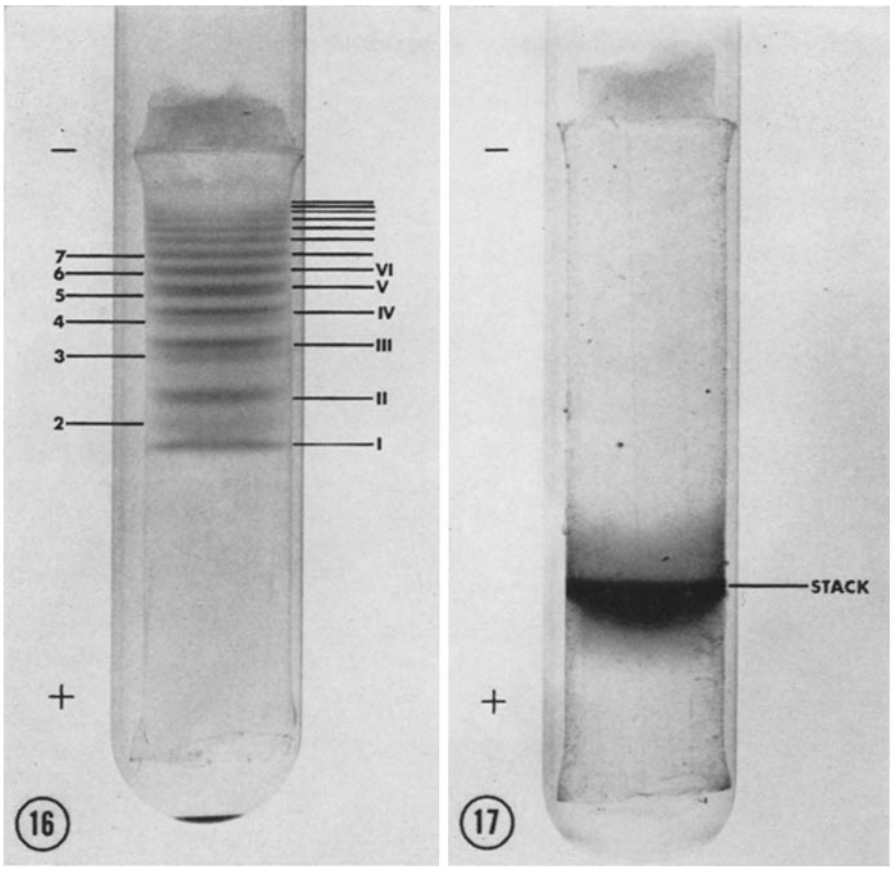


FIGURE 16 Fractionation of purified oligomers on a 9% T/3% C polyacrylamide gel stained with amido black. Bands XIV and XV are not present. Roman numerals, slower moving member of each doublet; Arabic numerals, faster moving member.

FIGURE 17 Fractionation of purified oligomers on a 3% T/3% C polyacrylamide gel stained with amido black. Bands XIV and XV are not present.

or physical-chemical studies, and therefore cannot be compared to hoof keratohyalin.

After ribonuclease digestion, staining by the basic dyes toluidine blue, methyl green–pyronin, and acridine orange is abolished, suggesting removal of an acidic polyribonucleotide. However, acid dyes (hematoxylin and Congo red), diazotized sulfanilic acid, and sodium alizarin sulfonate continue to stain the ribonuclease-treated specimens. These results support the above explanation for the histochemical staining of keratohyalin granules or isolated macroaggregates by acidic and basic dyes, and, because of the staining of ribonuclease-treated specimens by sodium alizarin sulfonate, also indicate that calcium or magnesium are bound to the basic protein, possibly at the carboxy groups of aspartic and glutamic acids.

Continued aggregation after ribonuclease digestion also indicates that interaction between protein subunits is the major stabilizing factor in maintaining the macroaggregated state. Ribonuclease activity is greatest immediately below the stratum corneum (7), but failure of ribonuclease to disperse the basic protein of *in situ* keratohyalin granules or isolated macroaggregates indicates that additional mechanisms are involved in depolymerization.

The results of physical-chemical studies which indicate that the macroaggregates are highly purified and composed of a 25 member oligomeric series (47) are of assistance in interpreting the ultrastructure of isolated macroaggregates. These appear to be composed of two distinct substances, an inner dense core and a less dense, loosely packed margin. However, the loosely packed margin is

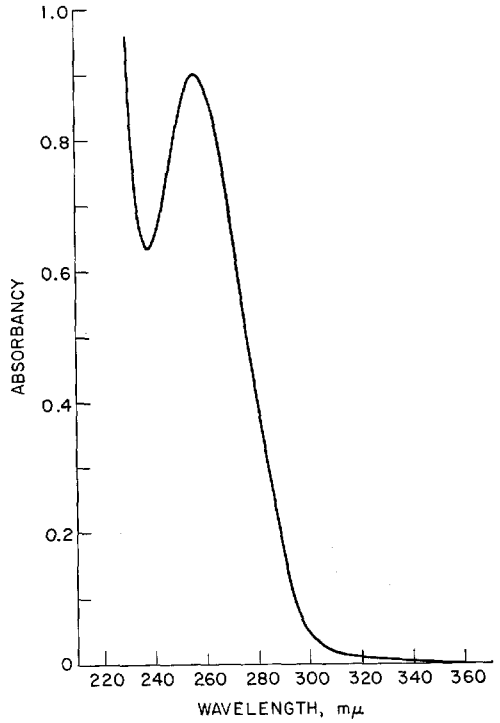


FIGURE 18 Spectrum of purified oligomers in 0.02 M sodium decylsulfate. Maximum absorbancy at 257 mμ. Protein concentration, 140 μg/ml.

similar in appearance to partially extracted keratohyalin, and further, the thickness of this margin can be increased by dialyzing isolated macroaggregates against 0.1 M potassium phosphate buffer (pH 7.0). These morphological observations indicate that the margin and core of isolated macroaggregates may be composed of the same material, but appear different due to artifacts of the aggregation technique.

The present studies on the behavior of the oligomeric series on polyacrylamide gel electrophoresis demonstrate that the oligomers undergo progressive stacking as the pore size of the polyacrylamide gel is increased. On 3% T/3% C polyacrylamide gels, there is sufficient retention of the two nonhomologous species (band Nos. 14 and 15) to permit easy separation from the stacked oligomeric series. By such fractionation of crude oligomers it is possible to isolate the entire oligomeric series from the nonhomologous species in one step.

The doublet pattern noted with fractionated epidermal oligomers has been previously observed with synthetic oligodeoxynucleotides (8) and calf thymus histone III oligomers (9). Calf thymus histone III oligomers contain two cysteinyl residues per monomer and reduction of the disulfide groups results in the conversion of oligomers to the monomer. Oxidation of the calf thymus histone III monomer results in the reformation of the oligomeric histones, suggesting that the formation of disulfide bridges between monomers is the mechanism of oligomer formation. With two cross-linking sites per monomer, structural isomers of the multimers could exist (9), explaining both the doublet pattern (structural isomers) as well as the oligomeric pattern (size isomers) noted with synthetic oligodeoxynucleotides, histone III oligomers, or epidermal oligomers. Except for the oligomeric series of synthetic oligodeoxynucleotides described by Elson and Jovin (8), and the calf thymus histone III oligomers described by Fambrough and Bonner (9), the present series of oligomers, migrating as doublets, appears to be unique in the literature. Oligomeric series have been demonstrated by treating proteins with diethyl pyrocarbonate and dissociating the proteins with sodium dodecylsulfate (51). The present oligomeric series, obtained by fractionating NaDS dissociated macroaggregates without the use of diethyl pyrocarbonate, appears unique and not artifactual. Further, such an oligomeric series of aggregating ribonucleoproteins is compatible with morphological interpretations.

Staining of fractionated oligomers on polyacrylamide gels by the various histochemical reagents indicates that the protein and RNA travel together as discrete species, and suggests that the histochemical characteristics of macroaggregates are due only to the unique composition of the ribonucleoprotein. The most sensitive and permanent stain was amido black, and the best method for rapid localization has proven to be precipitation of the oligomers on the gel with 7.5% acetic acid. Staining of both member of doublets by the various histochemical reagents indicates that each member is similar at a histochemical level.

Formation of ordered aggregates as described in this study is presumably dependent on a built-in set of specific bond sites and a tendency to form the maximum number of most stable bonds during the aggregation process. As such, the process is similar to crystallization and is governed by the laws of statistical mechanics (5). Therefore one might predict that the aggregation process is highly efficient in the purification of the 1.0 M potassium

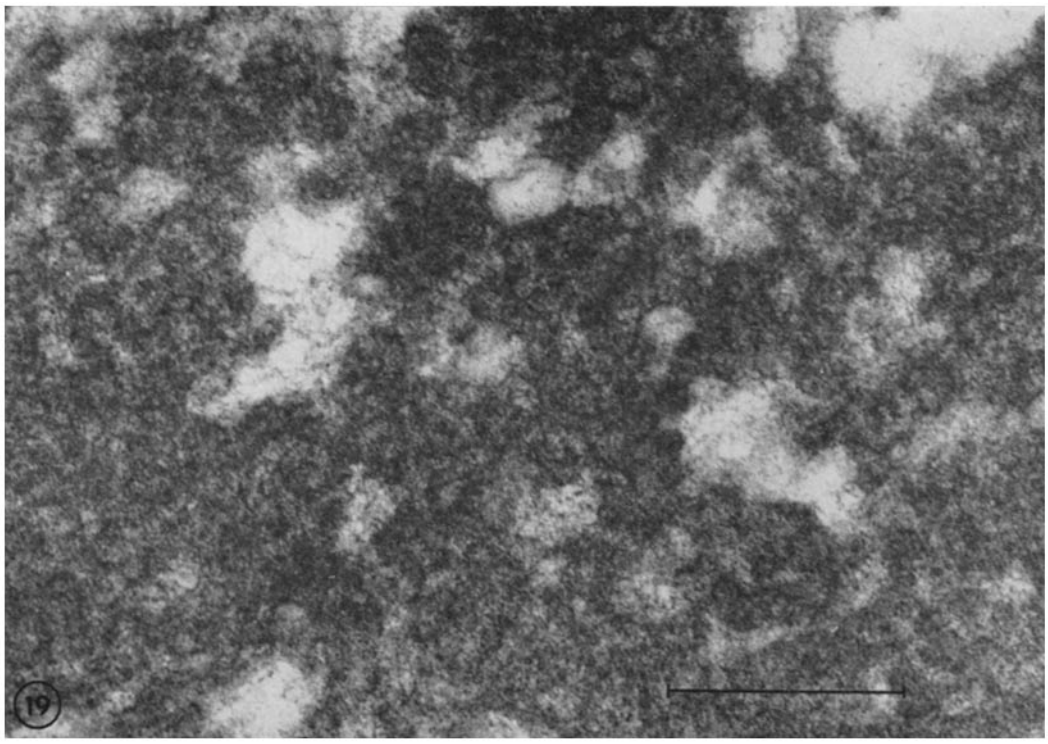


FIGURE 19 Aggregates of sodium decylsulfate denatured oligomers. The aggregates are highly disordered and reveal no substructure. Uranyl acetate and lead citrate. Scale marker, 0.5 μ. × 46,000.

phosphate buffer (pH 7.0) extract. The fractionation of crude oligomers demonstrates the efficiency of in vitro aggregation in purifying a crude extract of epidermis. As demonstrated in the present study, the nonhomologous species have histochemical characteristics similar to those of the oligomers. Preliminary studies indicate that these nonhomologous species also have similar RNA/protein ratios and spectra as the purified oligomers, and therefore it is possible that the nonhomologous species may be size or charge isomers of the oligomeric series. If this be the case, then crude oligomers have been extensively purified by the in vitro aggregation process. In this connection, at present it is not known whether the difference in per cent RNA in crude and purified oligomers is due to the separation of an RNA-rich fraction or if the difference is due to permanent dissociation of noncovalently linked ribonucleotides by NaDS. Since both the oligomers and the nonhomologous species have similar RNA/protein ratios, it would seem that the latter is the probable explanation for the difference in per cent RNA between crude and purified keratohyalin. Alternatively, it is possible that RNA-rich fractions were not able to penetrate the concentration gel and were lost during the purification procedure.

Because of the tendency for oligomers to aggregate in low salt solutions and the incompatibility of polyacrylamide gel electrophoresis with high salt concentrations, denaturation with detergent has been necessary in order to utilize the high resolving power (38) of polyacrylamide gel electrophoresis. Despite the irreversible denaturing effects of NaDS, aggregates formed from either crude or purified oligomers continue to display histochemical characteristics identical to those of *in situ* keratohyalin granules or macroaggregates isolated from 1.0 M potassium phosphate (pH 7.0) extracts. Because of the difficulty in removing detergent from protein (17, 26, 31, 34, 40), precise matching of corresponding groups during in vitro aggregation might be prevented, resulting in the failure to reconstitute aggregates or the formation of morphologically altered particles, as seen with NaDS-denatured oligomers in these studies. Since simi-

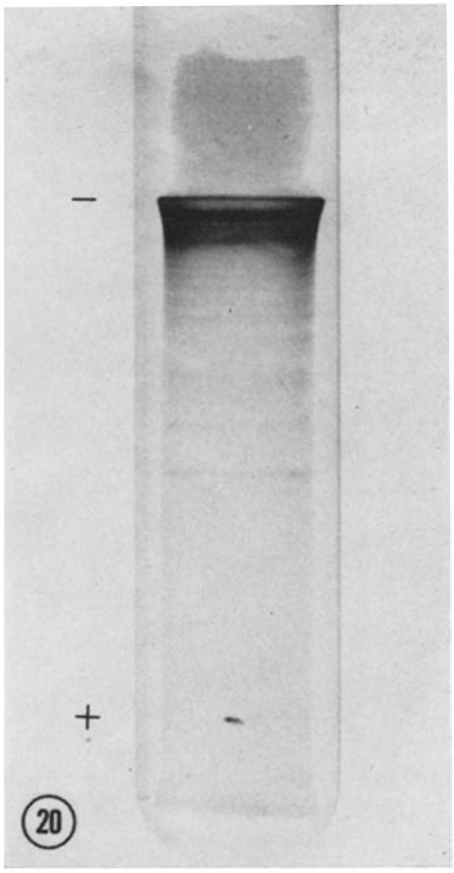


FIGURE 20 Fractionation of crude oligomers on a 9% T/3% C polyacrylamide gel stained with diazotized sulfanilic acid. Similar patterns are noted with gels stained with Harris' hematoxylin, sodium alizarin sulfonate, Congo red, pyronin, and toluidine blue (Table I).

lar aggregates are formed from both crude and purified denatured oligomers, the major factor appears to be the residually bound detergent, although dissociation of ribonucleotides may have had an influence.

In 1949 Smith and Parkhurst (42) presented histochemical evidence that keratohyalin was a ribonucleoprotein, and this was later confirmed by Leuchtenberger and Lund (21). On the basis of ultrastructural studies, it has been suggested that the small particles surrounding the keratohyalin granules were ribosomes (10, 37), and that keratohyalin was formed by enzymatic degradation of these ribosomes (14, 15, 28). However, failure to demonstrate rapid labeling by amino acids at the margins of keratohyalin granules (13) suggests that the small particles are not engaged in protein synthesis. In this case it is necessary to assume either that the particles are not ribosomes, or that they are inactivated ribosomes. Although it is difficult to relate the present denatured system to in vivo mechanisms, the formation of macroaggregates or keratohyalin granules in hoof epidermis by the polymerization of ribonucleoproteins is suggested from the present studies. Whether such polymerization represents an inactivation mechanism (11, 39, 43, 51) or a storage mechanism is unknown. Since isolated oligomers have an amino acid composition and physical-chemical properties which differ significantly from those of ribosomal ribonucleoproteins (30), it would seem that the oligomers are not formed by enzymatic degradation of ribosomes. Ultrastructurally, it is suggestive that the small particles surrounding the keratohyalin granule are degraded and incorporated into the granule; hence, the present data also indicate that the small particles are not ribosomes, but a stage of aggregation which occurs prior to macroaggregation.

Other hypotheses, which attempt to correlate ultrastructural data but have not had the benefit of correlation with observations on isolated keratohyalin, have been proposed for the function and composition of keratohyalin granules. Brody proposed that the small particles surrounding the keratohyalin granules are precursors of keratohyalin, and that keratohyalin is a stage in the development of tonofilaments (4). Rhodin and Reith hypothesized that the particles surrounding the keratohyalin granules are ribosomes probably engaged in the production of keratohyalin. They proposed that keratohyalin and tonofilaments consolidate to form keratin (37). Fukuyama has presented a similar theory, but proposed that a "histidine—rich" protein is needed to link tonofibrils and other substances to form keratohyalin granules (13). Lavker and Matoltsy suggest that keratohyalin granules disperse and mix with protein contained in endoplasmic reticulum. Subsequently, the keratohyalin–endoplasmic reticulum–protein complex infiltrates the tonofilaments to form the terminal fibrous-amorphous complex or keratin (20).

Within the stratum granulosum of the epidermis, unique biological events are constantly occurring, and presumably unique biological mechanisms are involved. Living epidermal cells undergo func-

tional and progressive cellular decay, as evidenced by dissolution of nuclei and other cellular constituents, and terminating in the transition of these living cells into the dead outermost layer of skin. During this transition, keratohyalin forms large intracytoplasmic aggregates and then disperses just beneath the outermost layer of the epidermis. At present it is not known whether keratohyalin has a functional role or whether keratohyalin forms as a consequence of cellular death. Since epidermal nuclei or nucleoli do not demonstrate histochemical characteristics similar to those of keratohyalin, it would seem that nuclear decay per se is not related to keratohyalin formation. Further, the apparent increased synthesis of keratohyalin in the stratum granulosum (3, 12, 13) suggests an active rather than a passive role.

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