

# FURTHER CHARACTERIZATION OF BOVINE KERATOHYALIN

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## ABSTRACT

Extraction of serial sections of cattle hoof epidermis with solutions of calcium chloride, magnesium chloride, potassium chloride, sodium chloride, guanidine hydrochloride, ammonium sulfate, and potassium phosphate buffer (pH 7.0) at varying salt concentrations demonstrates that keratohyalin (KH) is extracted by these salts at certain molarities. Under given conditions of time and temperature, each salt has a specific extraction pattern, and similar salts have similar extraction patterns. Dialysis of the salt extracts of hoof epidermis against distilled water results in the macroaggregation of KH, as assayed by histochemical methods. Although the various macroaggregates appear identical at the histochemical level, they display different ultrastructural characteristics. Polyacrylamide gel electrophoresis of the sodium decyl sulfate-solubilized macroaggregates results in the fractionation of a 20 (or more) member homologous series of oligomers. Isolation of the various oligomeric species of bovine keratohyalin and re-electrophoresis indicate that the various KH species can undergo depolymerization. Amino acid analyses of the unfractionated bovine macroaggregates and the various molecular weight species of bovine KH are similar, further demonstrating homology of the oligomers. The molecular weight of the subunit (monomer) of bovine KH is 14,955, estimated from the amino acid analyses.

## INTRODUCTION

Previous histochemical and ultrastructural studies have demonstrated that keratohyalin (KH) can be extracted from cattle hoof epidermis with 1.0 M potassium phosphate buffer (pH 7.0), and isolated by *in vitro* aggregation effected by dialysis against distilled water (24, 25). Physicochemical analysis of the resultant aggregates (termed "macroaggregates") (25), has indicated that they are composed of a 13 (or more) member homologous series of oligomers with a subunit (monomer) molecular weight of 16,900 (26). In agreement with histochemical (12, 15, 18, 21, 25) and radioautographical (6, 7, 9) studies of *in situ* KH granules, the protein species contain significant amounts of serine, arginine, glycine, and histidine, and appear

to be complexed to ribonucleotides (25). Further evidence for homology and identity of the isolated material as KH is the production of specific antibody to bovine KH after immunization of rabbits with the isolated bovine macroaggregates or specific molecular weight species of bovine KH.<sup>1</sup>

The present study was directed at defining the relationship between extraction of bovine KH and salt concentration, and further demonstrating the homology of the isolated KH by physicochemical methods and amino acid analyses of the various oligomeric states.

<sup>1</sup> Guss, S., and A. R. Ugel. 1972. Immunofluorescent antibodies to bovine keratohyalin and immunological confirmation of homology. *J. Histochem. Cytochem.* In press.

## MATERIALS AND METHODS

### *Serial Extractions of Bovine Hoof*

Epidermis was obtained from the posterior aspect of cattle hooves (27). Specimens were frozen in liquid nitrogen and sectioned with a cryostat set at 6  $\mu$ . Other specimens were fixed in 80% methanol, 10% buffered formaldehyde, or 6% buffered glutaraldehyde (25), embedded in paraffin under vacuum at 40°C, and serially sectioned at 6  $\mu$ . A single section of each preparation of cattle hoof epidermis (cryostat, methanol-fixed, formaldehyde-fixed, glutaraldehyde-fixed) was extracted in a solution of specified molarity for 15 min at 37°C. Solutions, varying at 10th molar intervals, were as follows: (a) sodium chloride (0.1 M–6.0 M); (b) potassium chloride (0.1 M–4.0 M); (c) guanidine hydrochloride (0.1 M–5.0 M); (d) magnesium chloride (0.05 M–6.0 M); (e) calcium chloride (0.05 M–6.0 M); (f) ammonium sulfate (0.1 M–5.0 M); (g) potassium phosphate buffer (pH 7.0) (0.1 M–2.0 M); (h) urea (0.1 M–8.0 M). Only potassium phosphate solutions were buffered; in all other cases the extractions were performed at the pH of the specified solution. All chemicals were reagent grade. Guanidine hydrochloride and urea were obtained from Mann Research Labs. Inc., New York. After extraction, sections were rinsed in distilled water for 2 min, then stained with either Harris' hematoxylin, Congo red, or diazotized sulfanilic acid (18, 25).

### *Preparation of Macroaggregates from Bovine Hoof Epidermis*

Cattle hoof was dissected, and the entire epidermis located beneath the stratum corneum was retained (26, 27). Dissected tissue was minced with scissors, then extracted with 1.0 M potassium phosphate buffer (pH 7.0) (24, 25). Additional samples of cattle hoof epidermis were extracted with 1.0 M solutions of the reagents described above (sodium chloride, potassium chloride, guanidine hydrochloride, magnesium chloride, calcium chloride, ammonium sulfate), or with 8 M urea. Cleared extracts were dialyzed against 32 volumes of distilled water, and onset of visible aggregation was recorded. Macroaggregates were harvested by centrifugation (24, 25). Additional samples of minced cattle hoof epidermis were fixed in 80% methanol for 12 hr at 4°C, rinsed in distilled water, then extracted with 1.0 M potassium phosphate buffer (pH 7.0) as described above. Extracts were dialyzed against distilled water, and macroaggregates were harvested as described above.

### *Light Microscopy*

Macroaggregates were collected on Millipore filters (type HA Millipore filter, 0.45  $\mu$  pore size,

13 mm diameter, placed in a Swinnex filter holder [Millipore Corp., Bedford, Mass.] (25). Macroaggregates (on the surface of Millipore filters) and specimens of cattle hoof epidermis were fixed in 80% methanol or Carnoy's solution (25), then stained with Harris' hematoxylin, Congo red, diazotized sulfanilic acid, sodium alizarin sulfonate, toluidine blue, methyl green-pyronin, and acridine orange (18, 22, 25). Additional specimens were stained with fast green (1).

### *Electron Microscopy*

Macroaggregates (on the surface of Millipore filters) were fixed in phosphate-buffered glutaraldehyde, thin sectioned, then stained with uranyl acetate and lead citrate (24, 25). Specimens were examined in a Siemens electron microscope at 80 kv.

### *Polyacrylamide Gel Electrophoresis of Solubilized Macroaggregates*

Macroaggregates were solubilized in sodium decyl sulfate (NaDS) (Eastman Organic Chemicals, Rochester, N. Y.) (25). Polyacrylamide gel electrophoresis of solubilized macroaggregates was performed on 9% separation gels (3% cross-linked) as previously described (19, 25, 26), except that the time of electrophoresis was extended until the glycinate-decyl sulfate boundary (26) approached gel bottom (in order to further separate the various oligomers). Protein was determined by the method of Lowry et al., using bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard (16).

Gels were stained with amido black, and destained electrophoretically (26). Additional samples of bovine macroaggregates (formed from nonphosphate buffer extracts) were subjected to electrophoresis on 9% polyacrylamide gels, then stained with Harris' hematoxylin, Congo red, diazotized sulfanilic acid, sodium alizarin sulfonate, toluidine blue, and pyronin (25). Additional gels were stained with fast green (11).

### *Preparation of Bovine Keratohyalin Fractions*

300–500  $\mu$ g samples of solubilized bovine KH were fractionated on 9% polyacrylamide gels as described above. A sample gel was placed in 7.5% acetic acid to rapidly precipitate the fractionated species (25). Visualization was aided by passing a beam of light through the gel which resulted in the scattering of light at the sites of precipitation. Areas corresponding to the monomer were excised and extracted (25). Oligomeric species 2, 3, 4, and 5 were excised together and extracted as above, and are referred to as "fraction II", and oligomeric species 6 through 20

and nonhomologous species were similarly isolated and are referred to as "fraction III". Additional samples of solubilized bovine macroaggregates were fractionated on 9% polyacrylamide gels, and the area corresponding to the nonhomologous species and the largest oligomers (approximately species 18, 19, and 20) were also excised and extracted. These species are referred to as "fraction IV." Purified oligomers were prepared by selective unstacking of nonhomologous species on 3% polyacrylamide gels (3% cross-linked) (25). Gel slices were homogenized with a high-speed shearing device (Polytron, Brinkman Instruments Inc., Westbury, N. Y.) in the presence of an equal volume of 0.02 M NaDS, and extracted for 48 hr at room temperature. Extracts were centrifuged at 75,000 *g* to remove gel particles (25). 100 to 200- $\mu$ g samples of the above preparations were subjected to electrophoresis on 9% polyacrylamide gels, then stained with amido black as described above. Additional samples of the KH monomer, fractions II, III, IV, and purified oligomers were precipitated from the extract by the addition of an equal volume of 10% trichloroacetic acid. The resultant turbid suspensions were stored at 4°C for 24 hr, then collected by centrifugation at 75,000 *g* for 30 min. Pellets were washed in 90% methanol, lyophilized, and stored at -90°C.

#### *Amino Acid Analyses*

Samples of bovine monomer, fractions II and III were prepared by dissociating fraction IV as described above. These fractions and additional samples of fraction IV, purified oligomers, and unfractionated bovine macroaggregates were hydrolyzed in 6 N hydrochloric acid under nitrogen for 24 hr at 110°C. Amino acid analyses were performed by an automated amino acid analyzer (Beckman, Model 117). All values are corrected for losses of threonine, serine, and tyrosine by extrapolation to zero hydrolysis time, as determined by hydrolysis of macroaggregates for 24, 48, and 72 hr.

Tryptophan was estimated colorimetrically by the method of Gaitonde and Dovey (10).

## RESULTS

#### *Extraction of Bovine Keratohyalin from Tissue Sections*

In all cases, extraction was similar with either cryostat or methanol-fixed, vacuum-embedded sections (see below). Formaldehyde or glutaraldehyde fixation of tissue, however, prevented extraction by all salts at any molarity.

Guanidine hydrochloride, sodium chloride, and potassium chloride completely extracted bovine

KH at similar salt concentrations (0.7 M, 0.7 M, and 0.8 M, respectively). These salts also completely extracted bovine KH at all higher molarities (Fig. 1).

Magnesium and calcium chloride completely extracted bovine KH at the lowest salt concentrations of the salts studied (both between 0.05 M and 0.10 M). As noted with guanidine hydrochloride, sodium chloride, and potassium chloride, extraction of bovine KH also occurred at all higher molarities of magnesium and calcium chloride (Fig. 2).

Ammonium sulfate completely extracted bovine KH at a salt concentration of 0.5 M and continued to extract KH up to a salt concentration of 2.9 M. Above 2.9 M ammonium sulfate, extraction of bovine KH was greatly reduced (Fig. 3).

Potassium phosphate buffer (pH 7.0) completely extracted bovine KH at a salt concentration of 0.5 M and continued to extract KH up to 1.3 M. Above a salt concentration of 1.3 M, extraction was greatly reduced, although KH granules appeared swollen and in certain areas appeared to have coalesced (Fig. 4).

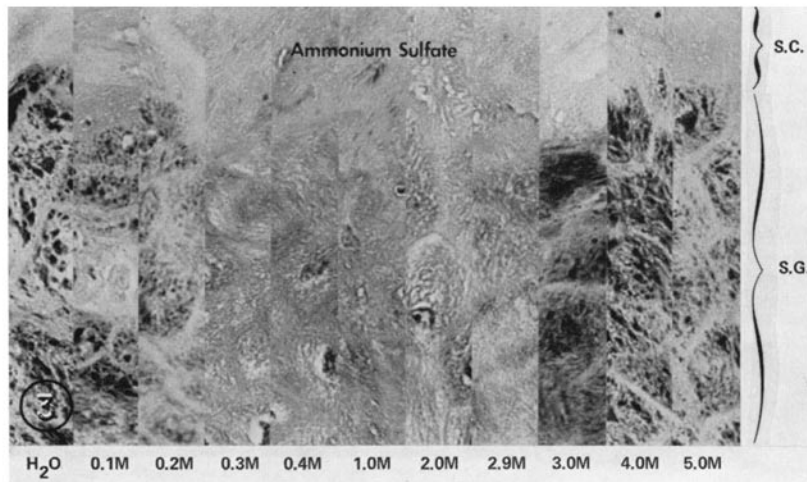
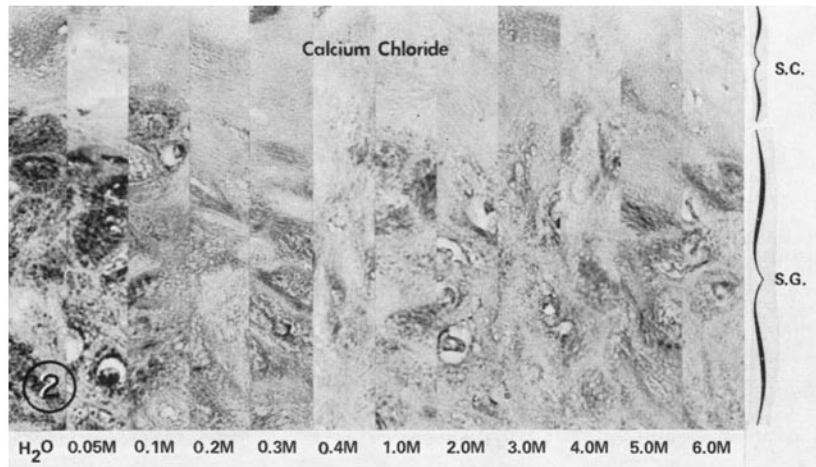
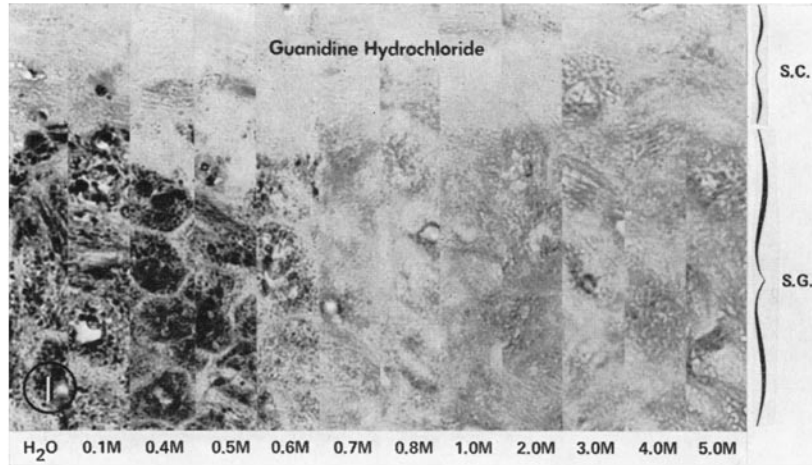
Urea solutions (0.1 M-8.0 M) failed to extract bovine KH under the given conditions (Fig. 5).

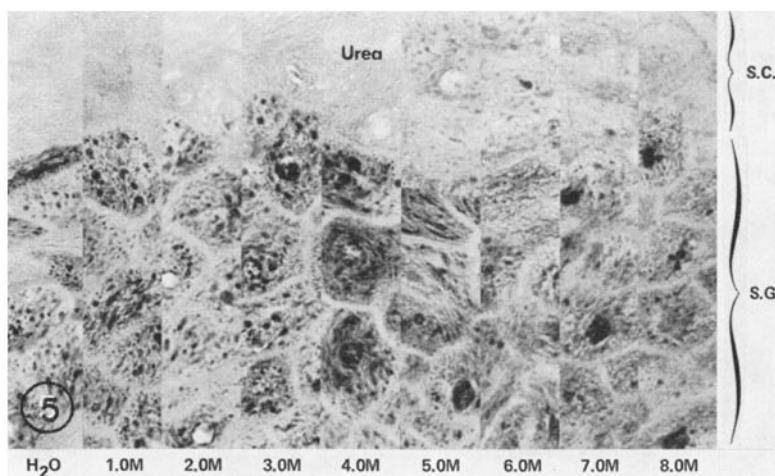
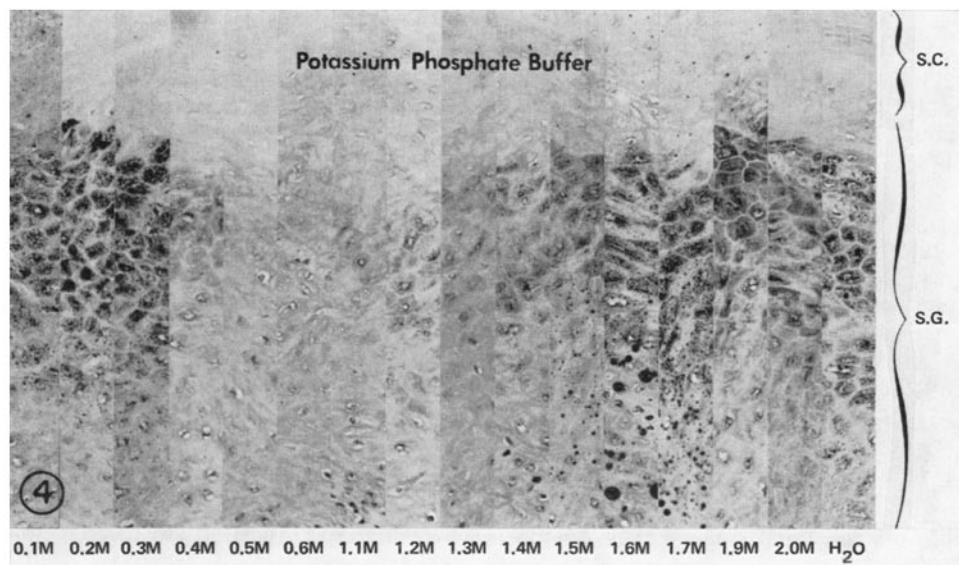
#### *In Vitro Aggregation of the Salt-Solubilized Keratohyalin*

In all cases, dialysis of the high salt extracts of cattle hoof epidermis resulted in the aggregation of protein(s) (see below). Grossly visible aggregation occurred in the sequence: (guanidine hydrochloride, sodium chloride, potassium chloride), (potassium phosphate buffer [pH 7.0]), (ammonium sulfate), (magnesium chloride, calcium chloride). Dialysis of 8 M urea extracts of cattle hoof epidermis against 32 volumes of distilled water resulted in the formation of a gel which maintained the configuration of the dialysis bag.

#### *Light Microscopy*

Bovine macroaggregates formed from the various salt extracts were similar at the histochemical level to those formed from potassium phosphate buffer (pH 7.0) extracts (25), staining with Harris' hematoxylin, Congo red, diazotized sulfanilic acid, sodium alizarin sulfonate, toluidine blue, and pyronin. Fast green stained *in situ* KH granules and macroaggregates, but diffusely stained other epidermal structures.





FIGURES 1-5 Composites of serial sections of cattle hoof epidermis which have been extracted in solutions of guanidine hydrochloride, calcium chloride, ammonium sulfate, potassium phosphate buffer (pH 7.0), and urea, respectively. Concentrations of extracting solutions were varied at 0.1 M intervals, and extraction was carried out for 15 min at 37°C. Sections were fixed in 80% methanol before extraction. Sodium chloride and potassium chloride have extraction patterns similar to that seen in Fig. 1, and magnesium chloride has a pattern similar to that seen in Fig. 2. Darkly stained cytoplasmic particles are KH granules. *S.C.*, stratum corneum; *S.G.*, stratum granulosum;  $H_2O$ , water control; *M*, molarity. Harris' hematoxylin. Figs. 1, 2, 3, and 5:  $\times 460$ ; Fig. 4:  $\times 140$ .

### Electron Microscopy

Although bovine macroaggregates formed from the various salt solutions appeared identical at the histochemical level, finite differences were noted at the ultrastructural level (Figs. 6, 7, 8). The most homogeneous-appearing macroaggregate was that

formed from the potassium phosphate buffer (pH 7.0) extract (Fig. 6). Macroaggregates formed from potassium and magnesium chloride extracts appeared to be composed of tightly packed smaller aggregates, somewhat similar in appearance to the small particles located at the margins of *in situ* KH granules (Fig. 7). Macroaggregates formed from

sodium chloride, guanidine hydrochloride, calcium chloride, or ammonium sulfate were more disordered in substructure, and had areas which appeared vacuolar (Fig. 8). These latter macroaggregates also varied considerably in size and shape.

### *Polyacrylamide Gel Electrophoresis of Solubilized Macroaggregates*

As previously described (25, 26), electrophoresis of solubilized bovine macroaggregates formed from 1.0 M potassium phosphate buffer (pH 7.0) extracts resulted in the fractionation of a 13 (or more) member homologous series of oligomers (which migrate as doublets), and two nonhomologous species. By extending the time of electrophoresis (as described in Methods), a total of 20 darkly staining oligomers were observed (Fig. 9). Electrophoresis of solubilized macroaggregates formed from the other salt solutions resulted in similar fractionation patterns. The homologous oligomers and the conformational isomers and nonhomologous species 21 and 22 (previously designated 14 and 15) (25) were stained by all the histochemical reagents, as has been previously described (25). Identical fractionation patterns were obtained with KH which had been extracted from methanol-fixed cattle hoof epidermis.

Electrophoresis of the solubilized gel formed after dialyzing the 8 M urea extract against distilled water resulted in the fractionation of numerous bands which were heterogeneous and not a homologous series.

### *Isolation of Bovine Keratohyalin Fractions*

Figs. 10, 11, 12, and 13 demonstrate the fractionation patterns of the monomer, fractions II, III, and IV obtained as described in Methods. In

all cases, the species originally excised from the gels are present in large amounts, but lesser amounts of the smaller oligomers and relatively larger amounts of the monomer are also present.

### *Amino Acid Analyses*

The amino acid analyses of the bovine KH monomer, fractions II, III, IV, purified oligomers, and unfractionated bovine macroaggregates are shown in Table I. All values are corrected for losses of threonine, serine, and tyrosine as described in Methods.

## DISCUSSION

### *Solubility of Keratohyalin*

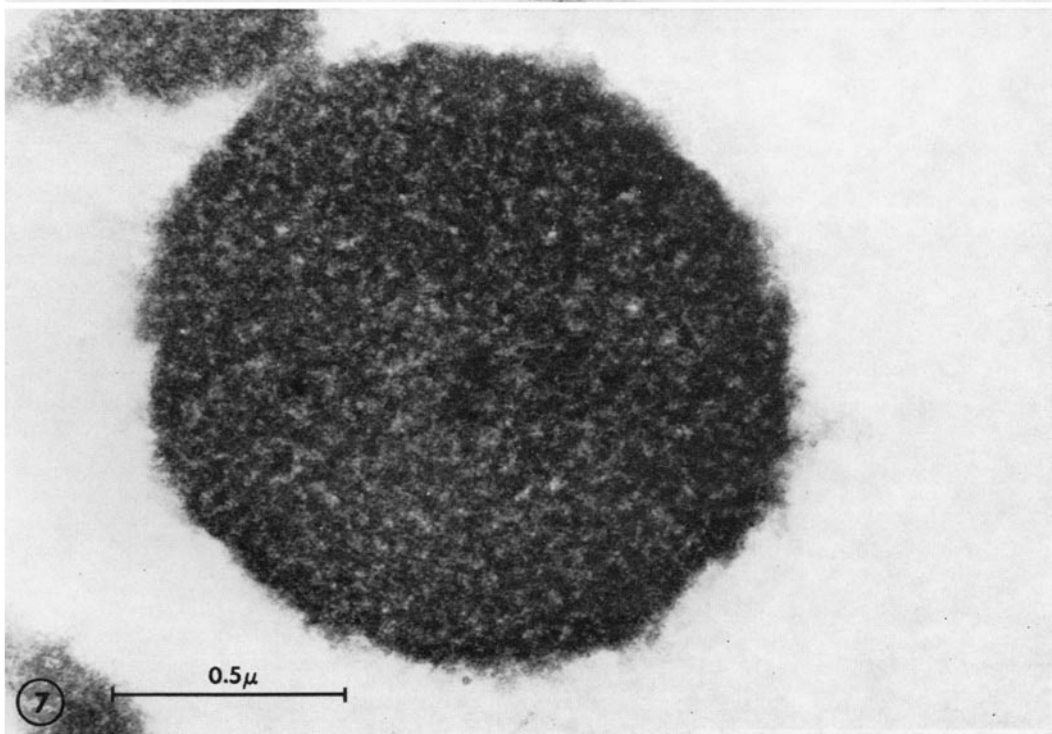
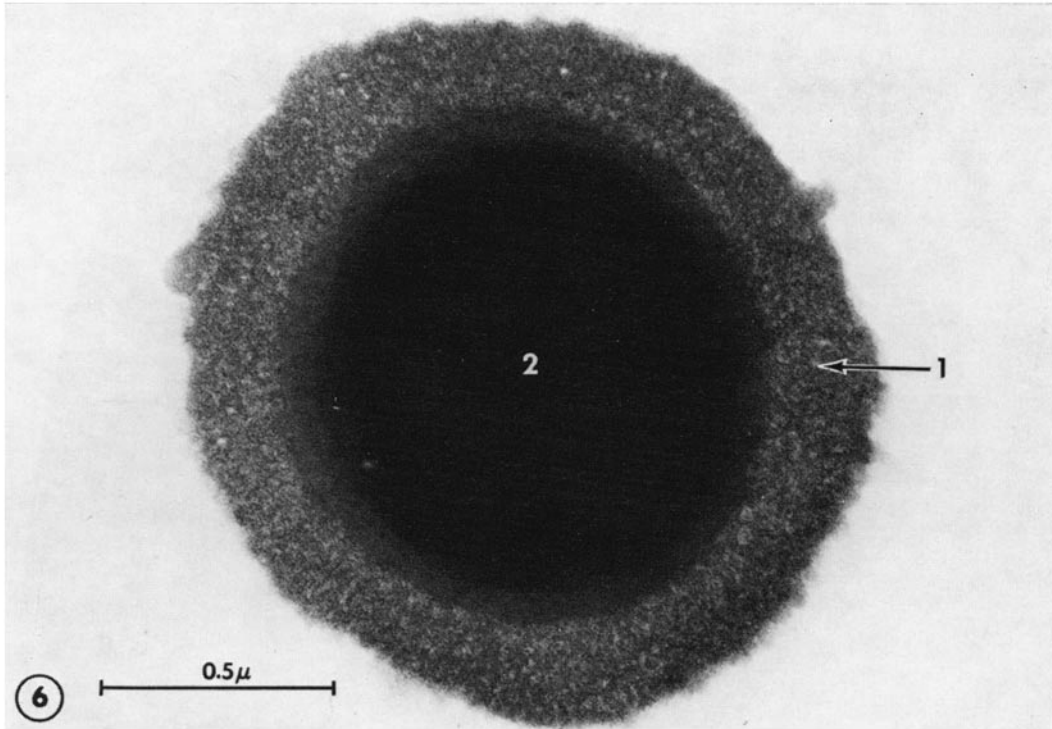
The isolation of bovine KH was predicated on extraction experiments performed on serial cryostat sections of hoof epidermis (23). These experiments demonstrated that bovine KH could be extracted from hoof epidermis by potassium phosphate buffer (pH 7.0) in the range of 0.5 M–1.3 M, and suggested that the solubilized KH would be precipitated by lowering the salt concentration of the high salt extract of hoof epidermis below 0.5 M.

The present studies demonstrate that bovine KH is extractable by numerous salt solutions. Similar extraction as a function of salt concentration has been noted with protein-polysaccharide complex of bovine nasal cartilage (including decreased extraction at high salt concentrations) (20), but a precise explanation for the decreased extraction at high salt concentration is not known. Urea solutions (up to 8 M) failed to solubilize KH (at least as monitored histologically), presumably because the reagent is nonionic. Again, similar results have been noted with protein-polysaccharide complex, although urea did act synergistically with other salts (20). Preliminary studies, however,

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FIGURE 6 Isolated macroaggregate which has been formed by dialysis of 1.0 M potassium phosphate buffer (pH 7.0) extract of cattle hoof epidermis against 32 volumes of distilled water. The core (2) appears electron-opaque and homogeneous, and is surrounded by a shell of less densely staining material (1). Uranyl acetate and lead citrate.  $\times 46,000$ .

FIGURE 7 Isolated macroaggregate formed by dialysis of 1.0 M magnesium chloride extract of cattle hoof epidermis against 32 volumes of distilled water. The macroaggregate appears to be composed of closely grouped smaller aggregates which appear somewhat like the shell surrounding the macroaggregate in Fig. 6, and like the particles which are seen at the margins of *in situ* KH granules. Similar macroaggregates are formed by dialyzing potassium chloride extracts of cattle hoof epidermis against distilled water. Uranyl acetate and lead citrate.  $\times 46,000$ .



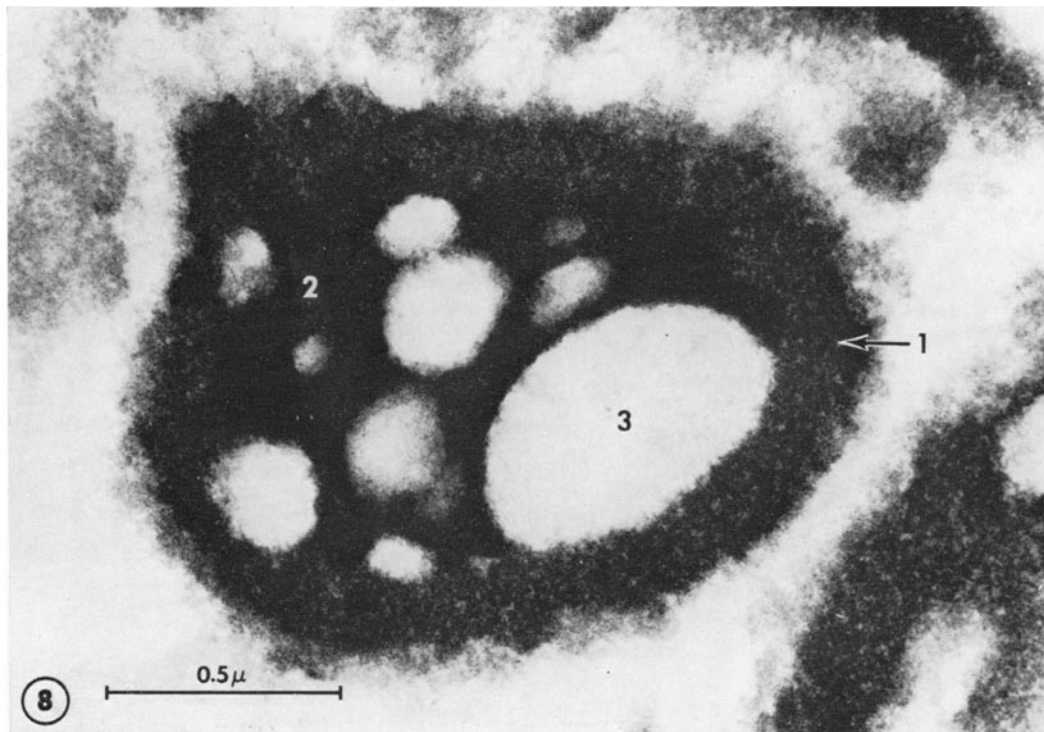


FIGURE 8 Isolated macroaggregate formed by dialysis of 1.0 M guanidine hydrochloride extract of cattle hoof epidermis against 32 volumes of distilled water. The macroaggregate has some characteristics of the particle described in Fig. 6 in that a dense central core (2) and a less dense shell (1) are apparent, but differs in that the central core has vacuolar areas (3). Similar macroaggregates are noted when sodium chloride, calcium chloride, or ammonium sulfate solutions are dialyzed against distilled water. Uranyl acetate and lead citrate.  $\times 46,000$ .

indicate that isolated bovine KH is also soluble in 8 M urea, provided sufficient time is allowed for solubilization.

The marked difference in the solubility of KH noted with small changes in salt concentration (at least as small as 0.05 M for magnesium or calcium chloride), as revealed by the extraction patterns, strongly suggests that macroaggregation is due to ionic or hydrogen bonding between the various KH oligomers. The formation of macroaggregates by *in vitro* aggregation as a function of salt concentration further supports this interpretation.

#### *Comparison of Macroaggregates formed from Different Salt Solutions*

For comparison, a salt concentration of 1.0 M for all salt extraction experiments was arbitrarily selected, even though the respective extraction patterns indicated that lower salt concentrations

could have been employed. The difference in appearance of the various macroaggregates at the ultrastructural level might indicate that salt-KH complexes are important in determining the macromolecular structure of the native KH granules and the isolated macroaggregates. The resemblance to *in situ* KH granules was most obvious with macroaggregates formed from potassium phosphate extracts, and may indicate that potassium and phosphate ions are complexed to the *in situ* KH granule.

#### *Isolation of Bovine Keratohyalin Fractions*

All oligomer fractions isolated from gels produced monomer and smaller oligomers on re-electrophoresis. Apparently, in the presence of NaDS, the oligomers break down by releasing monomeric units, and not by splitting. However, the relative stability of the oligomers as compared



to the macroaggregates in the presence of NaDS suggests that oligomer formation and macroaggregation probably occur by different mechanisms, or that the stability of bovine KH oligomers in the presence of anionic detergent decreases as the size of the oligomer increases. Presumably, the macroaggregate or the *in situ* KH granule, which may represent the largest polymeric state, is the most sensitive to salt or detergent concentration. Because the solubility of the macroaggregate in NaDS and the continued breakdown of the oligomers in NaDS, it is possible that the entire mechanism of polymerization or depolymerization is noncovalent.

### Amino Acid Analyses

The amino acid analyses of the bovine macroaggregates, monomer, fractions II, III, IV, and purified oligomers were similar (Table I). These

TABLE I  
Amino Acid Composition of Bovine  
Keratohyalin

Amino acid	Mean*	Standard deviation
Aspartic acid	7.9	0.3
Threonine‡	7.8	0.3
Serine‡	31.7	1.0
Glutamic acid	14.8	0.4
Proline	1.0	0.4
Glycine	18.3	0.5
Alanine	3.0	0.4
Half cystine§	1.1	0.4
Valine	3.9	0.2
Methionine	0	
Isoleucine	0	
Leucine	1.3	0.3
Tyrosine‡	1.1	0.3
Phenylalanine	0	
Lysine	2.1	0.6
Histidine	10.8	0.5
Arginine	14.7	0.5
Tryptophan	0	

\* Residues per subunit (monomer), as determined by analyses of bovine monomer, fractions II, III, IV, purified oligomers, and macroaggregates. A value of zero indicates less than 0.3 residue.

‡ Corrected for losses during hydrolyses as described in Methods.

§ Sum of cystine and cysteic acid.

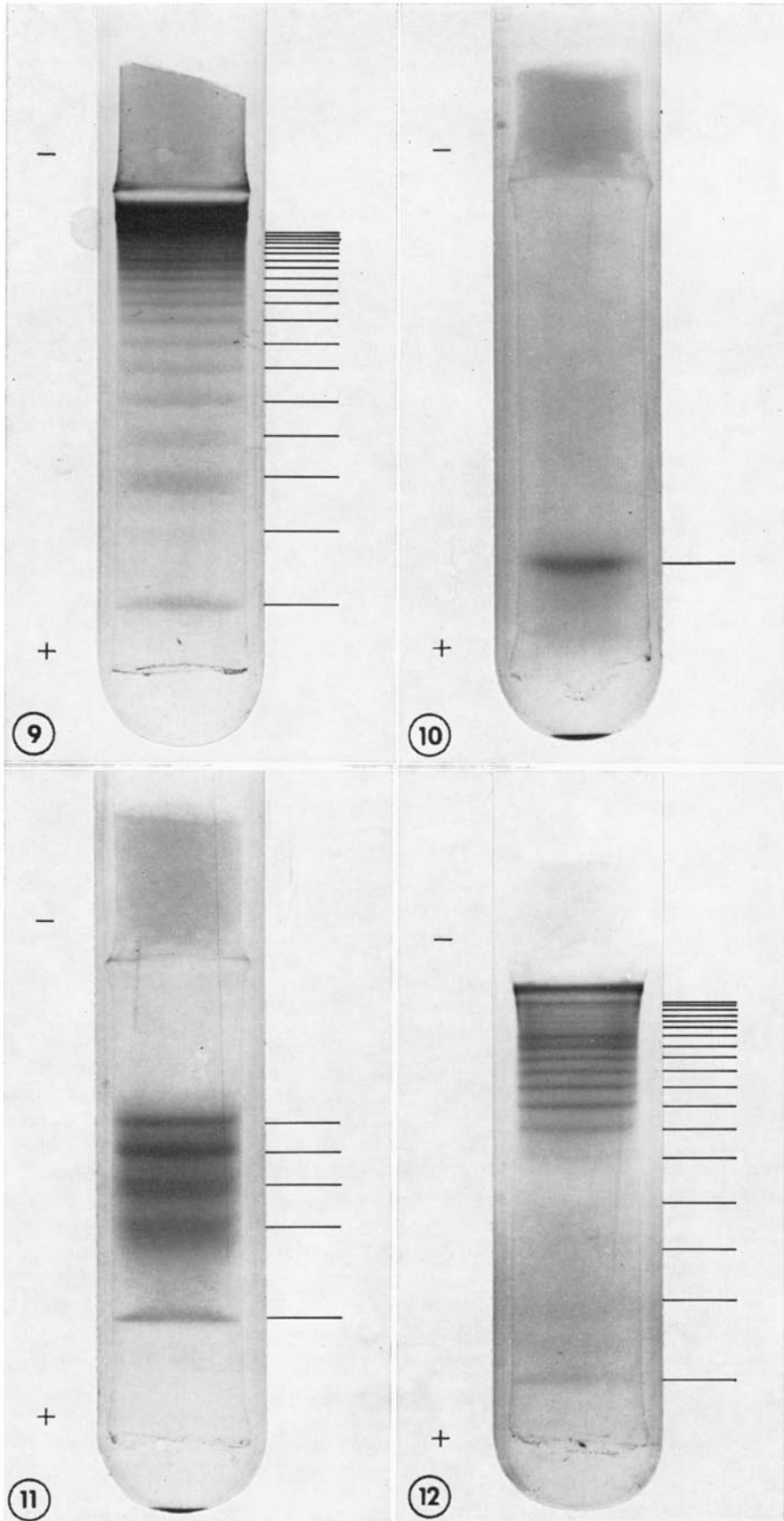
|| Determined colorimetrically by the method of Gaitonde and Dovey (10), using bovine macroaggregates.

results further suggest the homology of the species and also suggest that the nonhomologous species 21 and 22 are either size or charge isomers of KH, as has been previously indicated (25). The similar analyses also indicate that the entire macroaggregate is composed of a homogeneous group of proteins. The analyses resemble those reported for rat and human "histidine-protein" (2, 3, 4, 13, 28), but are markedly different from the analyses of rat KH as reported by Matoltsy and Matoltsy (17).

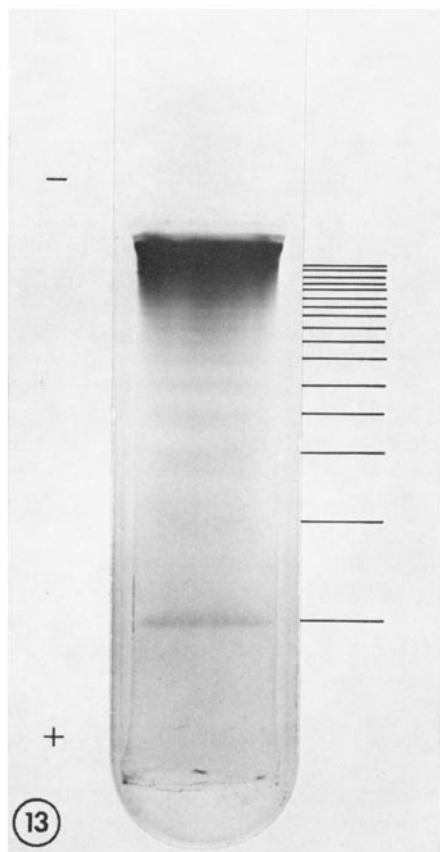
The molecular weight of bovine KH is 14,955 (after rounding off to nearest whole number). Adding 8% (due to the presence of ribonucleotides) (25), the molecular weight is 16,150, which is in fair agreement with the subunit (monomer) molecular weight of 16,900 as determined on 9% polyacrylamide gels by the linear regression of the logarithm of molecular weight on mobility (26).

### Definition of Keratohyalin

Explicit in any definition of KH is the assumption that the particular substance(s) being defined composes *in toto* the *in situ* KH granule. In defining the homologous series as bovine KH, therefore, it is necessary to assume that the *in situ* KH granule is composed entirely of these homologous oligomers, i.e., that the *in situ* KH granule is homogeneous. At present, there are no definitive data to indicate whether the *in situ* KH granule is homogeneous or heterogeneous. However, certain data favor a homogeneous nature for *in situ* KH granules. At the histochemical level, KH stains homogeneously with all stains employed (25). Ultrastructurally, most KH granules appear homogeneous (5, 24, 25), although some granules appear heterogeneous (8, 14). However, as previously demonstrated, macroaggregates of bovine KH may appear very heterogeneous at the ultrastructural level, but are homogeneous at the molecular level (25). Elias et al. have shown that *in situ* bovine KH granules are labeled in accord with the known amino acid composition of bovine KH (7). The possibility of making such a correlation would seem to be dependent upon the presence of a somewhat homogeneous group of proteins. Further, previous studies have demonstrated that all known morphological, histochemical, ultrastructural, and radioautographical characteristics of the *in situ* KH granule can be accounted for by the presence of a single species of ribonucleoprotein in which the



protein species has large amounts of serine, glycine, arginine, and histidine (25). Finally, immunization of rabbits with bovine macroaggregates produces antibody to the entire *in situ* KH granule.<sup>1</sup> The previous statement that there is no



FIGURES 9-13 Electrophoresis of bovine KH fractions on 9% polyacrylamide gels stained with amido black. Fig. 9, electrophoresis of solubilized macroaggregates, demonstrating 20 bands distributed in a geometric series. Nonhomologous species XXI and XXII are not sufficiently separated to be visualized. Fig. 10, electrophoresis of bovine monomer revealing a single prominently stained band. Fig. 11, electrophoresis of fraction II, demonstrating the presence of oligomers 2 through 5, as well as the monomer. Fig. 12, electrophoresis of fraction III, revealing relatively greater amounts of oligomers 6 through 20 and the nonhomologous species, and lesser amounts of the KH monomer and the smaller oligomers. Fig. 13, electrophoresis of fraction IV, demonstrating the presence of relatively greater amounts of oligomers 18 through 20 and the nonhomologous species, but lesser amounts of the other species are also present.

absolute method of identifying KH (at the molecular level), despite the present assumption of homogeneity, may still be true, depending on whether the definition is limited to a particular species (25). In the case of bovine KH, however, it is possible to speculate that KH exists *in situ* as a biopolymer, probably stabilized by salt or hydrogen bonds. Relatively weak salt or hydrogen bonding seems to be involved in macroaggregation whereas stronger noncovalent linkages may be involved in formation of the molecular species which range in size from the monomer to the 20-mer. The subunit (monomer) molecular weight is 16,000-17,000, and is composed of a complex consisting of 8% RNA and 92% protein, the protein being basic and containing large amounts of serine, glycine, arginine, and histidine. The function of KH remains unknown.

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