at *p*H 4.5. The experimental results shown in Fig. 1 and Table 1 are in agreement with this proposition, since this is also the approximate *p*H at which it becomes impossible to isolate the RNA-complex previously formed inside TYMV at alkaline *p*H. Hence it is tempting to see the alkaline treatment of TYMV and its subsequent reversion to *p*H 4.5 as a model of the dissociation and reassociation of the RNA and protein components of this virus. At first there would be a breakage of carboxylate-amino links due to removal of protons at a *p*H exceeding neutrality and a subsequent formation of bonds between RNA fragments leading to complex formation, after which, at *p*H 4.5, the linkage with protein is restored at the expense of the interfragment interactions and leading to the destruction of the complex.

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Keratohyalin: Extraction and in vitro Aggregation

Abstract. *Keratohyalin can be extracted from epidermis by 1.0 molar phosphate buffer (pH 7.0), as demonstrated histochemically and ultrastructurally. If the extracted material is dialyzed against water, it aggregates to form granules that are similar to keratohyalin granules in size, shape, histochemical staining, and electron opacity.*

Several lines of evidence suggest that keratohyalin is a structural protein or protein complex of the epidermis (*1*). This unique substance gradually accumulates in cells just beneath the outermost layer (stratum corneum) of the skin as discrete cytoplasmic granules without a limiting membrane (*2*). As cells containing keratohyalin granules mature and enter the stratum corneum, these discrete granules lose their identity, but little is known about the eventual function of keratohyalin (*3*). Furthermore, keratohyalin granules have not been isolated in sufficient quantity to permit direct physical and chemical studies (*4*).

Studies of nonepidermal tissues have demonstrated that relatively insoluble proteins and protein complexes (such as collagen and myosin) can be partially solubilized by salt solutions or buffers and then aggregated by manipulating the ionic strength of the extracting solution (*5*). Such aggregates may resemble the protein or protein complex in the natural state both morphologically and biologically; study of these aggregates has resulted in considerable data on structure and function.

Using previous extraction and aggregation studies as a model, I have developed a technique (extraction by phosphate buffer and aggregation by dialysis against water) for obtaining granules from the epidermis, which are morphologically and histochemically similar to keratohyalin granules.

Cattle-hoof epidermis was employed in these studies because of the large amount of keratohyalin present. Fresh tissue (1 g, wet weight) obtained from

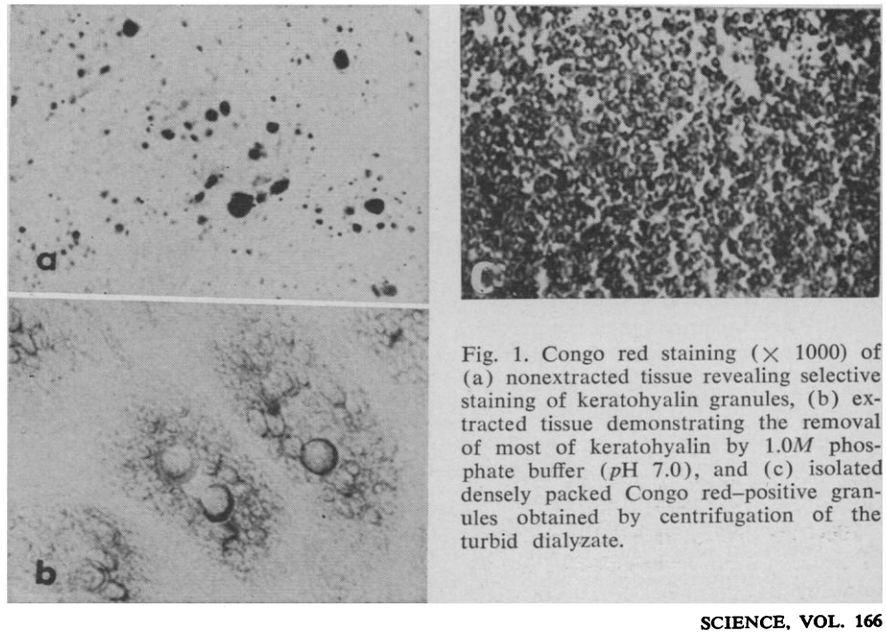

Fig. 1. Congo red staining (× 1000) of (a) nonextracted tissue revealing selective staining of keratohyalin granules, (b) extracted tissue demonstrating the removal of most of keratohyalin by 1.0*M* phosphate buffer (*p*H 7.0), and (c) isolated densely packed Congo red–positive granules obtained by centrifugation of the turbid dialyzate.

just beneath the stratum corneum, was extracted in 10 ml of 1.0*M* phosphate buffer (*p*H 7.0) for 15 minutes at 37°C with gentle stirring. The suspension was decanted, and the extracted tissue was reserved for histochemical and ultrastructural study. The suspension was then clarified by centrifugation at 50,000*g* for 30 minutes at 4°C. The clear supernatant was dialyzed against 100 volumes of distilled water for 48 hours at 4°C. The resultant turbid dialyzate was collected, and pellets were obtained by centrifugation at 50,000*g* for 30 minutes at 4°C.

For light-microscopic examination, extracted and nonextracted tissue and pellets were fixed in 10 percent buffered formaldehyde and then stained with hematoxylin, Pauly's reagent (*6*), or the modified Congo red method without counterstaining (*7*). For electron-microscopic examination, specimens were fixed in 6 percent phosphate-buffered glutaraldehyde for 2 hours at 4°C, postfixed in 1 percent osmium tetroxide, dehydrated in upgraded alcohols, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. Grid preparations were made by placing one drop of the clear supernatant obtained before dialysis or of the turbid dialyzate on a 200-mesh copper grid supported with carbon-coated Parlodion. After 30 seconds, excess liquid was withdrawn with filter paper, and grids were stained with 3.5 percent aqueous uranyl acetate for 15 minutes at room temperature, washed in distilled water for 30 seconds, then dried in air prior to examination of ultrastructure. Additional samples of the supernatant obtained before dialysis and of the dialyzate were studied by phase microscopy.

Light microscopy of nonextracted tissue specimens (Fig. 1a) revealed numerous keratohyalin granules of varying size and shape, which were stained by hematoxylin, Pauly's reagent, and Congo red (*8*). After incubation in 1.0*M* phosphate buffer (*p*H 7.0), most of the material stained by hematoxylin, Pauly's reagent, or Congo red had been extracted (Fig. 1b). Pellets obtained by centrifugation of the turbid dialyzate were composed of densely packed granules which were stained by hematoxylin, Pauly's reagent, and Congo red with the same color and intensity as keratohyalin granules (Fig. 1c).

Electron micrographs of nonextracted tissue specimens revealed numerous keratohyalin granules of varying size and shape. The central mass was finely granular and electron opaque, and no limiting membrane was present. Tonofilaments surrounded portions of the keratohyalin granules (Fig. 2a). Extracted tissue revealed the absence of most of the finely granular electron-opaque material (Fig. 2b). Pellets were composed of granules of varying size and shape. The central mass was finely granular and electron opaque, and no limiting membrane was present (Fig. 2c).

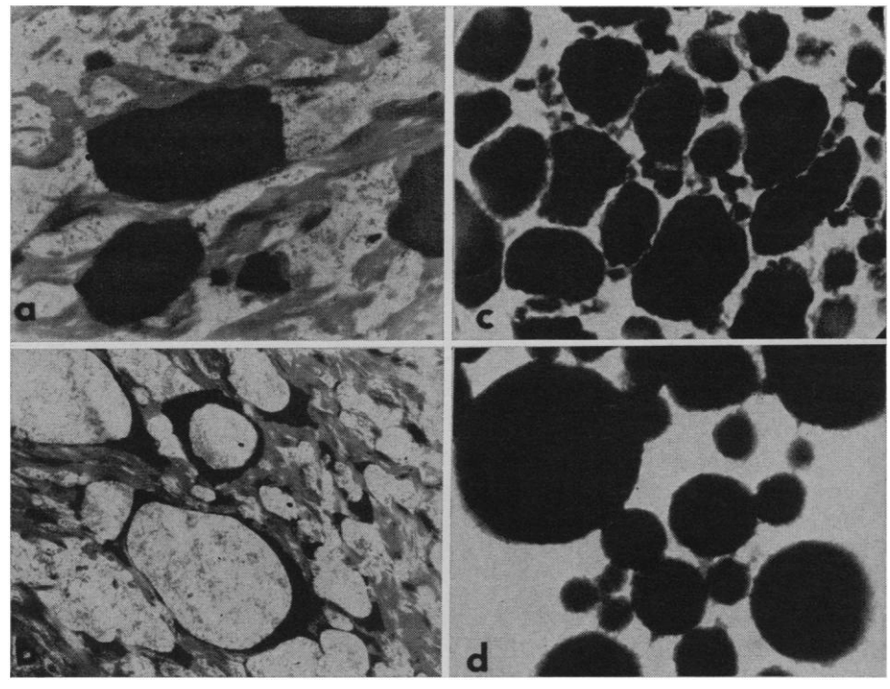

Fig. 2. Uranyl acetate staining (× 35,000) of (a) nonextracted tissue revealing electron-opaque keratohyalin granules of varying sizes and shapes partially surrounded by tonofilaments, (b) extracted tissue demonstrating the selective removal of the majority of the electron-opaque material and a residual fibrillar network, (c) isolated electron-opaque granules of varying sizes and shapes, and (d) grid preparation of granules of varying sizes and shapes obtained from the turbid dialyzate.

When the extracted keratohyalin in the clear supernatant was dialyzed and the resultant turbid dialyzate was examined by phase-microscopy, electron-microscopy (grid preparations), and centrifugation studies, intact granules were evident. However, by the same techniques, granules cannot be demonstrated in the clear supernatant prior to dialysis, an indication of in vitro aggregation of keratohyalin.

Although the isolated granules resemble keratohyalin granules in size, shape, histochemical staining, and electron density, the following should be noted. (i) Keratohyalin was not extracted completely, as evidenced by examination of extracted tissue which revealed residual keratohyalin and a background of fibrillar material within extracted areas (Fig. 2b); (ii) margins of the isolated granules were less densely packed than those of keratohyalin granules (Fig. 2c); and (iii) most important, chemical comparisons between the isolated granules and keratohyalin granules are not possible at this time since the composition of both remain unknown.

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9. I thank M. A. Lutzner and R. G. Crounse for advice and criticism, and W. Idler, V. Hearing, and A. Patterson for technical assistance.

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