A STAINING METHOD FOR KERATOHYALIN GRANULES*1

BY

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ABSTRACT

A selective staining method for keratohyalin granules was examined and reported. This method includes treatment with metal salts and then staining with unoxidized Hematoxylin. Various kinds of metal salts were compared for their specificity and stainability of the granules. The treatment with cobalt salts was considered to be the most satisfactory.

INTRODUCTION

The keratohyalin granules are stained with Hematoxylin, Toluidine Blue, Alizarin Red S, Congo Red, Gentian Violet, Thionin, Acridine Orange, Methyl Green-pyronine, and others, but their identification with staining is not always easy in pathologic lesions of keratinizing squamous epithelium. Pizzolato and Lillie1) revealed that keratohyalin granules in fixed tissue had the property of combining in vitro with a large number of metal salts and forming deeply colored chelate complexed with unoxidized Hematoxylin. On the basis of their report, a method of selectively demonstrating keratohyalin granules was examined in the present study.

MATERIALS AND METHODS

The keratinizing squamous epithelium was obtained from the tongue of mice, and from squamous cell carcinoma, leukoplakia, and keratocyst of human beings. The specimens were fixed 2–3 days in 10% neutral buffered Formalin, dehydrated in increasing concentration of ethanol, and embedded in paraffin as usual. They were sectioned at 4–5 μ and the sections were dewaxed routinely.

The staining procedures were as follows: (1) Treatment for 3–4 hr with

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0.01 M solution of various metal salts. (2) Washed 15 min in distilled water. (3) Stained for 3-4 hr in unoxidized Hematoxylin (1/10,000 dilution in 0.01 M phosphate buffer, pH 7.0). (4) Dehydrated in graded ethanol and mounted in synthetic resin.

Various metal salts used in the staining were chlorides of Al, Ca, Co\(^{2+}\), Cu\(^{+}\), Fe\(^{2+}\), Fe\(^{3+}\), Mn, Ni, Pb, Zn, nitrates of Al, Ca, Co\(^{2+}\), Cr, Fe\(^{2+}\), Ni\(^{2+}\), Pb, Zn, and sulfates of Cu\(^{+}\), Fe\(^{3+}\), Mn. All these chemicals were standard laboratory reagents of the highest purity available.

To determine if metal-containing fixatives might have any effect on staining, some specimens were fixed in osmium tetroxide, Zenker solution, and basic lead acetate.

For studying the mechanism of the above staining, methylation (Fisher and Lillie), acetylation (McManus and Cason), and sulfation (McManus and Mowry) were carried out before staining\(^2\). Effect of prior enzymic incubation on staining was also examined with saliva, trypsin (Difco), and ribonuclease (Ribonuclease A from bovine pancreas, Sigma)\(^3\).

**Results**

In this method, the use of most metals resulted in blue staining of keratohyalin granules, but in the case of some metals the granules were stained violet or green. The preparations treated with various metal salts were compared with each other for the degree of specificity of the keratohyalin granules from other tissue elements of the epidermis, especially keratin and prekeratin, and of stainability, that is, intensity of staining. The preparations treated with Fe\(^{2+}\), Fe\(^{3+}\), Mn, and Pb salts were poor in specificity and stainability (Fig. 1). Those treated with Al salts were good in intensity of staining, but specificity was unsatisfactory (Fig. 2). Treatment with Cu\(^{1+}\) salts stained the entire tissue greenish (Fig. 3). Specificity and stainability proved to be good in those treated with Co\(^{2+}\), Ni\(^{2+}\), and Ca salts (Figs. 4, 5 and 6). Of the metal salts examined, Co salt was considered to be the most satisfactory in both specificity and stainability. The stainability was well preserved for 2 years after staining.

The granules were clearly demonstrated in the specimens fixed in the metal-containing fixatives, but the staining of granules was better after fixation with 10% neutral buffered Formalin.

A previous treatment with methylation, acetylation, and sulfation brought out slight changes in color, but did not inhibit staining. Staining disappeared after prior digestion with trypsin. Even after prolonged incubation with saliva and ribonuclease, the granules could be stained.
DISCUSSION

The possibility of a selective demonstration of keratozyalin granules was suggested by the characteristic feature of keratozyalin granules to combine with metal salts. Hematoxylin was usually used in previous conjunction with metallic mordants, such as aluminum, iron, and chromium. In this method, however, the metal salts combined with the granules probably acted as mordants in the formation of Hematoxylin lake. Seki and Baker explained by ionic bond the mechanism of Hematoxylin staining with formation of the tissue-mordant dye complex.

The unoxidized Hematoxylin usually stained the keratozyalin granules in differing shades of color depending on the metal salt used. Of various kinds of metal salts examined, salts of cobalt proved to be the most excellent in stainability and specificity for these granules.

The exact mechanism of metal binding with keratozyalin granules has not been explained, but some ideas were reported. Lansing and Opdyke speculated, from observations of complete dissolution of keratozyalin granules on incubation with trypsin, the possibility of the binding of protein in the granules with metals. Our study also showed disappearance of staining after digestion with trypsin. Jessen divided electron microscopically keratozyalin granules into a single granule and a composite granule, saying that one of the two components of the composite granule was digestable with pepsin.

Pizzolato and Lillie stated that the histidine hypothesis was attractive in view of Eichler and Mayer’s findings that histamine formed dark blue complexes with copper, nickel, and cobalt but not with iron, manganese, or zinc. Reaven and Cox believed that histidine might be largely responsible for the selective in vitro zinc-binding property of the keratozyalin granules. It has been known that keratozyalin granules contain metals. Smith and Parkhurst reported that the deep color in the keratozyalin granules produced by unoxidized Hematoxylin and Alizarin Red S was due to a large amount of metallic mordant in these granules.

By incubation with ribonuclease the staining was not abolished in our experiment, but many authors reported that Toluidine Blue staining of keratozyalin granules was abolished or partly abolished by previous incubation with ribonuclease, suggesting that the cornifying epithelium had a large ribonucleic acid content. Hicks thought that the incomplete abolition of the Toluidine Blue reaction and persistence of strong Hematoxylin staining after ribonuclease digestion indicated the presence of some additional basophilic material, probably protein, in the keratozyalin granules. Fukuyama and Epstein showed that the granules were actually the site of synthesis of protein forming keratin.
Ugel demonstrated that keratohyalin could be extracted as macroaggregates with 0.1 M potassium phosphate buffer (pH 7.0), and Guss and Ugel verified antigenicity of macroaggregates formed \textit{in vitro} when dialyzed against distilled water. It is suggested from these experiments that \textit{in vitro} examination of binding mechanism of the macroaggregates with metal is meaningful.

\textbf{References}

EXPLANATION OF FIGURES

The photographs show keratinizing squamous epithelium of the tongues of mice previously treated with 0.01M solution of various metal salts. Magnification was ×300 in all pictures.

Plate 1

Fig. 1: Pb(NO₃)₂, Fig. 2: AlCl₃·6H₂O. Fig. 3: CuSO₄·5H₂O

Plate 2

Fig. 4: Co(NO₃)₂·6H₂O, Fig. 5: Ni(NO₃)₂·6H₂O. Fig. 6: Ca(NO₃)₂·4H₂O