

ERYTHROPOIETIC PROTOPORPHYRIA

Histochemical Study of Hyaline Material

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Abstract. The histochemical property of the hyaline material was studied in a case of erythropoietic protoporphyria. Alcian blue, azure A and periodic acid Schiff were used to stain mucopolysaccharides. Bromphenol blue was employed for detection of basic proteins. In a further attempt to identify various polyanions histochemically, staining was carried out with alcian blue containing various concentrations of electrolytes. Methylation, saponification, mild acid hydrolysis and digestion with streptomycetes or testicular hyaluronidase, or chondroitinase ABC were also employed. The results obtained indicate that the hyaline material found in this case of erythropoietic protoporphyria contained sialic acid-containing mucoprotein, and appeared to be histochemically similar to that in hyalinosis cutis et mucosae.

Porphyria has been classified as follows: 1) porphyria congenita, 2) erythropoietic protoporphyria, 3) acute intermittent porphyria, 4) porphyria cutanea tarda, and 5) combined porphyria.

Erythropoietic protoporphyria was overlooked for a long time in contrast to the other four types, since the urine contains no porphyrines. However, protoporphyrine and occasionally also coproporphyrine are present in increased amounts in the stool, serum, erythrocytes, and the bone marrow. Hornstein & Klingmüller (4, 6) found a homogeneous, basophilic material in the upper dermis of such a patient, and drew attention to the histological resemblance to hyalinosis cutis et mucosae. The same observation was made by Redeka et al. (12) who considered the homogeneous material to be degenerated collagen. With histochemical techniques, some workers (1, 14) showed that this material contains carbohydrate-protein-lipid complexes. Recently, van der Walt & Heyl (20) compared, histochemically, erythropoietic protoporphyria with hyalinosis cutis et mucosae, and found that the distribution and

eventual degree of hyalinization is quite different in the two conditions. However, no report has been presented on the precise nature of the carbohydrate component. This study was designed to analyse histochemically the carbohydrate component of the homogeneous material by applying the concept of the critical electrolyte concentration to the differentiation of polyanions.

MATERIALS AND METHODS

Biopsy skin specimens were obtained from the exposed and unexposed areas of a 5-year-old boy with erythropoietic protoporphyria. Each specimen was fixed in 10% neutral formalin for 24 hours. After being dehydrated and paraffin-embedded in the usual fashion, they were cut at 6 μ in thickness. In addition to routine staining methods (hematoxylin and eosin, picrofuchsin, trichrome, phosphotungstic acid hematoxylin, and resorcin fuchsin), the following techniques were employed.

1. *Alcian blue.* Alcian blue was dissolved to make a 0.05% solution in 0.05 M acetate buffer of pH 5.8. The solution was distributed in several dye baths and to these magnesium chloride or sodium chloride was added sequentially so as to give molarities from 0.025 to 2.0. Sections were stained for 12 hours in respective dye baths, then rinsed three times for 5 minutes in baths containing the same buffer, and same concentrations of same electrolytes as used for staining. After rinsing, the sections were washed three times with distilled water in 3-minute baths and dehydrated, cleared and mounted.

2. *Azure A.* Sections were stained for 30 minutes with 0.05% azure A in a 0.1 M phosphate-HCl or phosphate-citrate buffer of a selected pH at room temperature. The sections were blotted dry (not dehydrated in alcohol) and cleared in acetone-xylol. Some sections were stained with 3% azure A heated at 70°C.

3. *Periodic acid Schiff (PAS).* Sections were stained according to the method of Lillie (8). In some sections, a two-step PAS method was carried out as suggested by Scott & Dorling (19), i.e., sections were oxidized for 1 hour at 30°C in 2% sodium metaperiodate solution,

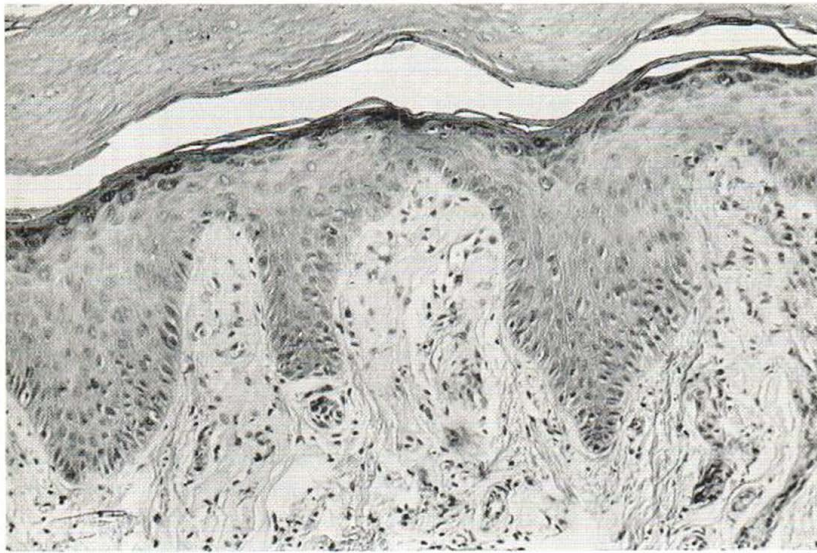


Fig. 1. Abundant hyaline material in the upper dermis. Hematoxylin and eosin stain, $\times 105$.

and washed with distilled water. Then the sections were immersed for 3 minutes in 1% sodium borohydride solution, washed with distilled water, and oxidized for 24 hours at 30°C in 2% osmium metaperiodate. After washing with distilled water, the sections were immersed for 30 minutes in Lillie's cold Schiff reagent, followed by three rinses of 1, 2 and 3 minutes in sulfite solution, and washed finally with distilled water.

4. *Mercuric bromphenol blue*. Sections were stained with the technique of Mazia et al. (10).

5. *Lipid extraction*. Sections were immersed into a 1:1 chloroform/ethanol mixture for 12 hours at 70°C.

6. *Methylation*. Sections were placed in 0.1 N HCl in absolute methanol for 4 hours at 60°C.

7. *Saponification*. Sections were immersed in a 1% potassium hydroxide solution in 70% ethanol for 30 minutes at 25°C.

8. *Acetylation*. Sections were placed in a 2:3 mixture of acetic anhydride/pyridine for 6 hours at 22°C.

9. *Mild acid hydrolysis*. Sections were placed in a 0.1 M acetate buffer at pH 2.5 for 1 to 4 hours at 37, 55 or 70°C.

10. *Diastase digestion*. Malt-diastase was dissolved in 0.02 M phosphate buffer at pH 6.0, in a concentration of 1 mg per ml. Sections were incubated for 1 hour at 37°C.

11. *Hyaluronidase digestion*. Testicular hyaluronidase was dissolved in a 0.1 M phosphate buffer at pH 6.5, in a concentration of 0.5 mg per ml. Sections were incubated for 1 hour at 37°C. Streptomyces hyaluronidase was dissolved in a 0.02 M acetate buffer at pH 5.0, in a concentration of 30 TRU per ml. Sections were incubated for 1 hour at 40°C.

12. *Chondroitinase ABC digestion*. Chondroitinase ABC was dissolved in a 2.5 mM Tris-HCl buffer at pH 8.0, in a concentration of 5 U per 0.3 ml. Sections were incubated for 2 hours at 37°C.

13. *Sialidase digestion*. Sialidase digestion was carried out as described by Luna (9).

Malt-diastase and testicular hyaluronidase were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A., streptomyces hyaluronidase from Amano Pharmaceutical Co., Nagoya, Japan, chondroitinase ABC from Seikagaku Kogyo Co., Tokyo, Japan, and sialidase from General Biochemical Co., Chagrin Falls, Ohio, U.S.A., respectively.

RESULTS

No abnormality was found in the sections obtained from the clothed areas. In the sections from the exposed areas, the epidermis and appendages appeared normal. In the upper dermis, there was abundant, faintly eosinophilic hyaline material (Fig. 1). The tissue in this area included numerous distended or slit-like capillary spaces, which were lined with flat endothelial cells and did not show distinct walls. Elastic fibers in the hyaline areas (Fig. 2) were reduced in number.

The hyaline material stained yellow with picrofuchsin, greenish grey with trichrome, brownish grey with phosphotungstic acid hematoxylin, and showed a positive reaction for PAS with routine or two-step procedure (Fig. 3). When acetylation was followed by routine procedure, the reaction was completely abolished. However, acetylation prior to two-step procedure had almost no effect on the reaction, and the reaction also persisted even after lipid extraction.

The hyaline material showed a strong affinity for alcian blue at pH 5.8. When the sections were immersed in alcian blue solutions containing

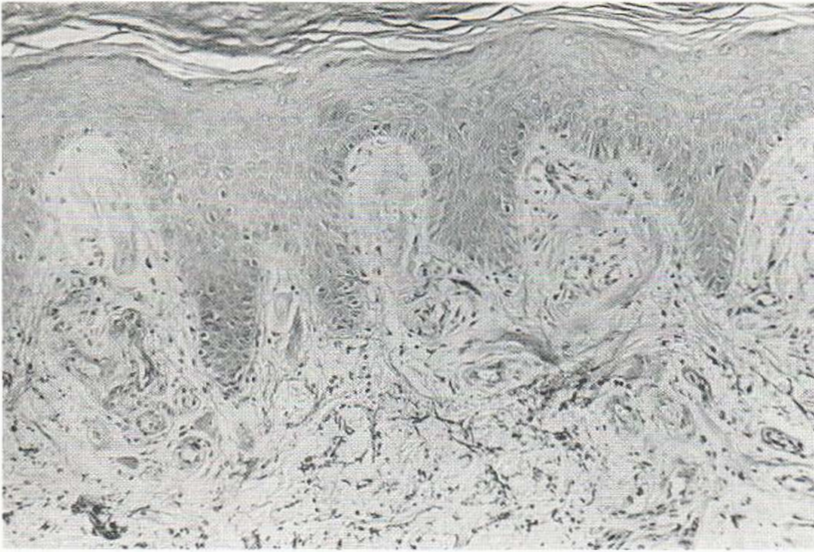


Fig. 2. Elastic fibers are less numerous in the hyaline areas. Resorcin fuchsin stain, $\times 105$.

different concentrations of magnesium chloride from 0.01 to 0.15 M, no variation was found in the affinity for alcian blue (Fig. 4). The affinity was slightly reduced in the presence of magnesium chloride at 0.2 M, and markedly at 0.4 M (Fig. 5). When alcian blue solutions were buffered at pH 2.5, the hyaline material showed only weak affinity for alcian blue, but this was greatly enhanced by addition of magnesium chloride. The affinity persisted at higher concentrations of magnesium chloride, although diminished at 0.8 M or higher. When sodium chloride was added to alcian blue solution, the affinity weakened slightly

at 0.5 M and markedly at 0.8 M. On the other hand, the material showed an azurophilia at pH 3.0 or a higher pH range. At pH 4.0 or a higher pH range, the material was metachromatic. When the sections were stained with 3% azure A at 70°C, it showed a strong metachromasia at pH 2.5 or a higher.

Methylation for 4 hours at 60°C slightly reduced the PAS reaction of the hyaline material. The 30-minute saponification following methylation failed to change the reaction intensity. The affinity for alcian blue completely disappeared after methylation. When saponification was car-

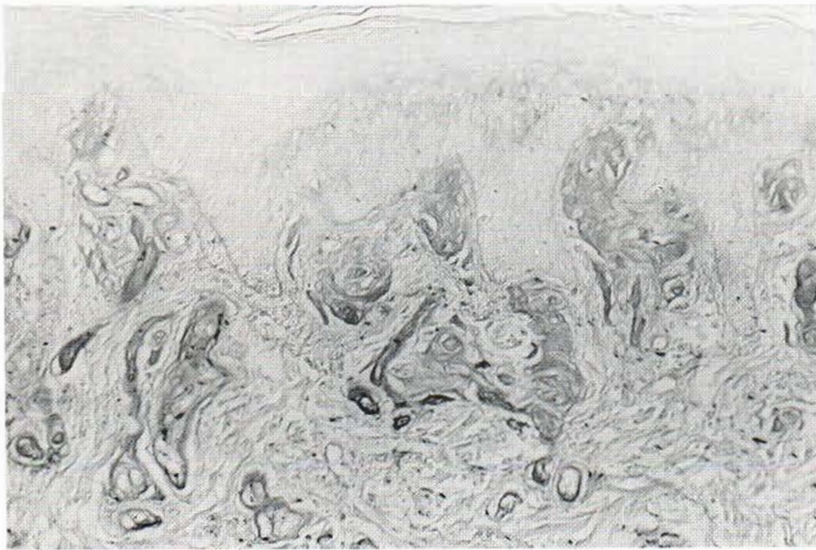


Fig. 3. The hyaline material is strongly reactive to PAS. PAS stain, $\times 105$.

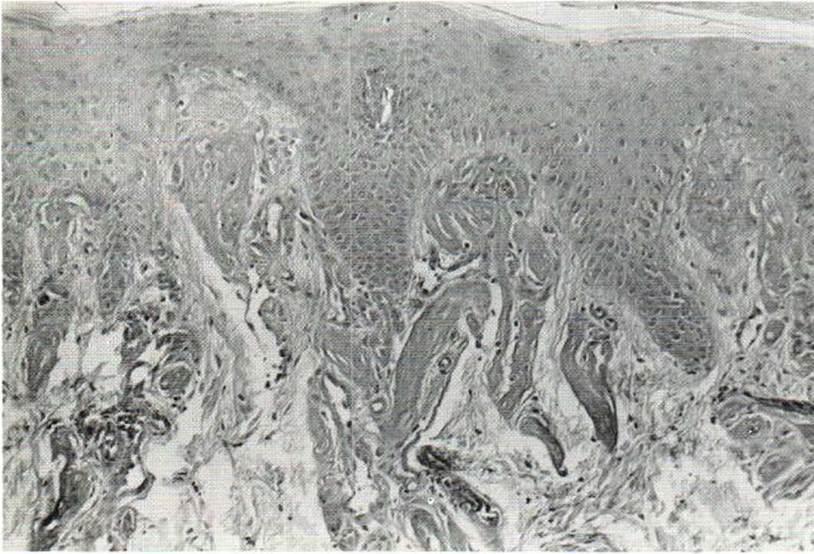


Fig. 4. In the absence of $MgCl_2$, the hyaline material shows a strong affinity for alcian blue. Alcian blue stain at pH 5.8, $\times 105$.

ried out after methylation, the affinity was partly recovered in the presence of magnesium chloride at 0.1 M or less. Mild acid hydrolysis for 4 hours at 55°C failed to change the staining behavior with any of the methods. However, when the sections were incubated in acid solution for 4 hours at 70°C, both the PAS reaction and affinity for alcian blue showed a decrease in intensity.

Malt-diastase or streptomyces hyaluronidase digestion failed to change the staining with any of the methods used. Treatment with testicular hyaluronidase, chondroitinase ABC or sialidase weakened the intensity of staining, although the

degree differed from one staining to another. The staining did not completely disappear, even when treatment with chondroitinase ABC followed by sialidase was carried out.

The hyaline material showed a weak reaction with mercuric bromphenol blue, and after methylation for 4 hours at 60°C, it was enhanced.

DISCUSSION

In general, carbohydrates are mainly responsible for PAS reaction. Theoretically, all periodate-oxidizable polysaccharides, whether acid or neu-



Fig. 5. The affinity of the hyaline material for alcian blue is markedly reduced in the presence of $MgCl_2$ at 0.4 M. Alcian blue stain at pH 5.8, $\times 105$.

tral, should stain. In practice, however, it has been considered that acid mucopolysaccharides fail to show a positive PAS reaction. Recently, Scott & Dorling (19) found that polysaccharides containing 1:4 linked uronic acids are specifically oxidizable in the 2,3-glycol group of the uronic acid by periodate, even though it is slow in comparison with neutral polysaccharides, and that the oxidation products react strongly with Schiff reagent. Furthermore, some lipids are capable of reacting with periodate because they contain a primary acylated amino group adjacent to a hydroxyl group. In contrast to carbohydrate, however, the PAS reactivity of lipids cannot be prevented by prolonged acetylation, though it disappears after lipid extraction. In the present study, the hyaline material was shown to be reactive by means of the routine or two-step PAS method. Treatment with chloroform-ethanol or diastase did not affect the PAS reactivity of the hyaline material, which result suggests that the PAS reactivity of the hyaline material is related to the presence of neutral and acid mucopolysaccharides.

Acid mucopolysaccharides are demonstrated histologically by showing a strong affinity for basic dyes. Dempsey et al. (2), taking into account the pK values of the carboxyl, phosphate and sulfate groups, suggested that the type of acid group which is responsible for the staining reaction might be determined by means of basic dyes buffered at different pHs. At high pH values, however, the carboxyl, phosphate and sulfate groups are all ionized. When basic dyes are buffered at low pH, the anionic sites of the substrate interact with the cations of protein, thus preventing a complete binding with the dye cations. Some workers (14, 20) indicated that the hyaline material showed a weak affinity for alcian blue at pH 2.5, and stained metachromatically with basic dyes at pH 4.0 or higher. Larsen (7) showed that at a high temperature, highly concentrated dye cations can exchange with the blocking protein. Electrolyte is known to be capable of splitting polyanion-polycation complexes at pH 2.5, thereby increasing the availability of the polyanion to alcian blue (11). Under these conditions, the present material showed a strong affinity for alcian blue, and stained metachromatically with azure A at pH 2.5 or higher. These results suggest the coexistence

of acid mucopolysaccharides (which are azurophilic at pH 2.5 or a higher pH range) and proteins.

All polyanions are precipitated by quaternary ammonium salts such as cetylpyridinium chloride, and the water-insoluble polyanion-cetylpyridinium chloride complexes are soluble in salt solutions, concentration of which is maintained above a certain concentration. The author's earlier study (17), using spots of acid mucopolysaccharide solution on filter paper, indicated that hyaluronic acid shows an affinity for alcian blue in the presence of magnesium chloride at 0.1 M, chondroitin sulfate B and heparitin sulfate at 0.6 M, chondroitin sulfates A and C at 0.7 M, and heparin at 0.9 M. On the other hand, when sodium chloride was used as an added electrolyte, the affinity of acid mucopolysaccharides for alcian blue disappeared at 0.2 M (hyaluronic acid), 0.7 M (heparitin sulfate), 0.8 M (chondroitin sulfate B), 0.9 M (chondroitin sulfate A) and 1.0 M (chondroitin sulfate C and heparin), respectively. Furthermore, the pattern of the critical electrolyte concentration of each acid mucopolysaccharide in tissue sections was shown to be similar to that in the model experiment, although the former was about 30% higher than the latter (17). An affinity of the hyaline material studied here for alcian blue reduced in the presence of magnesium chloride at 0.1 M or of sodium chloride at 0.5 M, and disappeared at 0.4 M (magnesium chloride) or at 0.3 M (sodium chloride). The affinity disappeared after methylation, and was partly restored by subsequent saponification. On the other hand, treatment with testicular hyaluronidase or chondroitinase ABC reduced the affinity, though streptomycetes hyaluronidase digestion failed to change it. Streptomycetes hyaluronidase degrades only hyaluronic acid, while testicular hyaluronidase attacks not only hyaluronic acid but also chondroitin sulfates A and C (18). Also, chondroitinase ABC degrades hyaluronic acid, chondroitin and chondroitin sulfates A, B and C (18). Therefore, the results mentioned above suggest the presence of sulfated mucopolysaccharides and of polycarboxylates other than hyaluronic acid. In addition, treatment with sialidase reduced the affinity for alcian blue and the PAS reactivity. Similar results were obtained by the mild acid hydrolysis procedure. Accordingly, it is likely that some reactive carboxyl

groups of the hyaline material is related to those of sialic acid.

On the nature of the hyaline material in hyalinosis cutis et mucosae, many histochemical reports have been presented. Some workers (1, 3-6, 16, 20) indicated the similarity of the histochemical and ultrastructural features between hyalinosis cutis et mucosae and erythropoietic protoporphyria. And the results obtained from the present study were similar to those in hyalinosis cutis et mucosae. Concerning the origin of the hyaline material, it has not been clarified. Rodermund & Klingmüller (13), studying hyalinosis cutis et mucosae with electron microscope, considered that the primary changes seem to occur at the site of the deposits followed by the transudation of plasma-proteins. In erythropoietic protoporphyria, Ryan & Madill (15) described how the hyaline material is derived from constituents of the blood or from the vessels themselves rather than resulting from degeneration of dermal connective tissue. However, Kint (5) indicated that the hyaline material which was found among the collagen bundles by Ryan & Madill, has a similar structure to that in hyalinosis cutis et mucosae, and he suspected the disturbance in the metabolism of the fibroblasts in these diseases. The problem requires a more precise investigation of the composition of the hyaline material.

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