THE EFFECT OF METHYLATION ON BASOPHILIA (METACHROMASIA)

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The methylation reaction is widely used in histochemistry to study the nature of the basophilia of various tissues [7, 8, 25]. However, because there is more than one chemical basis for this reaction [9, 22, 23] the mechanism of the suppression of the basophilia in each instance requires a separate analysis. In the present work an attempt has been made to carry out such a histochemical analysis based on certain chromotropic structures of the intestinal wall (vermiform appendix of the rabbit) whose basophilia is associated with the presence in them of mineral and organic anionic groups.

EXPERIMENTAL METHOD

The tissue was fixed in Shabadash, and after dehydration it was embedded in paraffin. Sections were stained in a 0.01% solution of toluidine blue prepared with citrate buffer (pH 1.9-6.6), with basic brown [3], with the Schiff-iodic acid reaction [2, 16], with a 0.05% solution of Nile blue sulfate [12]. Methylation was carried out with a mixture of methyl alcohol and a 0.1 N solution of HCl at 55° for $2^1/_2$ h. Demethylation by a 20 min treatment with a 1% solution of KOH in ethyl alcohol at 80° [15]. Sulfating of the free hydroxyl groups was carried out by treatment of the preparations with a mixture of concentrated sulfuric acid and diethyl ether for 30 min at room temperature [10, 19]. The stained preparations were placed in glycerine-gelatine and after dehydration in a mixture of acetone and alcohol were embedded in Canada balsam.

EXPERIMENTAL RESULTS

Staining with the 0.01% Solution of Toluidine Blue. In the unsulfated sections a proportion of the flask-shaped cells of the epithelium of the crypt showed a marked metachromasia (pH 6.6-1.9 and less). At pH 6.6 a small proportion of the cells lying deep in the crypt was stained weakly, or remained unstained. The granules of a basophil pigment in the reticular cells, and cytoplasm of the macrophages of the lymphatic follicles were stained orthochromatically in various shades of green (pH 6.6-4.0). In some reticular cells forming groups at the base of the follicles the small pigment granules acquired a weak metachromatic stain. At pH 1.9 the cytoplasm of the macrophages as well as a considerable proportion of the pigment granules remained unstained; only some of them retained the ability to acquire a weak stain. The weak metachromasia of the separate granules remained practically unchanged. Methylation completely blocked the metachromasia in the flask-shaped cells and reduced the basophilia of the cytoplasm of the macrophages and of the pigment granules. Nevertheless to eliminate it completely, in the latter case a more prolonged treatment of up to 5 days or more was required.

If the methylated sections were treated with an alkaline alcoholic solution before they had been stained, the basophilia which had been weakened or completely eliminated in the structures described was renewed. Also, the initial relationship to the reaction of the medium was maintained. In sections which had been sulfated before they had been stained the extent of the basophil material was increased. All the flask-shaped cells began to show a metachromasia (pH 6.6-1.9 or less). It is important to note that after such treatment the numerous microbial cells which

were consistently found to be present in the submucosa of the appendix began to show up clearly owing to the metachromasia developed (pH 6.6-1.9 or less).

After sulfation the chromatism of the pigment granules became more marked and more resistant even than at low pH values. The procedure had no influence on the weak metachromasia of any part. Methylation of the sulfated sections completely suppressed the metachromasia in the flask-shaped cells, and in the bacteria. The basophilia of the pigment was then greatly weakened, but not entirely eliminated. Treatment with alkali after methylation restored metachromasia of such sections to the flask-shaped cells (pH 6.6-1.9 and below), and the pigment granules showed a marked basophilia (orthochromasia) (pH 6.6-4.0); there was an increased tendency to metachromasia of some portion, and it became well marked also in the dehydrated preparations. After treatment of the sections with alkali the microbial cells whose metachromasia was blocked by methylation remained as before unstained and invisible in the preparations.

Stain with Basic Brown. In all sections only a very small proportion of the flask-shaped cells of the intestinal epithelium were stained. The stain was completely blocked by previous methylation, but was restored by treatment with alkali. In the sulfated sections all the flask-shaped cells were stained (including those lying deep in the crypts); also the bacteria, cytoplasm, macrophages, and pigment were stained. The time for which the stain lasted after sulfation was reduced from 1-2 h to 20-25 min. Methylation of sulfated sections blocked staining in all the structures mentioned but subsequent treatment with alkali restored it in the flask-shaped cells of the epithelium only. In this case the duration of the stain was increased to 1-2 h.

PAS-Reaction. A positive reaction was found in the mucus of every single flask-shaped cell, in the bacteria, and also diffusely in the cytoplasm of the macrophages, and in most of the large pigment granules of the reticular cells of the lymphatic follicles. Methylation by itself or combined with subsequent treatment with alkali did not influence the PAS-reaction in these structures. Previous sulfation completely blocked the reaction in the mucus of the flask-shaped cells and in the bacteria, but some action remained in the pigment granules. A weak staining of the pigment by the Schiff reagent developed under these conditions without oxidation in potassium periodate, whereas its natural color remained unchanged. After methylation of such sections the mucus of the flask-shaped cells and the bacteria once more became PAS-positive. However, in the pigment granules the PAS-reaction developed more weakly than usual. Treatment with alkali did not change the distribution of the PAS-positive material in the sections, nor did it affect the intensity of this reaction in any case.

Staining with a 0.05 Nile Blue Sulfate. Most granules stained various shades of blue. After methylation a portion of the pigment material, consisting mainly of the small granules, took on a purple shade. After prolonged methylation for $6\frac{1}{2}$-7 h the color changed into a well-marked pink. Also in the large pigment granules the blue color was greatly reduced in intensity, and changed into a greenish hue. Treatment in alkali had the effect that the pigment again assumed a blue color.

The results obtained indicate that the mucus of the flask-shaped cells of the intestinal epithelium contains acid (sulfated) mucopolysaccharides. The alcohol-resistent metachromasia, and staining of these cells with basic brown due to the presence of mucopolysaccharides were reversibly suppressed by methylation. This result indicates that the nature of the interaction of the $-SO_3H$-groups of acid mucopolysaccharide with the methyl group consists in the formation of an easily saponifiable ester bond. Evidently the PAS-positive properties of mucus cannot be directly associated with the acid mucopolysaccharides [1, 11, 21], and probably depend upon the presence in it of a mucoprotein component. The reactive hydroxyls of the latter are readily esterified by sulfuric acid, and the substrate then acquires chromotropic properties but loses the PAS-reaction [10, 15, 18, 19]. The reciprocal relationship between these two properties are preserved even after methylation. Because as has been shown under these circumstances the $-SO_3H$-groups of native acid mucopolysaccharides are blocked by the methyl group, the loss of chromotropic properties and the restoration of PAS positively indicate the liberation of reactive hydroxyl groups, at the base of which lies the mechanism of methanolitic desulfation.

The subsequent treatment of such sections with alkali does not influence the recovery of the PAS-reaction of the epithelial mucus but does restore its metachromasia and ability to stain with basic brown. This reaction mechanism is shown still more clearly in microbial cells, whose cytoplasm contains easily sulfated PAS-positive polysaccharides.

The dependence of the chromotropic properties of the pigment on the reaction of the medium, and its relationship to staining by Nile blue sulfate after methylation and demethylation indicate that the chromotropic properties

are to a large extent associated with the presence of carboxyl radicals which may be reversibly blocked by a methyl group, with formation of neutral esters [6, 12-14]. In addition the presence of a slight metachromasia in a certain portion of the pigment which is preserved at low pH values cannot depend on carboxyl groups, and appears to be due to condensation products of acetal-phosphatides, whose presence in certain PAS-positive fat pigments (chromolipids) together with ethylene bonds has previously been pointed out by several authors [4, 20, 21].

Typically the sulfation of reactive hydroxyls does not confer upon the pigment the property of metachromasia, and in this respect the pigment differs from the substrates where there is no doubt about the presence of a carbohydrate component. This result once more illustrates the importance of structural (steric) properties of the substrate in the mechanism of the development of metachromasia [4, 5, 22, 24]. Evidently if the ideas we have put forward are correct in connection with not only polysaccharides but also chromolipids, as has been demonstrated in this particular case, and if the ideas may be extended to other classes of PAS-positive substances also, yet one more method applicable to the differential analysis of these substances will have been added to the chemical armoury.

SUMMARY

A study was made of the effect of methylation on the chromotropic properties of certain structural elements of the intestinal wall (rabbit appendix) under normal conditions and after previous sulfation. It was found that the cause of the reversibility of natural basophilia due to the presence in the substrate of carboxyl radicals and sulfuric acid polyesters (acid mucopolysaccharides of the intestinal epithelium) was the mechanism of ester linkage formation between the latter and the methyl group. The depression of basophilia occurring after sulfation (and connected with the formation of artificially produced sulfur esters) was reversible and in the nature of a methanolytic desulfation. We discussed the significance of these tests in connection with histological differentiation.

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