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# Histochemical Significance of Green Metachromasia to Toluidine Blue

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*Summary.* The significance of green metachromasia to Toluidine blue O, exhibited by vitelline cells of monogeneans and epicuticle of millipedes is elucidated, as due to phenol-acid mucopolysaccharide complex, as synthetic phenols or synthetic phenol in combination with heparin or hyaluronic acid gave likewise a green colour to Toluidine blue. The green colour is indicative of a metachromatic reaction is suggested, by its being eliminated or suppressed on treatment with metallic cations or detergent like Sodium oleate, as in other metachromatic reactions. A probable involvement of -OH groups in phenol-acid mucopolysaccharide complex is suggested.

## Introduction

Toluidine blue O is known to exhibit metachromasia with sulphatide and acrolein fixed RNA (Feder and Wolf, 1965), as well as with acid mucopolysaccharide (see Walton and Ricketts, 1954). Red and purple metachromasia to Toluidine blue are suggested by Pearse (1961) to indicate the presence of acid mucopolysaccharide and acid mucopolysaccharide-protein complex respectively. The metachromasia caused due to sulphated acid mucopolysaccharides (red) are not extinguished at or below pH 2.0 (Johnson and Helwig, 1963); but with hyaluronic acid the metachromasia was extinguished below pH 4.0 (Adams, 1967). Thus, red and purple metachromasia to Toluidine blue in histochemistry are currently in vogue in aiding in the determination of chemical nature of the substances in tissues.

However, there are reports in the literature to the occurrence of green metachromasia to Toluidine blue by earlier workers. But, a considerable ambiguity exists as to its significance and nature of the substances that are responsible for the metachromasia. Thus the fresh moult epicuticle of *Orconectes virillis* (Travis, 1963a, b), the concrements of hepatopancreatic cells of *Helix pomatia* (Abolins-Krogis, 1960) and the fungal infected endocuticle of queen termite *Odontotermes obesus* (Sannasi, 1969) exhibiting green metachromasia were suggested, as due to the presence of aromatic amino acids, or proteins rich in them, or their derivatives on the basis of correlated occurrence of Millon's-positive substances in this region. Besides, green metachromasia was reported in the organic cups of *Proneomenia agalaophaeriae* and inner cuticle of *Acanthchitona cirinitus* (Beedham and Trueman, 1968), in the exocuticle of scorpion *Palamneus swammerdami* (Krishnan, 1965) and on the vitelline cells of *Parastrigea mexicanus* (Coil, 1969), but the significance in these has neither been discussed nor considered specific.

During the course of an investigation on the fresh, unfixed vitelline cells of monogenetic trematodes *Pricea* and *Protomicrocotyle* by one of us (K.R.) and on

fresh, unfixed epicuticle of fresh moult millipedes *Spirostreptus asthenus* and *Thyropygus poseidon* by the other (M.H.R.), a green metachromasia to Toluidine blue was observed in the regions which were positive to Millon's, argentaffin and alcian blue at pH 2.8, suggesting thereby the probable presence of phenol and acid mucopolysaccharide in the regions referred to above.

In view of the non-uniformity of opinion expressed by previous workers referred to above, and in order to verify whether Millon's-positive substances existing either free or bound with acid mucopolysaccharide as reported by Monné (1959), can give such a metachromatic response to Toluidine blue, a detailed investigation was carried out with Toluidine blue on monophenols, diphenols, and polyphenols, as they were positive to Millon's (Pearse, 1961), as well as phenols in combination with acid mucopolysaccharides.

## Materials and Methods

Toluidine blue O used in the study was supplied by B.D.H. (34077). A 0.1% dilution prepared in distilled water, adjusted to pH 7.0 was used throughout. The following phenolic substances were used: p-cresol (B.D.H. 27821), pyrocatechol (o-dihydroxy benzene B.D.H. 27563), 4-methyl catechol (3-4-dihydroxytoluene B.D.H. 28174), "dopa" DL-3-4-(dihydroxyphenylalanine B.D.H. 37078), resorcinol (B.D.H. 33019), α-naphthol, naphthocresol, pyrogallol (B.D.H. 10226) and hydroquinone (B.D.H. 10312). Besides, the following amino acids were used: DL-B-phenylalanine, DL-tyrosine (B.D.H. 37157) and tryptophane (B.D.H. 37154). Tests were carried out on crystals of the above synthetic substances using a drop or two of 0.1% of Toluidine blue on a porcelaine-tile and the colour reactions were recorded. Solutions of metallicions like 2% ferric chloride and saturated solution of calcium chloride were employed in blocking the reactions of the dye as according to Gersh (1959), Simkiss (1960) and Bresnick and Schwartz (1968) metallic cations are known to block the reaction. In addition, the dye was subjected to the influence of detergent like 0.2% aqueous sodium oleate as it is known to suppress or eliminate metachromasia (Gersh, 1959; Bresnick and Schwartz, 1968).

## Results and Discussion

The results obtained with different phenols are given in Table 1.

It is seen from the Table that p-cresol and all phenols gave an intense green colour, whereas hydroquinone did not show any metachromasia. In the light of the above observation, it is probable that the green metachromasia observed by earlier workers may be due to the presence of one form or the other of the phenol.

Table 1. *Results obtained with the synthetic phenols using 0.1% Toluidine blue*

| Phenols | Colour reactions obtained |
|---|---|
| p-cresol | ++ (green) |
| Pyrocatechol | +++ (green) |
| 4-Methyl catechol | +++ (green) |
| Dihydroxyphenylalanine | + (green) |
| Resorcinol | +++ (green) |
| α napthol | ++ (green) |
| Napthocresol | ++ (green) |
| Pyrogallol | ++ (green) |
| Hydroquinone | − (blue) |

Work of Beedham and Trueman (1968), may show a correlation between green metachromasia and the positivity of such regions to argentaffin test for phenols in the organic cups present in the integument of aplacophorans. Similarly, the green metachromasia exhibited by fungal infected endocuticle of *Odonototermes* may be due to phenol, as the fungal infections could result in the liberation of phenol from other side chains (Sannasi, 1969).

Green metachromasia may not be due to tyrosine or tyrosine containing substances or phenylalanine as suggested by Travis (1963a, b) and Abolins-Krogis (1961), in view of the absence of any metachromasia by the dye with aromatic amino acids (Table 2).

Table 2. *Results obtained with aromatic aminoacids using 0.1% Toluidine blue*

| Aromatic amino acids | Colour reactions obtained |
|---|---|
| Phenylalanine | — |
| Tyrosine | — |
| Tryptophane | — |

—: no colour formed.

Although the exact mechanism underlying the metachromatic reaction is unknown, it is believed to involve dye association or aggregation. This hypothesis is predicted upon the elimination or reduction of metachromasia by low dye concentration, high temperature, high ion concentration or the addition of detergents (Bresnick and Schwartz, 1968). The green metachromasia, as observed here with phenols, is likewise inferred to involve a dye association or aggregation, as the metachromasia was eliminated, when the substrate was pretreated with, solutions of metallic cations like ferric chloride and calcium chloride as well as by detergents like sodium oleate (Table 3).

Table 3. *Results obtained on using 0.1% Toluidine blue with pyrocatechol pretreated with solutions of metallic cations and detergents*

| Substrates | Colour reactions obtained |
|---|---|
| Pyrocatechol | green |
| Pyrocatechol + Ferric chloride | blue |
| Pyrocatechol + Calcium chloride | blue |
| Pyrocatechol + Sodium oleate | blue |

In addition to the elimination of the metachromasia referred to above, it was observed that the metachromasia may be altered under certain conditions. As seen from the Table 4, a mixture of heparin and Toluidine blue exhibiting red colour showed a spectral shift from red to green on addition of a diphenol, pyrocatechol.

Table 4. *Results obtained on pyrocatechol, heparin, hyaluronic acid, and in combinations of these using 0.1% Toluidine blue*

| Substrates | Colour reactions obtained |
|---|---|
| Pyrocatechol + Toluidine blue | green |
| Heparin + Toluidine blue | red |
| Hyaluronic acid + Toluidine blue | red |
| Heparin + Toluidine blue + Pyrocatechol | red to green |
| Hyaluronic acid + Toluidine blue + Pyrocatechol | red to green |
| Heparin + Toluidine blue + Pyrocatechol + Heparin | green |
| Hyaluronic acid + Toluidine blue + Pyrocatechol + Hyaluronic acid | green |
| Heparin + Toluidine blue + Pyrocatechol + Heparin + Toluidine blue | red |
| Hyaluronic acid + Toluidine blue + Pyrocatechol + Hyaluronic acid + Toluidine blue | red |

But, subsequent addition of heparin did not restore red metachromasia which suggests the non-availability of cations of the dye which has already combined with the phenol. The above observation may explain as to why green metachromasia occurs even in the presence of Hale's and alcian blue positive substances in the tissues (Beedham and Trueman, 1968), in the vitelline cells of monogenetic trematodes and in the fresh moult epicuticle of millipedes referred to here (vide infra). That the nonavailability of the cationic dye is responsible for the absence of spectral shift from green to red, is further supported by the reappearance of the red colour, subsequent to the addition of the dye to the above mixture (vide Table 4). Thus the degree of metachromasia is influenced by the ratio of the concentration of the chromotrope to the concentration of the dye, besides others factors as suggested by Gersh (1959). Similar results were obtained with hyaluronic acid substituting heparin (vide Table 4).

That the non-availability of the cationic dye is responsible for the absence of the spectral shift from green to red, is further strengthened from the observation made on a mixture of p-cresol and heparin to Toluidine blue (Table 5).

p-cresol, a monophenol, showed intense green metachromasia to Toluidine blue (Table 1), but the dye when added to p-cresol-heparin mixture showed neither green nor red metachromasia but remained orthochromatic; thus indicating that the only -OH radical of p-cresol is bound with acid mucopolysaccharide, probably by O-glycosidic linkage and not available to react with the dye. Possibly this suggests that the additional -OH in diphenol is responsible in reacting with the dye in producing the green metachromasia, whereas the other -OH possibly combines with acid mucopolysaccharide. In this context the findings regarding the nature of phenolic material in the vitelline cells of monogenetic trematodes reported by us elsewhere (in press) is of interest in that it is suggested to be a diphenol.

Table 5. *Results obtained on p-cresol and in combination with acid mucopolysaccharide using Toluidine blue (0.1%)*

| Substrates | Colour reactions obtained |
|---|---|
| p-cresol + Toluidine blue | green |
| Heparin + Toluidine blue | red |
| Hyaluronic acid + Toluidine blue | red |
| Heparin + Toluidine blue + p-cresol | blue |
| Hyaluronic acid + Toluidine blue + p-cresol | blue |
| Heparin + Toluidine blue + p-cresol + Heparin | blue |
| Hyaluronic acid + Toluidine blue + p-cresol + Hyaluronic acid | blue |
| Heparin + Toluidine blue + p-cresol + Heparin + Toluidine blue | red |
| Hyaluronic acid + Toluidine blue + p-cresol + Hyaluronic acid + Toluidine blue | red |

## Conclusion

Experimental evidences obtained with synthetic phenols and phenol-acid mucopolysaccharide combinations reveal, the green metachromasia to Toluidine blue observed in the vitelline cells of monogeneans and in the epicuticle of fresh moult millipedes, as due to phenol-acid mucopolysaccharide complex.

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