153. **Dimerization of Toluidine Blue Induced by MN-Active Sialoglycopeptides**

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Treatment of human erythrocytes with proteinases trypsin,\(^1\) pronase,\(^2\) and ficin\(^3\) resulted in release of M-, N-, and MN-active sialoglycopeptides which interacted, in aqueous solution, with toluidine blue\(^{1,4}\) to form sialoglycopeptide–toluidine blue complexes exhibiting an absorption maximum on the short wavelength side of the \(\alpha\)-band of the dye. The sialic acid residues\(^{1,4}\) of the sialoglycopeptides play as receptor sites for toluidine blue. This paper describes the binding of toluidine blue by the sialoglycopeptides and the dimer formation of the bound dye molecules by intermolecular reaction.

**Experimental.** The absorption spectra of toluidine blue (Toluidine blue O, E. Merck AG., Germany) in aqueous solutions were measured with a Hitachi recording spectrophotometer EPS-3T in 1 cm quartz cells within 20 min at room temperature.

![Absorption spectra of toluidine blue (TB).](image)

**Fig. 1.** Absorption spectra of toluidine blue (TB).

---: TB (0.001%).
---: TB (0.00125%).
---: TB (0.0015%).
---: TB (0.00175%).
---: TB (0.002%).
Results and discussion. Figs. 1 and 2 show the absorption spectra of toluidine blue aqueous solutions of varying concentrations. The absorption maxima of the spectra are presented in Table I. A peak at 640 m\(\mu\) of the toluidine blue solutions (0.001\%–0.0015\%) corresponds to the \(\alpha\)-band which indicates the presence of the dye monomer.\(^5,6\) The first peak at 640 m\(\mu\) or 638 m\(\mu\) of the toluidine blue solutions (0.00175\%–0.00275\%) corresponds to the \(\alpha\)-band and the second peak at 610–600 m\(\mu\) to the \(\beta\)-band which suggests the presence of the dye dimer.\(^5,6\) The spectra of the combined solutions\(^4\) of toluidine blue (0.001\%) and MN system sialoglycopeptides (each 0.05\%) released by proteinase treatment from human erythrocytes each showed a single maximum at 608–600 m\(\mu\) and had a shoulder at near 640 m\(\mu\) as shown in Table I. Since toluidine blue in 0.001\% shows only the \(\alpha\)-band at 640 m\(\mu\) as described above, the single peak of the sialoglycopeptide (0.05\%)–toluidine blue (0.001\%) systems seems to be the \(\beta\)-band which was formed in the interaction.

Bradley and Wolf,\(^7\) in a spectrophotometric study of interaction between acridine orange and linear polyanions, have presented an aggregation theory. If one molecule of acridine orange reacts with a binding site of the polyanion and both adjacent sites are empty, the dye molecule cannot stack and therefore exhibits the \(\alpha\)-band indicating a monomer. When neighboring two binding sites are occupied by two molecules of the dye, the dyes are stacked and are therefore a dimer exhibiting the \(\beta\)-band. When every site is occupied by the dye, all the dyes are stacked and the spectrum consists of the \(\gamma\)-band.
On the basis of this aggregation theory and of the present results that the sialoglycopeptide–toluidine blue systems each shows the β-band, it is presumed that two molecules of toluidine blue are attracted, in a dimer form, to the neighboring two binding sites of the sialoglycopeptides to form toluidine blue–sialoglycopeptide complexes which show the β-band. The negative binding sites seem to be the carboxylates of two sialic acid residues which are linked on terminals of the carbohydrate prosthetic groups, since the de-sialated sialoglycopeptides (i.e. glycopeptides) and free sialic acid do not react with toluidine blue. Therefore, it is concluded that the sialoglycopeptides each contains sterically adjacent two sialic acid residues which are capable of producing toluidine blue dimer. An absorption shoulder at wavelength around 640 mμ of the sialoglycopeptide–
toluidine blue systems seems to be due to the monomer of the dye either in a complex form or in free form.

Shepard and Geddes\(^8\) have suggested, from a quantitative examination with thiazine dyes in aqueous solution, that aggregation of the dyes may involve the bonding of one molecule of water between neighboring resonating dye ions. This observation has been supported by Weissman \textit{et al.}\(^5\) and Schoenberg and Moor.\(^10\) Therefore, to complete the dimerization of toluidine blue in the interaction with the sialoglycopeptides, one molecule of water must be involved between parallel apposition of the two dye cations which are attached, through an ionic bond,\(^11,4\) to the two carboxylate anions of the sialic acid residues. Linkage between the dye molecules may be stabilized by hydrogen bond\(^10\) through the water molecule.

From the view of Bradley and Wolf\(^7\) on the polymer formation of cationic dye and the present result that the sialoglycopeptide–toluidine blue systems did not exhibit the \(\gamma\)-band, it is possible to presume that the carbohydrate prosthetic groups of the sialoglycopeptides do not contain consecutively regular-spaced sialic acids more than three residues.

Recently, Adamany and Kathan\(^11\) isolated a common tetrasaccharide from M-, N-, and MN-active erythrocyte sialoglycoproteins subsequent to degradation with alkaline sodium borohydride, and tentatively proposed N-acetyleneuraminyl-(2\(\rightarrow\)2/4)-galactosyl-(1\(\rightarrow\)3) [N-acetyleneuraminyl-(2\(\rightarrow\)6)]-N-acetylgalactosaminol structure for the tetrasaccharide. Thomas and Winzler\(^12\) simultaneously reported that treatment of M- or N-active sialoglycopeptide, derived from human erythrocytes by trypsin treatment, with alkaline borohydride released a common tetrasaccharide, N-acetyleneuraminyl-(2\(\rightarrow\)3)-\(\beta\)-D-galactopyranosyl-(1\(\rightarrow\)3) [N-acetyleneuraminyl-(2\(\rightarrow\)6)]-D-N-acetylgalactosaminitol. They\(^12\) also described that the tetrasaccharide is the major, alkali-labile oligosaccharide. Both the studies\(^11,12\) clearly show that the carbohydrate prosthetic groups linked, through O-glycosidic bonds, to the serine or threonine of polypeptide core contain the N-acetyleneuraminyl-(2\(\rightarrow\)3) or (2\(\rightarrow\)2/4)-\(\beta\)-D-galactopyranosyl-(1\(\rightarrow\)3) [N-acetyleneuraminyl-(2\(\rightarrow\)6)]-D-N-acetylgalactosaminyl residue. This observation supports the above-mentioned conclusion that the carbohydrate prosthetic groups of the sialoglycopeptides in this work contain sterically adjacent two sialic acid residues which are capable of producing toluidine blue dimer.

For binding form between two molecules of toluidine blue and the two sialic acid residues of the M-, N-, and MN-active sialoglycopeptides, the schematic illustration as shown in Fig. 3 is proposed. The dimerization process of toluidine blue consists of two consecu-
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**Summary.** Spectrophotometric examination of aqueous solutions of toluidine blue of varying concentrations and of the combined solutions of toluidine blue and M-, N-, and MN-active erythrocyte sialoglycopeptides suggests that the presence of the sialoglycopeptides results in dimerization of the dye. On the basis of Bradley and Wolf's theory on the aggregation of cationic dyes and the present results, it is concluded that each of the sialoglycopeptides contains sterically adjacent two sialic acid residues which are capable of producing toluidine blue dimer. A schematic illustration is proposed for linkage steps, first the ionic bond reaction between two cations of toluidine blue and carboxylate anions of the two sialic acid residues, and second the intermolecular reaction between the two bound dye molecules which included one molecule of water. The schema may be available for complex formation between methylene blue and the M- and N-active sialoglycopeptides. Since the combined solutions of toluidine blue (0.001%) and sialoglycopeptides (each 0.05%) liberated from human erythrocytes by treatment with chymotrypsin and trypsin in low concentrations give a single peak at 606 m_{\mu} and 604 m_{\mu}, respectively, these sialoglycopeptides as well as the M-, N-, and MN-active sialoglycopeptides, may have sterically neighboring two sialic acid residues which are capable of producing the dimer of the dye.

![Diagram](image-url)

Fig. 3. Binding form between toluidine blue and M-, N-, and MN-active sialoglycopeptides.

between two molecules of toluidine blue and the two sialic acid residues.

References