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Note

Acid–base properties of the reaction product of sialic acid with fluorogenic reagent, 1,2-diamino-4,5-methylenedioxybenzene (DMB)

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Abstract

N-Acetylneuraminic acid (Neu5Ac) forms the highly fluorophoric quinoxalinone derivative (**Q**) when treated with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Effects of protonation and deprotonation on the fluorescence of **Q** were examined at room temperature. The strong fluorescence was found to be caused by the neutral form **Q** but not the protonated form of its excited state [**Q**]* and at pH below 1 the emission was completely quenched. The deprotonated singlet form [**Q**$^-$]* was a less efficient fluorescer than [**Q**]*. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *N*-Acetylneuraminic acid (Neu5Ac); 1,2-Diamino-4,5-methylenedioxybenzene (DMB); Quinozalinone derivative; Acid–base properties; Absorption–emission spectra

Sialic acids (Sia) are a family of 9-carbon carboxylated sugars having the nonulosonate skeletal structure [1]. About 40 different molecular species of Sia are known to occur in Nature and the most common Sia is *N*-acetylneuraminic acid (Neu5Ac) [1–3]. The Sia residues are generally found on animal oligosaccharide chains of a variety of glycoconjugate molecules, and are known to play key roles in biological events [1–5]. It is thus essentially important to develop highly sensitive, selective and reliable methods for identification and quantitation of Sia. A simple, rapid, and highly sensitive method was introduced by Hara et al. for the determination of sialic acid monomer [6]. This method consists of derivatization of Sia by reacting with DMB and separation of the products (quinoxalinones) by high-performance liquid chromatography with a fluorometric detector. The detection limits are about 25 fmol (7.5 pg) for *N*-acylneuraminic acid monomers.

To answer the question, 'What are the species responsible for the fluorescence of DMB–Neu5Ac (**Q**)?', the quinoxalinone derivative (**Q** in Scheme 1) was prepared in pure form and its fluorescence spectra were determined at different pH values (Fig. 1). Since all the hydroxypolyazanaphthalenes exist mainly in the –CO–NH– form both in the solid state and in aqueous solution [7], they are cyclic amides (lactams). Protonation and deprotonation in such a heterocycle can be expected to lead to measurable changes in the physical properties of the molecule, especially in its

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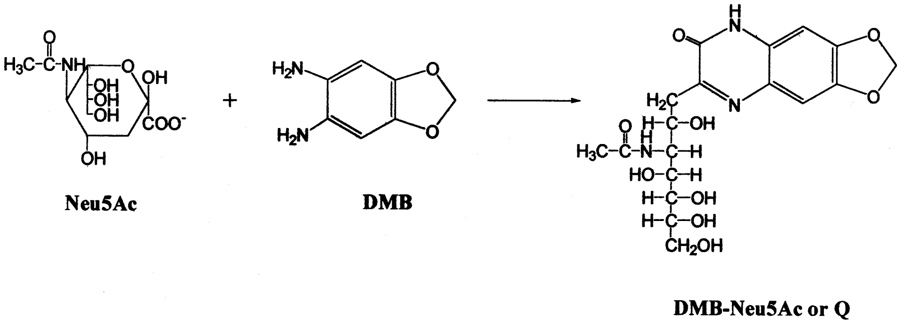
absorption and emission spectra [8,9]. On going from neutral pH to acidic pH the fluorescence emission was remarkably diminished though the shape of the emission spectrum remained almost unchanged (Fig. 1(A)). Thus, it is suggested that the same molecular species is responsible for the fluorescence of **Q** in its lower pH solutions as that in the neutral solution. At extremes ($pH < 1$) emission was almost completely quenched, and either the protonated species, $\mathbf{QH}^+$ was not fluorescent or the protonated species in the excited state, i.e., $[\mathbf{QH}^+]^*$ underwent deactivation by proton transfer at room temperature, ending up in the ground state. On going from pH 8 to 13, fluorescence spectral changes defined isobestic points (Fig. 1(B)). The appearance of an isobestic point indicated the presence of pH-dependent equilibrium between two fluorescent species neutral and anionic forms at room temperature.

The pH/fluorescence intensity profile for **Q** plotted at 455 nm is shown in Fig. 2. The fluorescence intensity was found to decrease at higher pH, reflecting ionization of the strongly fluorescent neutral species to weakly fluorescent anionic species in the excited state [$pK_a(S_1) \approx 9.5$]. Residual fluorescence observed above pH 11 would not be due to the remaining unionized molecule but must be ascribed to the less strongly fluorescent nature of the deprotonated species.

It seemed of interest to examine the somewhat different situation encountered in the absorption spectra of neutral, protonated and deprotonated forms of **Q** when compared with their fluorescence spectral behavior. Detailed spectra of **Q** determined at different pH values are recorded in Fig. 3. The protonation of **Q** was considered to occur at the cyclic amide oxygen atom, giving a cation such as $\mathbf{QH}^+$ in Scheme 2. The acidic and basic ionization constants determined for **Q** were 9.5 and ≤ 1, respectively. There was a marked change of absorption spectrum in passing from unionized form, **Q** (pH 7), to protonated species, $\mathbf{QH}^+$ ($pH < 0.7$), and a particular feature of the change of UV–Vis spectrum with pH shown by **Q** was the decrease in absorbance at 371 nm until pH decreases from 7 to about 0.72 (0.5 M TFA), concomitant with an appearance of a new broad peak at about 435 nm (Fig. 3(A)). From the structural similarities of the quinoxalinone derivative and 6-hydroxypteridine, it was expected that **Q** would undergo deprotonation to form the conjugate base ($\mathbf{Q}^-$). Fig. 3(B) shows serial spectra of a partially ionized DMB–Neu5Ac solution. The spectrum of the conjugate base of **Q** was hypsochromically displaced by 6 ~ 7 nm from that of the neutral form.

The results presented above are consistent with the existence of more than one species responsible for the fluorescence of DMB–Neu5Ac (**Q**), depending on the environment of the molecule in aqueous solution (Scheme 2). At present alternative possibilities we cannot be ruled out: a collisional quenching of $[\mathbf{Q}]^*$ by H_3O^+, a collisional quenching of $[\mathbf{QH}^+]^*$ by H_3O^+, and other radiationless transitions of them. It should be noted that reversibility was checked and confirmed by identification of the original absorption spectrum of **Q** at pH 7 when each of the solutions was restored to neutral pH after they had been allowed to stand separately at acidic and basic pH values for 30 min.



Scheme 1. Reaction of Neu5Ac and DMB to form the fluorescent quinoxalinone derivative (**Q**).

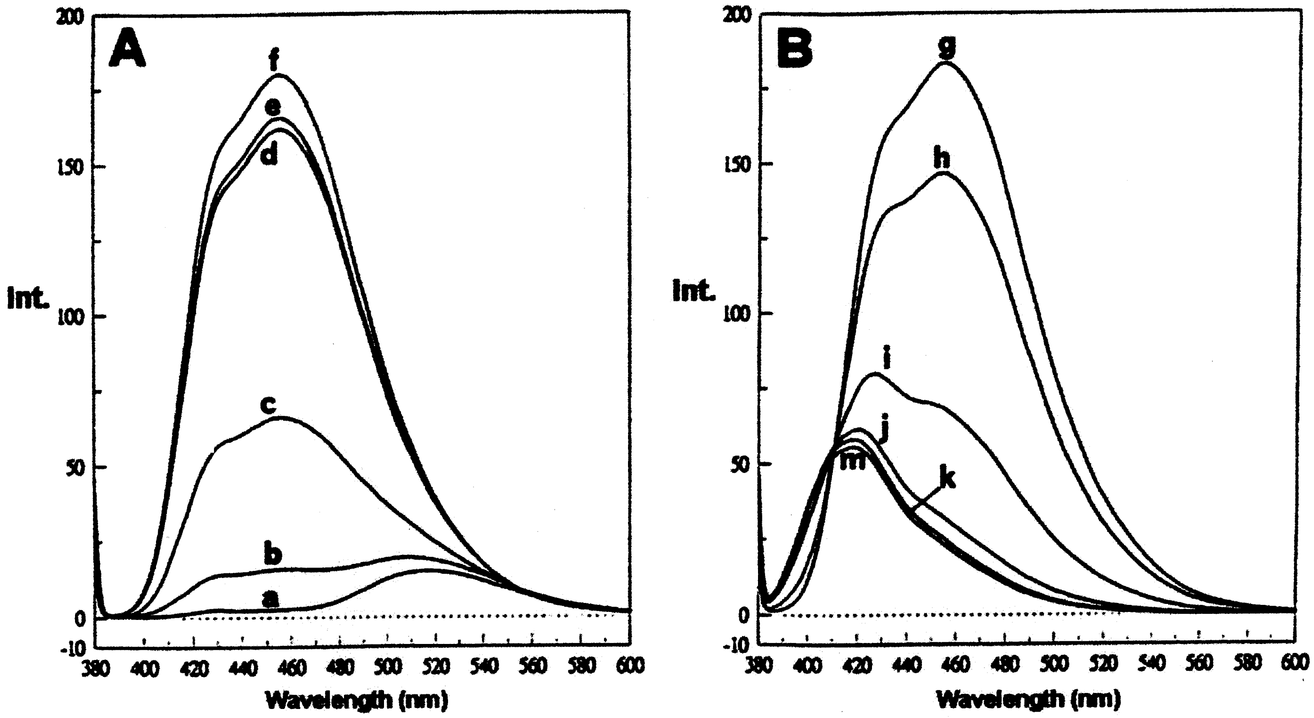


Fig. 1. Fluorescence emission spectra of DMB–Neu5Ac (**Q**; the product of reaction of Neu5Ac with DMB) at selected (A) acidic pH values: (a) pH 0.72; (b) pH 1.31; (c) pH 2.03; (d) pH 4.03; (e) pH 5.00; (f) pH 7.00, and (B) basic pH values: (g) pH 8.00; (h) pH 9.01; (i) pH 10.01; (j) pH 11.00; (k) pH 11.83; (m) pH 13.42.

In view of the biological significance and structural diversity of oligo/polySia glycotopes, development of highly selective, sensitive and reliable analytical method for detection, identification and quantitation is essential and urgent. Recently, two different chromatographic methods were developed, i.e., (a) highly efficient and specific separation of oligo/polymers of diverse types of sialic acid by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-100 column with pulsed amperometric detector [10], and (b) detection and identification of fmol levels of oligosialic acids after tagging their reducing termini with DMB followed by fluorometric high-performance liquid chromatography [11,12]. We showed that the latter method can be applicable for determination of degree of polymerization (DP) of a given oligo/polySia chain with DP up to about 25. Starting with an oligo/polySia chain with $DP = N$, we can observe a series of peaks of homologous DMB-tagged oligo/polySia chains with $DP \leq N$ ([12]; S. Inoue, to be reported). The present paper can be considered as a basic chemical study which provides for the first time the sound chemical background for the DMB method useful in sialoglycobiology.

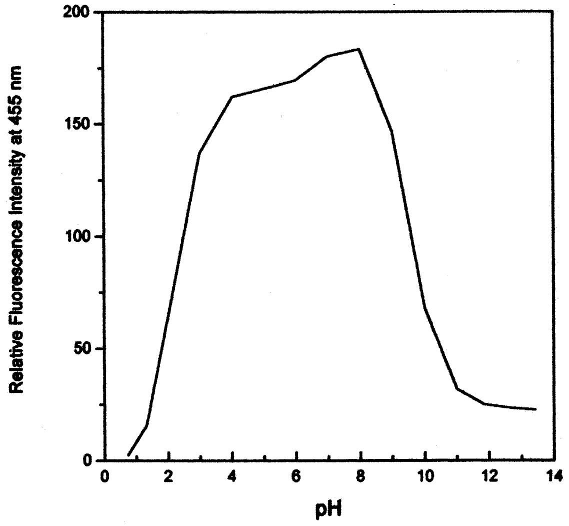


Fig. 2. Variation of fluorescence intensity of **Q** with pH at rt. Excitation was at 372 nm and fluorescence was measured at 455 nm.

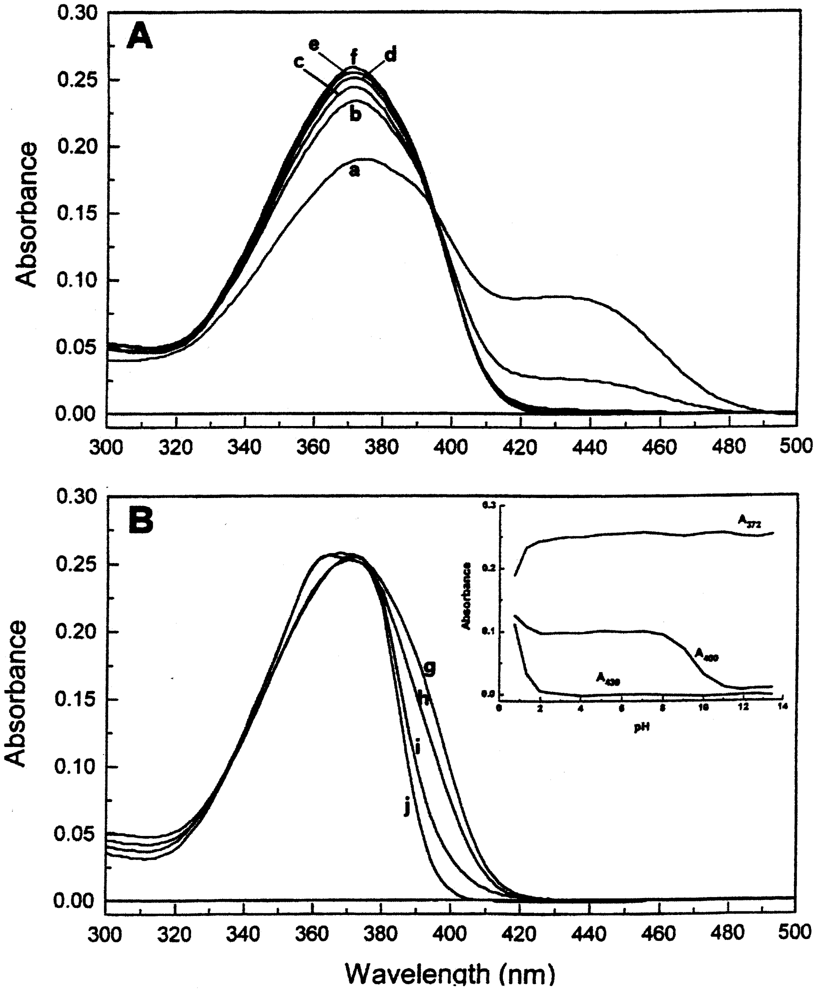
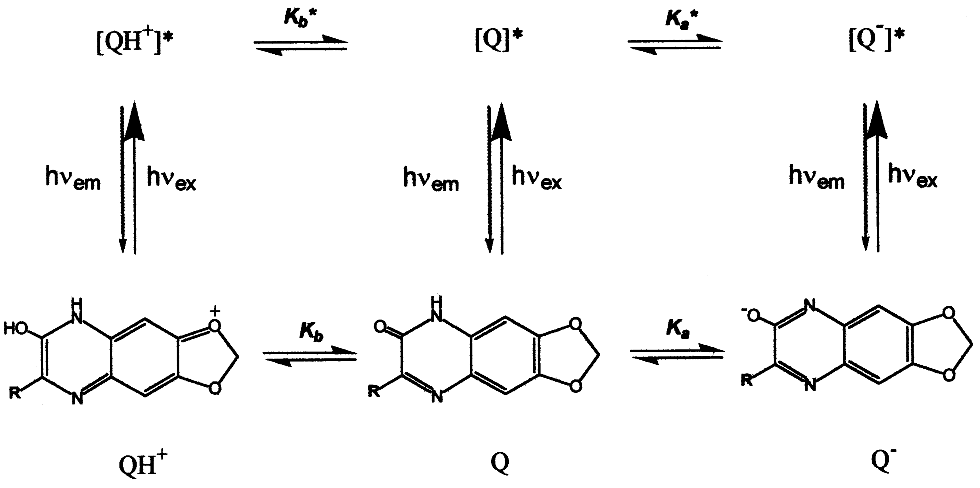


Fig. 3. UV–Vis absorption spectra of **Q** at (A) acidic pH values: (a) pH 0.72; (b) pH 1.31; (c) pH 2.03; (d) pH 4.03; (e) pH 5.0; (f) pH 7.0, and (B) basic pH values: (g) pH 8.0; (h) pH 9.0; (i) pH 10.01; (j) pH 11.83.



Scheme 2. Protonation and deprotonation equilibria of DMB–Neu5Ac (**Q**) and proposed mechanism for fluorescence and quenching of the fluorescent species, DMB–Neu5Ac (**Q**) in aqueous solution over the pH range 0–13. R = $-CH_2-CHOH-CHNHCOCH_3-CHOH-CHOH-CHOH-CH_2OH$.

1. Experimental

Materials.—DMB was a product of Dojinbo, Japan, and Neu5Ac was purchased from Nacalai Tesque (Kyoto, Japan). The quinoxalinone derivative, **Q**, was obtained by the reaction of Neu5Ac with DMB essentially according to Hara et al. [6] and purified using a HPLC system equipped with a Jasco UV-970 UV–Vis detector (monitored at 372 nm) and a Microsorb C18 column (RAININ Instrument Co., Inc.) with a 7:5:88 eluting solution of CH_3CN–CH_3OH–water.

Spectral measurements.—The UV–Vis absorption spectra of the purified sample of **Q** were taken with a Hitachi model U-2000 spectrophotometer at rt. Fluorescence emission spectra were measured on a Jasco FP-750 fluorescence spectrophotometer at rt. Phosphate buffer solutions (0.1 M) were used for pH $2 \sim 13$, and measurements at other acidic and basic pH values were made using sample solutions in 0.02, 0.1 and 0.5 M trifluoroacetic acid, and 0.02, 0.1 and 0.5 M NaOH solutions.

Acknowledgements

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