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UV–visible absorption, fluorescence, and optical rotatory study of the amylose–Rose Bengal complex *

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ABSTRACT

An extensive spectroscopic study of the amylose–Rose Bengal complex in aqueous solution has been undertaken in order to explain the physical and chemical mechanism of the complexation phenomenon. UV–visible absorption, fluorescence, and optical rotatory measurements provided information on the complex formation. Two mechanisms, one connected with structural changes of amylose induced by Rose Bengal and the other connected with the formation of the nonfluorescent complex of amylose and Rose Bengal, have been found and are discussed quantitatively. A simple model for amylose–Rose Bengal complex formation has been proposed.

1. INTRODUCTION

A number of extensive studies[1–4] have shown that amylose can form complexes with a variety of substrates. It is well established that, in the case of agents of low molecular size, amylose forms helical inclusion complexes, with the complexing agents entering the centre of the amylose helix; these are known as V-amylose complexes[5–7]. The major contribution to the complex formation comes from hydrophobic–lipophilic interactions[8,9]. It is also noteworthy that interturn hydrogen bonding strongly influences the stability of the helical inclusion complexes. One of the best known examples of an inclusion complex in aqueous solution is the reaction of amylose with iodine. The cooperativity of the iodine bonding process has been accepted in many reports[10–12]. It is assumed that one helical turn constitutes a binding site.

Earlier studies concerning the physical properties of dyes associated with polymers indicate changes in many optical parameters of the dye. For example, in some of them, the fluorescence is quenched while others show fluorescence only in the associated form[13,14].

* Amylose–Rose Bengal Complexes, Part I.

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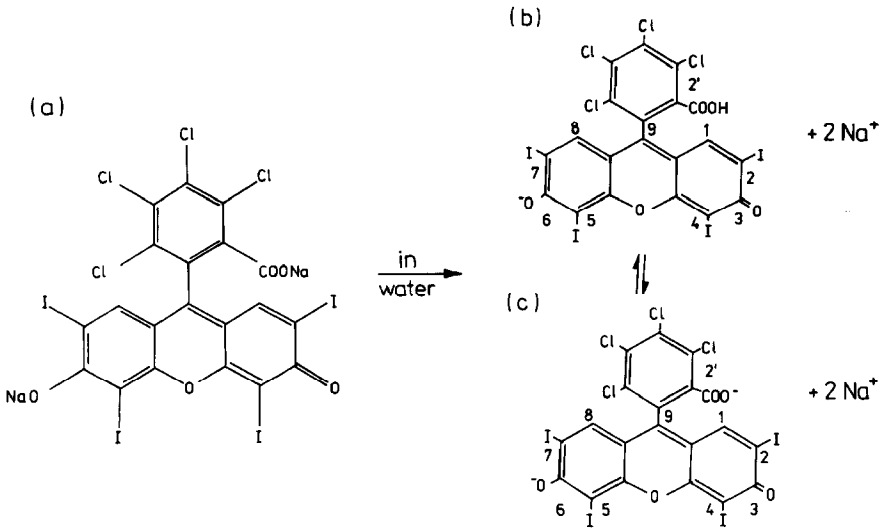

Fig. 1. Molecular structures of Rose Bengal: (a), in the solid state; (b) and (c), mono- and di-anionic forms in solution, respectively.

Taking into account the high reactivity of amylose towards different kinds of molecules, it seemed possible that amylose might be suitable as a natural polymer matrix for intentionally introduced dyes. Such applications are important in pharmacology and the food industry.

In our previous paper[15], we presented spectroscopic results for the amylose–Rose Bengal complex. Rose Bengal is a polynuclear heterocycle and, as such, is a member of a group of compounds of interest in the study of the interaction between dyes and biopolymers. In aqueous solution, Rose Bengal may occur in mono- or di-anionic forms, available for interaction with amylose (see Fig. 1).

The aim of our work is to determine the physical and chemical properties of the complex, to find out what are the physical mechanisms responsible for complex formation, and to give the quantitative characteristics of the complex. The complexing effect between amylose and Rose Bengal has been studied by visible spectroscopy comprising absorption, fluorescence, and optical rotatory methods. On the basis of our results, we propose a simple model of the complex formation.

EXPERIMENTAL

Our study of the interaction between amylose and Rose Bengal was carried out in aqueous, non-buffered solutions. Rose Bengal (sodium salt) was purchased from SIGMA and potato amylose (dp 120, as indicated by light scattering) was obtained from Poland Chemical Reagents. The water was doubly distilled and deionised. Solutions prepared by digesting amylose powder for 20 min in boiling water were cooled to 25°C at 12°C/min and mixed with a solution of Rose Bengal to give the

desired concentration. The concentration of Rose Bengal (C_{RB}) was 1.5×10^{-4} M and that (C_a) of amylose varied from 0.05 to 8%.

Spectroscopy.—Absorption spectra were recorded with a SPECORD UV–VIS Spectrometer, using a 1-cm cell, and fluorescence spectra with a Perkin–Elmer MPF-66 fluorescence spectrophotometer.

Optical rotation.—A POLAMAT-A polarimeter was used with a 10-cm cell and at 366 nm.

RESULTS AND DISCUSSION

UV-Absorption results.—The visible absorption spectrum of Rose Bengal in water shows two peaks[15], one at 545 nm and the other a shoulder at 507 nm. The anionic forms of the dye are assumed to be responsible for all observed changes in the absorption spectrum in the given range of dye and amylose concentration. In the presence of amylose, a bathochromic shift of both maxima is observed (Fig. 2). A similar bathochromic effect in the visible spectrum of Rose Bengal has been observed for solvents of lower dielectric constant. Such behaviour may suggest that, during the interaction between Rose Bengal and amylose, the dye, or at least part of it, is built into the helix, where the environment is much more hydrophobic than outside it. Because the ratio of the 545- and 507-nm band intensities, I_{545}/I_{507}, does not change when compared to the spectrum of Rose Bengal without amylose, there is no dimerization process to consider.

The absorption spectrum of the dye with increasing amylose concentration shows an isosbestic point at 556 nm[15], which indicates the equilibrium between

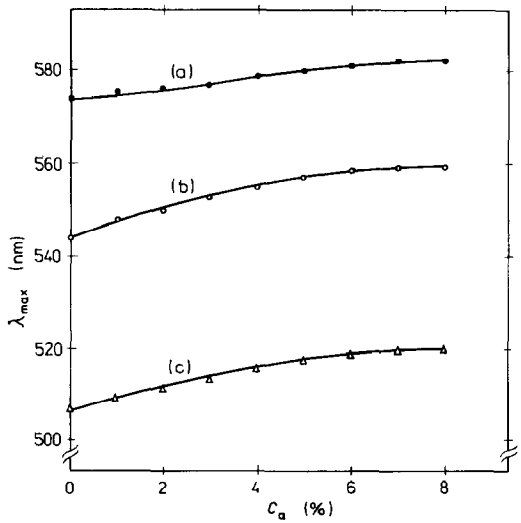

Fig. 2. Changes of λ_{max} for the spectra of Rose Bengal in solution vs. amylose concentration: (a), fluorescence; (b) and (c), UV–visible absorption spectra.

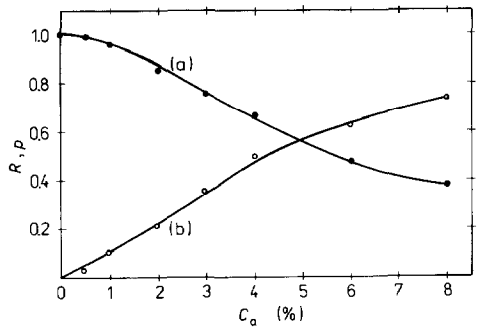

Fig. 3. (a) Relative intensity of the fluorescence (R; relative to the intensity in pure Rose Bengal solution), and (b) the degree of fluorescence polarization (p), for Rose Bengal in aqueous solution vs. amylose concentration (C_a).

free dye in aqueous solution and that interacting with the amylose chain. This point corresponds to the amylose concentration $C_a = 1.5 \times 10^{-4}$ M and Rose Bengal $C_{RB} = 1.5 \times 10^{-4}$ M. The difference seen in the absorption spectrum between the nominal concentration and that in the presence of amylose was assumed to correspond to the amount of the dye which forms the chemical complex.

Fluorescence results.—Fig. 3 depicts the quenching of the Rose Bengal solution fluorescence by amylose. A bathochromic shift of the fluorescence spectra occurs upon increasing progressively the amylose concentration to 8% (Fig. 2). This suggests an interaction of amylose with Rose Bengal, whereby a Rose Bengal molecule preferentially binds within the relatively nonpolar amylose helix. As may be expected, a decrease of the fluorescence intensity with increasing amylose concentration also depends on the initial dye concentration. The behaviour observed in our case appears to be in contrast to that reported by Hui and Gai[8], where growth of fluorescence of the 2-chloro-*N*-dodecylpyridinium ion upon complexation with amylose was observed.

In order to explain our results, two possible mechanisms have been considered. The first is the formation of a fluorescent complex, in which the quenching phenomenon results from the specific interaction of the amylose helix with Rose Bengal. Similar behaviour has been reported by Matsui and Mochida[16], where the quenching of Methyl Orange fluorescence by α-cyclodextrin was observed. The other mechanism is the formation of a nonfluorescent complex of amylose and Rose Bengal. In that case, the excitation energy is dissipated into the environment.

A Stern–Volmer plot of the fluorescence data[17] reveals two positively sloped lines with a correlation coefficient of 0.992 (Fig. 4). While a linear Stern–Volmer plot generally indicates an equivalent accessibility of all species of fluorophores present in a particular system to the quencher, it cannot indicate clearly the exact mode of quenching. Consequently, contributions from both static and dynamic quenching must be considered unless additional information is available. Two

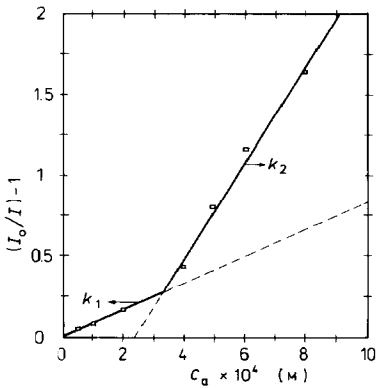

Fig. 4. Stern–Volmer plot of quenching of fluorescence for Rose Bengal in aqueous amylose solution (I_0 and I, intensities of fluorescence of Rose Bengal in pure water and amylose solution, respectively; C_a, amylose concentration in the solution).

quenching constants, $k_1 = 75\ M^{-1}$ and $k_2 = 320\ M^{-1}$, obtained from the Stern–Volmer plot, describe our process as follows. At a low concentration of amylose, the formation of the inclusion complex is observed, accompanied by an instant shift of the fluorescence maximum toward longer wavelength. With increasing amylose concentration ($C_a > 3 \times 10^{-4}$ M), we observe formation of complexes of a different kind in which Rose Bengal molecules do not show fluorescent properties.

Our results obtained from fluorescence measurements suggest that the Rose Bengal molecule can bind to amylose in two ways. The first is the formation of the inclusion or intercalation complex, which places the Rose Bengal molecule in a more hydrophobic environment. The dimensions of the Rose Bengal molecule are such that it fits into the groove formed between two helices. The second is the formation of the nonfluorescent complex, probably by an adsorption process on to the amylose. Thus, for solutions of amylose concentration greater than 3×10^{-4} M, the number of adsorbed dye molecules per amylose segment has been calculated.

Fluorescence polarization results.—To calculate the number of adsorbed dye molecules per amylose molecule segment and the fraction of bound dye molecules, we applied the fluorescence polarization method. This method is based on the fact that the fluorescence of the dye adsorbed on the macromolecule is partially polarized, while that of a free dye is usually unpolarized. Therefore, for any mixture of a fluorescent dye with a macromolecule, where part of a dye is bound to the matrix, the polarization P_d observed will be less than that for completely bound dye, P_b.

The polarization emission anisotropy (P) was calculated according to eq 1[18],

$$P = \frac{P_d}{P_b} = \frac{I_c - GI_p}{I_c + 2GI_p}, \qquad (1)$$

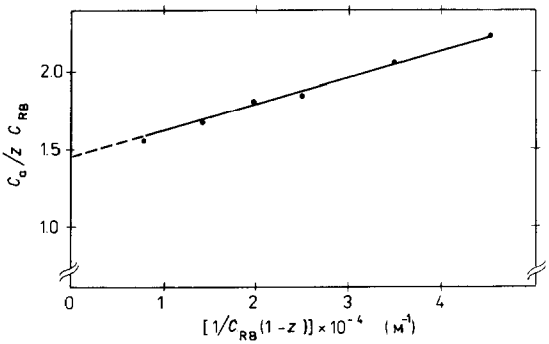

Fig. 5. Modified Klotz plot for Rose Bengal in aqueous amylose solution (C_a and C_{RB}, amylose and Rose Bengal concentrations, respectively; z, fraction of bound Rose Bengal molecules).

where I_c and I_p are the intensities of fluorescence for the incident light polarized perpendicular and parallel to the sample, respectively, and G is the correction coefficient including system polarization:

$$G = \frac{I_{pc}}{I_{pp}}. \tag{2}$$

In order to calculate the number of dye molecules adsorbed per amylose segment (n), fraction of bound dye (z), and equilibrium constant K, we used the modified Klotz equation[19], namely:

$$\frac{C_a}{zC_{RB}} = \frac{1}{n} + \frac{K}{nC_{RB}(1-z)}, \tag{3}$$

where $z = pR - (R + 1)$ (4)

and $R = I_b/I_0$ is the ratio of the fluorescence intensity of the dye bound to the macromolecule (I_b) to that of free dye (I_0).

The plot of C_a/zC_{RB} versus $1/C_{RB}(1-z)$, with data taken from Fig. 3 and eq 2, is shown in Fig. 5. The calculated fractions of bound dye molecules (z) as a function of amylose concentration are given in Table 1.

We found that the values of the equilibrium constant K and the number of dye molecules binding per amylose segment (n) are equal to $K = 0.17 \times 10^{-4}$ M^{-1} and $n = 0.7$.

The changes of the fraction of the molecule of bound dye (z) with increasing amylose concentration implies an increase of the helical form in the amylose chain. In order to prove this suggestion, we used the optical rotatory method.

TABLE I

The fraction (z) of Rose Bengal molecules bound to amylose versus amylose concentration in the solution (C_a)

C_a (10^{-4} M)	0.5	1.0	2.0	4.0	6.0	8.0
z	0.03	0.10	0.33	0.66	0.80	0.89

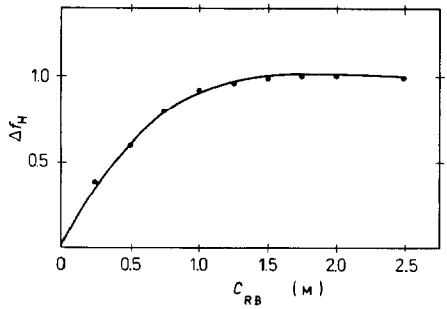

Fig. 6. Content of the helix form in the amylose chain (Δf_H) vs. concentration of Rose Bengal in the solution (C_{RB}) (amylose concentration, 0.2%).

Optical rotatory results.—Our earlier study[15] of the optical rotatory behaviour of amylose solutions in the presence of Rose Bengal molecules showed a plateau region of molecular rotation $[\alpha]_{366}$ for Rose Bengal concentrations higher than 1.5×10^{-5} M. It enables us to calculate the changes in the content of the helix form in the amylose chain[18] (Δf_H) induced by Rose Bengal molecules. As may be seen from Fig. 6, the presence of Rose Bengal in amylose solution leads to an increase of the amount of the helical form in amylose. These results correspond with the data from the Stern–Volmer plot where the presence of Rose Bengal induced the formation of a helical form as was predicted.

On the basis of these results, we may start to draw conclusions as to a possible mechanism for the interaction between amylose and Rose Bengal in aqueous solution, but the question which still remains is the precise way in which the dye molecules interact with amylose. We do not know whether they intercalate or are included into helixes, or whether dye molecules prefer the formation of "domains" where the ordered amylose molecules are forced to form helical structures. This problem is discussed in the following paper[20].

CONCLUSIONS

As follows from the optical rotatory measurements[15], the presence of Rose Bengal in amylose solution induces some structural changes in amylose and it seems clear that the dye is chemically and/or physically bound to the polymer structure and not simply trapped in the polymer matrix. The results from the fluorescence study suggest that the Rose Bengal molecule can bind to amylose by a "helical inclusion" complex and probably by an adsorption process on the amylose chain. An apparent isosbestic point observed at 556 nm in the absorption spectrum also confirms the contribution from a nonfluorescent complex to the proposed mechanism. The red shift observed in the absorption spectrum of Rose Bengal, however, supports some H-bonding interaction of the dye with amylose. The fact that both acidic groups are ionized may facilitate such interaction.

On the basis of dimensional considerations of Rose Bengal, which is approximately 8 Å in width by 9 Å in length, it is apparent that amylose with its molecular size is able to accommodate most of the Rose Bengal molecule inside the helix or into the groove. Even if a small part is exposed to the aqueous environment, it is not likely to be amenable to complexation with a second amylose molecule.

The fluorescence and polarization fluorescence results show that Rose Bengal in an amylose solution is partially incorporated into the amylose structure and induces conformational changes in the latter. As indicated by the fluorescence quenching and optical rotatory studies, the process of conformation change occurs after the dye and amylose are mixed, whereas the further step is a diffusion-controlled fluorescence-quenching process. In order to understand the quenching of Rose Bengal fluorescence, we shall consider the charge distribution in both molecules. The glycosyl residue does not show Π-electron activity but has high electron density provided by the glycosidic oxygen; according to our quantum mechanical calculation (unpublished data), similar electron density is also carried by the other hydroxyl oxygens. The electron density on the ring oxygen is lower, which suggests some contribution of lone-pair electrons to the pyranosyl ring. In such a case, we may expect that high electron-density spots in the glucopyranosyl residue interact with a lone pair of the unprotonated, phenolic oxygen of the dye. Because of the steric behaviour of amylose, we may also expect that the interactions will strongly depend on the actual conformation of amylose in solution. From that point of view, the enhanced quenching of Rose Bengal fluorescence may be attributed to specific inductive effects which involve direct interaction of the oxygen heteroatom with local high-density spots of amylose provided by the glycosidic oxygen. The probable enhancement of intersystem crossing as a result of interaction with amylose, in turn, decreases the number of fluorescing species in solution, resulting in the observed quenching. This ability of Rose Bengal to reflect changes in microenvironment polarity suggests its potential application as a probe in other polysaccharide systems.

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