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Two very distinct types of anthocyanin complexation: copigmentation and inclusion

O. DANGLES, C. STOECKEL, M.C. WIGAND and R. BROUILLARD*

Laboratoire de Chimie des Polyphénols associé au CNRS, Université Louis Pasteur,
Faculté de Chimie, 1 rue Blaise Pascal, 67 Strasbourg, France

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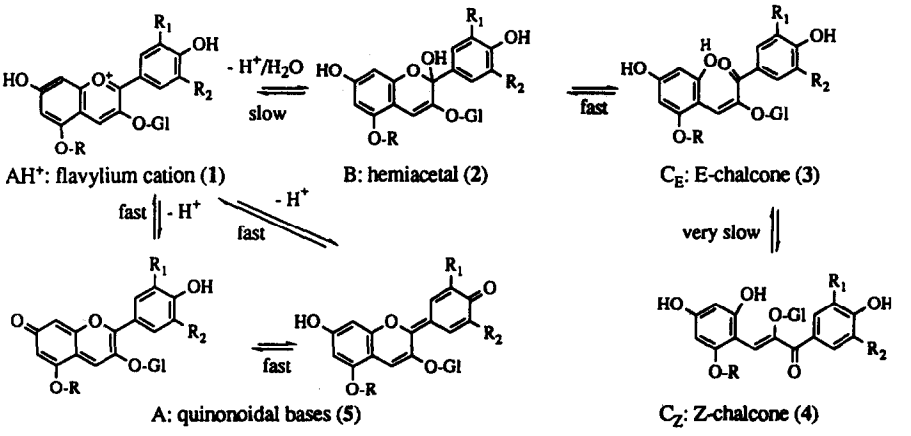
Abstract: Some natural and synthetic anthocyanins (pigments) are studied for their ability to give inclusion complexes into cyclodextrins. As demonstrated from thermodynamic and kinetic investigations, the effect of inclusion on colour (fading) is opposed to that of typical molecular interactions involving anthocyanins (copigmentation).

Polyphenols typically include in their structure hydrophobic aromatic moieties and hydrophilic polar groups like hydroxyl groups. This amphiphilic character makes polyphenols very good candidates for molecular complexation in water where hydrophobic bonding assisted by hydrogen bonding can take place.[1] Among polyphenols, anthocyanins are natural pigments which are responsible for most of the vivid colours of flowers and fruits.[2] The chemical structure of the flavylium cation, one of the most important pigment coloured forms, is reported on Scheme 1 (Gl=glycosyl). The flavylium structure consists of the chromophore called anthocyanidin, a 2-phenyl, 1-benzopyrylium system, and of glycosyl residues generally bound to the chromophore at the 3 position or at both 3 and 5 positions (R=glycosyl). In slightly acidic to neutral aqueous solutions i.e. in pH conditions close to those prevailing in their natural medium, anthocyanins exist under coloured (AH^+ (**1**) and A (**5**)) and colourless (B (**2**), C_E (**3**) and C_Z (**4**)) forms in thermodynamic equilibrium (Scheme 1). Except in strongly acidic solutions, the colourless forms are largely prevailing so that the corresponding solutions are poorly coloured. Fortunately, owing to their planar electronically unsaturated structure, the coloured forms (**1, 5**) exhibit a great propensity for associating selectively with numerous colourless molecules called copigments upon vertical stacking. Formation of such molecular complexes (copigmentation) stabilizes the coloured forms and efficiently competes with the nucleophilic attack of water onto the pyrylium system (hydration), thus restoring colour.

Copigmentation by polyphenolics is now believed to be one of the most efficient processes allowing colour stabilization *in vivo*. Curiously, interaction between cyclodextrins and suitable anthocyanins leads to effects which are opposed to those observed with copigments.[3,4] We now report on this unusual "anti-copigmentation" phenomenon which is expected to occur in the starch-rich plants whose colour is due to anthocyanins, in particular in the widespread coloured varieties of maize (Indian corn) known to contain large amounts of cyclodextrins.



Scheme 1

Addition of β-cyclodextrin to a fairly acidic solution of callistephin ($R=R_1=R_2=H$) brings about a strong decrease in the anthocyanin visible absorbance.[3,4] Steric requirements in both pigment and macrocycle molecules seem determining. Indeed, for a given pigment and at given pigment and cyclodextrin concentrations, β-cyclodextrin is the macrocycle which by far leads to the strongest hypochromic shift in the pigment visible absorption band. On the other hand, this hypochromic shift is negligible for sterically hindered pigments such as anthocyanidin 3,5-diglycosides (R=glycosyl) and anthocyanidin 3-monoglycosides heavily substituted on their terminal aromatic ring ($R_1=R_2=OMe$, for instance). By contrast, callistephin and chrysanthemin ($R=R_1=H$; $R_2=OH$), two very abundant naturally occurring anthocyanins, are very good candidates for inclusion into the β-cyclodextrin cavity. The large loss of colour following the addition of β-cyclodextrin to a solution of suitable pigment points to the preferential inclusion of the colourless forms (2, 3, 4) into the β-cyclodextrin cavity, a phenomenon leading to a net displacement of the hydration equilibrium toward the colourless forms. In fact, anthocyanin encapsulation constitutes the first reported example of "anti-copigmentation". A first theoretical treatment where the different colourless forms are taken as a whole can be derived.[3] The inclusion stability constants K_1 and K for the flavylium cation (1) (the only pigment coloured form to be considered at the experimental pH) and the colourless forms (2, 3, 4), respectively, have been computed for callistephin and chrysanthemin, at

25°C. Their values are as follows: K_1=30 (±20) M^{-1}, K=920 (±200) M^{-1} for chrysanthemin; K_1=130 (±30) M^{-1}, K=4000 (±400) M^{-1} for callistephin. In both cases, the K/K_1 ratio of about 30 suggests an almost complete selectivity of the inclusion process in favour of the colourless forms. In order to distinguish the different colourless forms (**2**, **3**, **4**) by their propensity for associating with β-cyclodextrin, a more sophisticated investigation based on relaxation kinetics has been undertaken. Among the structural transformations that an anthocyanin undergoes in aqueous solution, the proton transfer equilibria and the cycle-chain tautomerism are quite instantaneous. By contrast, the hydration reaction and the chalcone Z-E isomerization are relatively slow processes occurring on very distinct time scales. For instance, hydration is an acid-catalyzed process whose equilibrium state is achieved within about 10 seconds at pH 3-4 and about a few minutes at pH values closer to neutrality. The chalcone Z-E isomerization is much slower and pH-independent; its equilibrium state is fully established within about two hours. The kinetics of both latter phenomena and the way it is influenced by the presence of an anthocyanin ligand (copigment or macrocycle) can thus be easily studied using a standard UV-visible spectrometer. From the time-dependence of the pigment visible absorbance after quick addition of a ligand solution to an equal volume of anthocyanin equilibrated solution, the apparent first-order rate constants for both hydration and chalcone isomerization can be evaluated. For a good sensitivity, the study of hydration requires an additional pH jump which is achieved by using an alkaline (NaOH) ligand solution and a fairly acidic (citric solution at pH 2.0-2.5) pigment solution so that the final pH is about 3.5 i.e. in an acidity range where the major pigment coloured form is still the flavylium cation (**1**). Concerning the chalcone Z-E isomerization, both anthocyanin and ligand solutions are at the same pH (about 2). Copigmentation has been demonstrated to provide an efficient protection against hydration resulting in a strong slowing down of the fading process.[5] Once more, the difference with what happens on anthocyanin inclusion is striking, hydration being significantly speeded up by β-cyclodextrin. However, the inclusion-induced acceleration is quite modest, hydration appearing about twice as fast inside the β-cyclodextrin cavity. The kinetic investigation of inclusion has been performed on a synthetic, non-natural anthocyanin, the 3,4'-dimethoxy-7-hydroxyflavylium chloride, of easy access and thus available in much larger amounts than the natural pigments. Similarly, β-cyclodextrin catalyses the slow chalcone Z-E isomerization. From the increase in the isomerization apparent first-order rate constant on increasing macrocycle concentration (Figure 1), Z-E isomerization has been estimated to be about ten times as fast inside the β-cyclodextrin cavity. The absence of a clearcut saturation at high cyclodextrin concentration probably points to the taking part of a ternary complex involving two cyclodextrins and one anthocyanin in the catalytic process. From the absorbances at equilibrium of the first kinetic step (hydration), the overall inclusion stability constant K_2 for the fast equilibrating hemiacetal (**2**) and E-chalcone (**3**) forms has been computed. K_2 (340 M^{-1}) appears about 3.5 times as large as K_1 (100 M^{-1}).

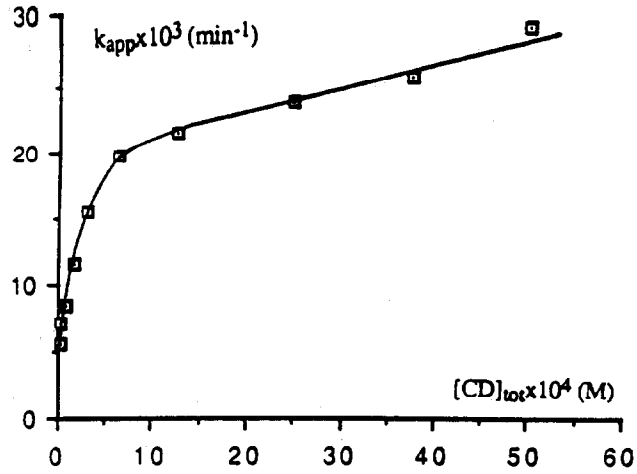


Figure 1: chalcone Z-E isomerization. Plot of the apparent first-order rate constant versus β-cyclodextrin (CD) total concentration. pH=2.3 (0.025 M citric solution); T=25°C; 3,4'-dimethoxy-7-hydroxyflavylium chloride concentration=$2.5x10^{-4}$ M.

Finally, from K_2 and K (930 M^{-1}), the value of the remaining inclusion stability constant K_3 for the Z-chalcone form (**4**) can be readily evaluated. K_3 (3880 M^{-1}) appears more than ten times as large as K_2. The stronger stabilization of the colourless forms in comparison with the flavylium cation (**1**) might arise from their larger flexibility. Indeed, the presence of a tetrahedral carbon atom in the hemiacetal (**2**) and of an open chain in the chalcone forms (**3**, **4**) makes them much less rigid than the almost planar flavylium cation and probably allows a closer fit to the macrocyclic cavity. In addition, solvation differences between the coloured and colourless forms might partially explain the differences in inclusion ability. The relative desolvation of the guest molecule when it enters the cyclodextrin cavity is probably more endothermic with the charged and highly polar flavylium cation (**1**). Among the anthocyanin forms, the Z-chalcone (**4**) possesses the structural characteristics by far the best adapted to inclusion into the cyclodextrin cavity. One of these characteristics might be the good linearity of the molecule with an unsaturated open chain connecting two aromatic moieties which makes it similar to some azoic dyes known to interact strongly with cyclodextrins.

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