Stability in Solution of Indolium Heptamethine Cyanines and Related pH-Sensitive Systems

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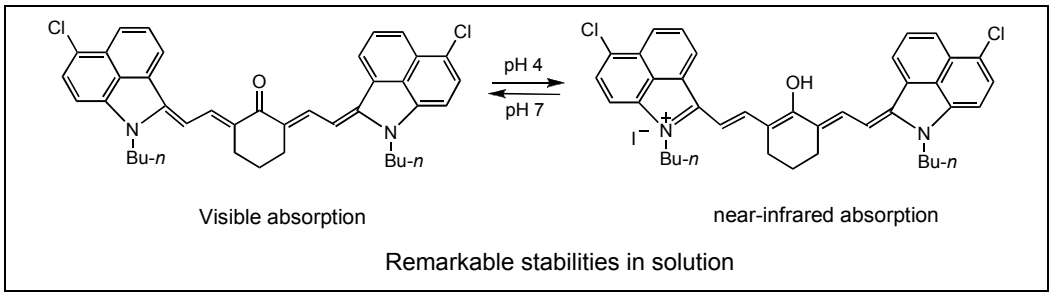

Relative stabilities of methanolic solutions of dyes that are potentially important for labeling of biological macromolecules with a visible-red or near-infrared fluorophore were evaluated in the presence of molecular oxygen under dark and light conditions.

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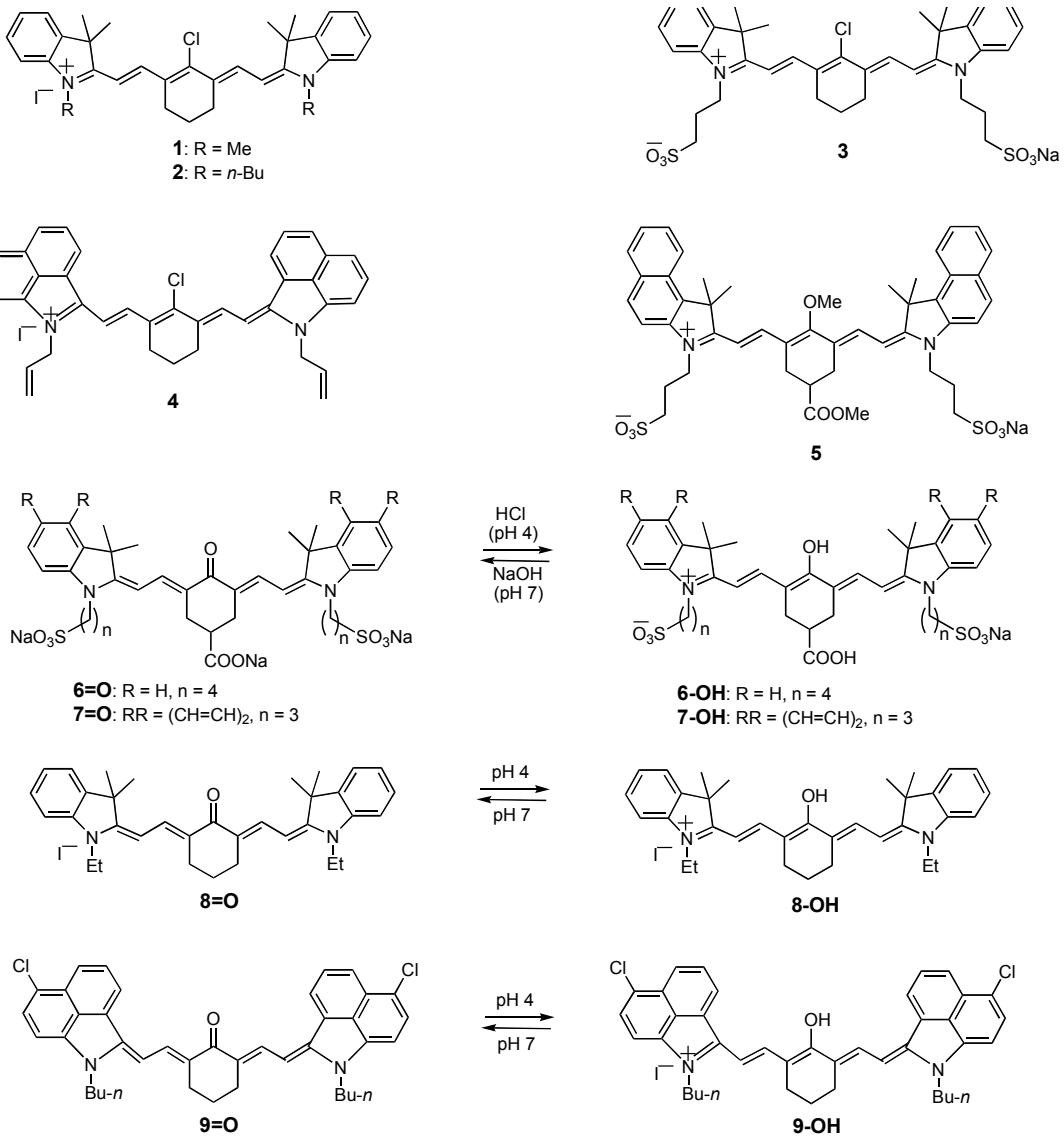
Cyanine dyes are important fluorescence labels for biological macromolecules [1,2]. The use of heptamethine cyanines which absorb and fluoresce in the near-infrared region (> 650 nm) is especially advantageous thanks to inherently low interference from biological media at these wavelengths. Both covalent and non-covalent labeling of biological macromolecules has become a firmly established trend in bioanalytical research [1-8]. The bioanalytical applications of near-infrared cyanines are hampered by instability of the dyes in solution. The instability under dark conditions may be related to the addition reaction of a nucleophilic solvent to the chromophore [9], On the other hand, the major pathway of the photodecomposition involves the reaction of singlet oxygen $^1O_2$ with the chromophore [10-12]. Specifically, upon light absorption the molecule undergoes intersystem-crossing from the excited singlet state S_1 to the excited triplet state T_1 followed by interaction of T_1 with endogeneous ground state triplet oxygen $^3O_2$ to generate the destructive singlet species $^1O_2$. Subsequent attack of the polymethine chain by $^1O_2$ results in fragmentation of the chromophore. The photobleaching is especially pronounced for dyes with structural features that increase efficiency of intersystem-crossing. These are the presence of a halogen atom such as chlorine in the molecule and the ability of the dye to form aggregates in solution. Aggregation and, consequently, photobleaching are decreased by encapsulation of the dye molecules in β-cyclodextrins, either covalently bound [13] or as inclusion agents in solution [14]. Aggregation is also decreased for dyes containing sulfonate or sulfonatoalkyl groups at the heterocyclic subunits [14]. An important class of the bioanalytical markers are cyanines containing a ring structure at the center of the chromophore (Chart). The partial rigidization of the molecule increases not only stability but also fluorescence efficiency [11,15,16]. The chloro-substituted dyes, such as **1** – **4**, are easily synthesized and purified by crystallization (no chromatography), and they serve as precursors to other meso-substituted dyes [17-19]. A new class of fluorescent markers, developed by us recently, is pH-sensitive dyes **6** – **9** [18,21-23]. The keto forms **6=O** – **9=O** absorb light in the visible region. Upon protonation, they are transformed into hydroxy cyanines **6-OH** – **9-OH** that absorb in the near-infrared region. Dyes **6** and **7** contain a carboxy function for covalent attachment to the amino group of proteins [21,22].

Dyes **1** – **9** are stable indefinitely when stored in the solid state in the absence of molecular oxygen and light. On the other hand, they undergo degradation in solution, especially when exposed to air and visible light. Due to the potential importance of dyes **1** – **9** in bioanalytical chemistry of proteins, it was of interest to evaluate relative stabilities of these dyes in solution. The results are described below. They are intended to provide practical guidelines in handling of these and similar dyes as fluorescent biomarkers.

Degradation of dyes in methanol was studied by monitoring decreases in absorbance at λ_{max} over time. In order to ensure comparable conditions a relatively high concentration (0.1 mM) was used, so that more than 95% of visible light was absorbed in all cases studied, and all solutions were exposed to excess of molecular oxygen (air). Identical solutions were also kept in the dark and their stabilities were monitored in a similar way. The results are given in Table.



Stability under dark conditions. All solutions are more stable when stored in the dark in comparison to the light conditions. Comparison of the data for **1** and **2** reveals that the *n*-butyl substitution provides little additional protection from degradation over the methyl substitution. However, compound **3** containing sulfonatopropyl groups is much more stable than **1** and **2**. Dye **5** that is substituted with the sulfonatopropyl groups and devoid of a chlorine atom is more resistant to degradation than all chloro-substituted compounds **1** – **4**.

The pH-sensitive dyes **6** – **8** are least stable, especially in the keto forms **6=O**, **7=O**, and **8=O**. The stabilizing effect of the sulfonatoalkyl substituents can be seen from analysis of **6** and **8**. Interestingly, benz[*c*,*d*]indolium dyes **9** show outstanding stabilities which are even greater than that of the analogous dye **4**. The effect of pH was analyzed briefly by using selected cyanine **5**. It was found

Table. Half-lifes ($t_{1/2}$) for decomposition of dyes **1-8** (0.1mM) in methanol under dark and light conditions (light intensity of 0.08 W/cm^2)

Dye	pH	$t_{½}$ (hours)	
		dark	light
1	7.0	150	49
2	7.0	160	52
3	7.0	400	133
4	7.0	600	192
5	7.0	1,150	525
5	4.0	825	383
6=O	7.0	45	2.3
6-OH	4.0	72	5.0
7=O	7.0	45	2.3
7-OH	4.0	72	5.0
8=O	7.0	21	1.8
8-OH	4.0	30	2.5
9=O	7.0	836	59
9-OH	4.0	1,100	78

that this dye is slightly less stable at pH 4 than at pH 7. This result is consistent with the facile protonation of cationic cyanines to give dications that show increased reactivity toward nucleophilic solvent [24].

Stability under light conditions. Within the two series of dyes, , **1** – **5** and **6** – **9**, the relative stabilities in solution under light conditions parallel those for the corresponding solutions stored in the dark. The stabilities of pH-sensitive dyes **6** – **8** are marginal. A notable exception is a relatively high resistance to photodegradation of benz[*c,d*]indolium derivatives **9**. For all pH-sensitive dyes **6** – **9** the hydroxy cyanine form is much more stable than the keto analog.

It should be noted that these experiments were conducted under a high intensity of 0.08 W/cm^2 of visible light. The light intensity is about an order of magnitude smaller in an average chemistry laboratory. Consequently, a greater photostability under normal laboratory operation than the data given in Table can be expected. Importantly, the resistance of any cyanine dye to degradation is increased upon binding of the dye with a macromolecule. This stabilizing effect is similar to that of encapsulation of a dye with cyclodextrins, as already discussed [13,14]. Accordingly, all dyes **1** – **9** can be used as near-infrared labels for macromolecules, provided fresh stock solutions are used and exposure to light is limited. The system **9=O/9-OH** shows the greatest resistance to degradation, including photodegradation, of all pH-sensitive dyes studied. It can be predicted that the analog of **9** substituted with sulfonatoalkyl groups and lacking chlorine atoms will be even more stable.

EXPERIMENTAL

Synthesis of dyes **1** – **9** has been reported previously [17-23]. The near-infrared absorption (λ_{max}) of the cyanines in methanol ranges from 709 nm for **8-OH** to 1026 nm for **4** with the extinction coefficient ε of 160,000 ± 40,000 M^{-1}cm^{-1}. The ketones **6=O** – **9=O** show intense absorption in the visible range. Their hydroxyl-substituted counterparts **6-OH** – **9-OH** and cyanines **1** – **5** exhibit an absorption minimum at around 450 nm with an extinction coefficient of about 500 M^{-1}cm^{-1} that gradually increases and reaches a value of at least 10,000 M^{-1}cm^{-1} at 700 nm.

For stability studies, a 0.1 mM solution in methanol of each dye was prepared using a 1L-volumetric flask; then the volume was equally divided (500 mL each) and placed into two 1L round-bottom flasks. One flask was kept in the dark and the second flask was illuminated with an incandescent light source so that the intensity of light reaching the solution was 0.08 W/cm^2. Immediately after the solutions had been prepared, aliquots were taken and quantitatively diluted with methanol to arrive at absorption at λ_{max} of the dye of about 1.0. Over the time of the experiments (up to several hours under light conditions and up to several days under dark conditions) additional measurements were made for identical dilutions. The apparent rate constant k for degradation was then determined using the integrated first-order law given in equation 1,

$$\ln[A_t] = - kt + \ln[A_0] \quad (1)$$

where A_0 is the absorbance at time t_0 and A_t is the absorbance at time t. The change in concentration of dye over time was obtained by plotting $\ln[A_0]/[A_t]$ versus t. A linear-fit of the data was obtained and provided the apparent rate constant k. The half-life $t_{1/2}$ was then calculated by using equation 2.

$$t_{1/2} = \ln[2]/k \quad (2)$$

Alternatively, for relatively unstable dyes the absorbance measurements were continued until the $t_{1/2}$ values were reached. Both methods gave virtually identical values of $t_{1/2}$.

Absorption measurements were conducted using a Perkin-Elmer Lambda 20 uv/vis/nir instrument. Light intensity was determined by using a Molechrom Power Max 5100 detector.

REFERENCES

[1] J. R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum, New York, 1999.

[2] A. R. Swamy, J. C. Mason, H. Lee, F. Meadows, M. Baars, L. Strekowski and G. Patonay, New Infrared Absorption/Luminescence Measurements, in Encyclopedia of Analytical Chemistry, A. Holyoake, ed., Wiley, London, 2000.

[3] R. J. Williams, M. Lipowska, G. Patonay and L. Strekowski, *Anal. Chem.*, **65**, 601 (1993).

[4] L. G. Lee, S. L. Woo, D. F. Head, R. S. Dubrow and T. M. Baer, *Cytometry*, **21**, 120 (1995).

[5] B. L. Legendre Jr., D. L. Moberg, D. C. Williams and S. A. Soper, *J. Chromatogr.*, **A779**, 185 (1997).

[6] D. B. Shealy, M. Lipowska, J. Lipowski, N. Narayanan, S. Sutter, L. Strekowski and G. Patonay, *Anal. Chem.*, **67**, 247 (1995).

[7] H. J. Gruber, C. D. Hahn, G. Kada, C. K. Riener, G. S. Harms, W. Ahrer, T. G. Dax and H.-G. Knaus, *Bioconjugate Chem.*, **11**, 696 (2000).

[8] J. H. Flanagan Jr., S. H. Khan, S. Menchen, S. A. Soper and R. P. Hammer, *Bioconjugate Chem.*, **8**, 75 (1997).

[9] L. Strekowski, J. C. Mason, J. E. Britton, H. Lee, K. Van Aken and G. Patonay, *Dyes Pigments*, **46**, 163 (2000).

[10] S. Lepaja, H. Strub and D. J. Lougnot, *Z. Naturforsch.*, **38A**, 56 (1982).

[11] N. Tyutyulkov, J. Fabian, A. Mehlhorn, F. Dietz and A. Tadjer, Polymethine Dyes: Structure and Properties, St. Kliment Ohridski University Press, Sofia, Bulgaria, 1991.

[12] P. Chen, J. Li, Z. Qian, D. Zheng, T. Okasaki and M. Hayami, *Dyes Pigments*, **37**, 213 (1998).

[13] R. Guether and M. V. Reddington, *Tetrahedron Lett.*, **38**, 6167 (1997).

[14] T. V. S. Rao, J. B. Huff and C. Bieniarz, *Tetrahedron*, **54**, 10627 (1998).

[15] N. V. Monich, Z. A. Krasnaya and V. F. Levkoev, *Khim. Geterotsikl. Soedin.*, **12**, 1631 (1980).

[16] G. A. Reynolds and K. H. Drexhage, *J. Org. Chem.*, **42**, 885 (1977).

[17] L. Strekowski, M. Lipowska and G. Patonay, *J. Org. Chem.*, **57**, 4578 (1992).

[18] L. Strekowski, M. Lipowska and G. Patonay, *Synth. Commun.*, **22**, 2593 (1992).

[19] M. Lipowska, G. Patonay and L. Strekowski, *Synth. Commun.*, **23**, 3087 (1993).

[20] J. C. Mason, G. Patonay and L. Strekowski, *Heterocyclic Commun.*, **3**, 409 (1997).

[21] L. Strekowski, J. C. Mason, H. Lee and G. Patonay, *Heterocyclic Commun.*, **10**, 381 (2004).

[22] L. Strekowski, J. C. Mason, H. Lee, M. Say and G. Patonay, *J. Heterocyclic Chem.*, **41**, 227 (2004).