Journal of Fluorescence, Vol. 3, No. 3, 1993

New Fluorescent Labels for Time-Resolved Detection of Biomolecules

M. Sauer,[1,3] K.-T. Han,[1] R. Müller,[1] A. Schulz,[1] R. Tadday,[1] S. Seeger,[1] J. Wolfrum,[1] J. Arden-Jacob,[2] G. Deltau,[2] N. J. Marx,[2] and K. H. Drexhage[2]

Received October 18, 1993

New dyes with characteristic fluorescence lifetimes have been developed for bioanalytical applications. Based upon the concept of "multiplex dyes," we have designed rhodamine dyes with nearly identical absorption and emission spectral characteristics but different fluorescence lifetimes. Extending this principle to applications with laser diodes, new rhodamines with functional groups for covalent coupling of analytes have been developed. The new labels exhibit absorption and fluorescence beyond 600 nm and have a high quantum efficiency, even in aqueous buffer systems.

KEY WORDS: Fluorescence; multiplex dyes; photochemistry; time-resolved spectroscopy.

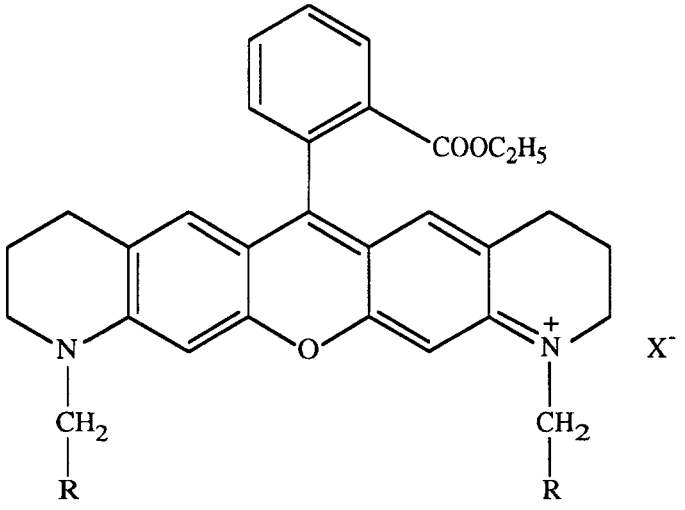
INTRODUCTION

Due to the enhanced demand for fluorescent markers in bioanalytical applications, the development of new fluorescent dyes has increased considerably in recent years [1–4]. Fluorescein as well as rhodamine dyes covalently coupled to biomolecules are most commonly used as fluorescent markers [5–7]. Typically the emission spectrum of the dye is used as a characteristic property. Meanwhile in routine diagnostics there is a growing need for multianalyte (multiparameter) analysis. Unfortunately the present methology does not allow the use of more than two or three simultaneous labels because the emission spectra of different fluorescent dyes overlap significantly. Consequently different analytes in a mixture are poorly separated.

It is the purpose of our investigations to increase the number of discernable fluorescent labels. Our concept consists of labeling the molecules or materials under investigation with different fluorescence lifetimes. With this method, not only can the spectral information of the fluorescent dye be utilized, but also the characteristic fluorescence lifetime. Here a difficulty may arise due to the fact that different dyes in general exhibit different absorption maxima. Therefore in most cases it is not possible to excite different dyes with the same efficiency. Hence a group of fluorescent dyes would be desirable in which all chromophores can be excited efficiently with only one monochromatic excitation source and detected at the same wavelength but can be distinguished due to their characteristic fluorescence lifetime. Fluorescent dyes whose absorption and emission wavelength are nearly identical but that differ in fluorescence lifetime are called "multiplex dyes" [8–10]. To label the analytes with an unequivocal lifetime, it is desirable to use fluorescent dyes, which show fluorescence properties nearly independent of the molecular environment. To test this principle we have designed two classes of rhodamine dyes with similar spectral characteristics but different fluorescence lifetimes. We started our investigations with rhodamine 630 (Fig. 1). Due to the incorporation in six-membered rings the amino groups do not induce nonradiative transitions. Therefore this dye shows

[1] Physikalisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany.
[2] Institut für Physikalische Chemie, Universität-Gesamthochschule Siegen, Adol-Reichwein Straße 3, 57068 Siegen, Germany.
[3] To whom correspondence should be addressed.

1053-0509/93/0900-0131$07.00/0



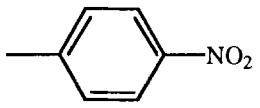
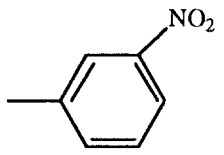
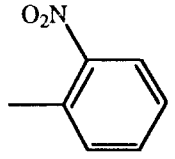
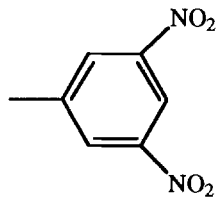
	rhodamine 630	pn-rhod.	mn-rhod.	on-rhod.	dn-rhod.
R	-CH_3				

Fig. 1. Molecular structures of rhodamine dyes with nitrophenyl groups.

a high fluorescence quantum yield and a lifetime of about 4 ns independent of the molecular environment and temperature [8,11,12]. Furthermore, the chosen dye rhodamine 630 offers the possibility to attach at the amino groups a fluorescence quencher for controlling the lifetime of the excited state as well as a functional group for covalent coupling to analytes. In the first class of multiplex dyes we have attached acceptors (nitrophenyl groups) at the amino position of the chromophore (Fig. 1). Due to the electron-accepting characteristics of the nitrophenyl group, intramolecular electron transfer occurs, leading to a well-defined reduction of the fluorescence lifetime. As predicted in the classical theory of electron transfer of Marcus [13,14], we were able to control the fluorescence lifetime through either the donor–acceptor distance or the acceptor reduction potential. Using redox potentials obtained in a separate measurement, we can explain the observed fluorescence quenching in various alcohols. In these solvents the fluorescence lifetime correlates well with the free-energy change calculated for photoinduced electron transfer between the excited donor (rhodamine 630) and the ground-state acceptor (nitrobenzene). However, for an application in biodiagnostics, e.g., DNA sequencing, the multiplex dyes should have discernible lifetimes, especially in aqueous systems. In this paper we present measurements on these nitrophenyl-rhodamines in various aqueous systems, showing their applicability also under biological conditions (Fig. 2). A second class of multiplex dyes discussed in this paper represents rhodamine derivatives with modified *o*-carboxyphenyl substituents, leading to a group of new dyes with a reduced fluorescence efficiency in fluid solvents such as ethanol, methanol, and water (Fig. 3). First applications with such rhodamine derivatives have shown the possibility of detecting two differently labeled antibodies only by way of the intrinsic fluorescence lifetime of the covalently coupled dye [15].

The increased sensitivity of laser-induced fluores-

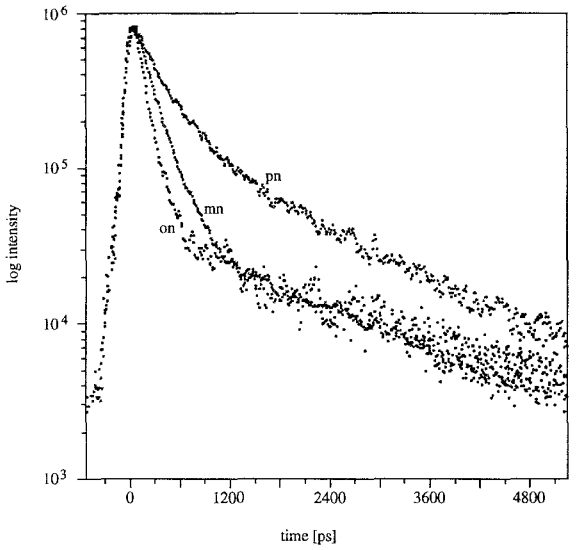


Fig. 2. Fluorescence decay curves for on-rhod., mn-rhod., and pn-rhod. in aqueous buffer, pH 7.0 (λ_{ex} = 532 nm; emission detected behind a bandpass filter, CWL, 585 nm).

cence in the visible/near-IR region, down to single-molecule detection [16,17], has prompted current efforts to develop fluorescent dyes with a high quantum efficiency that show absorption and emission beyond 600 nm. The use of such dyes in combination with low-cost optical sources and detectors such as laser diodes and photodiodes constitutes a new concept in biodiagnostics [9,18]. Ultrasensitive near-IR detection has been reported using a diode laser as the excitation source and a methanol solution of the dye IR-140 [18,19]. However, for application in aqueous environments, polymethine dyes do not appear to be the best choice, because the spectroscopic characteristics of these dyes are very sensitive to changes in the environment [20]. In this paper we report on new fluorescent dyes with absorption and emission in the red region of the spectrum (Fig. 4). The spectroscopic characteristics and stability parameters of these rhodamine derivatives have been investigated. The dyes appear to be well suited to time-resolved detection in aqueous systems.

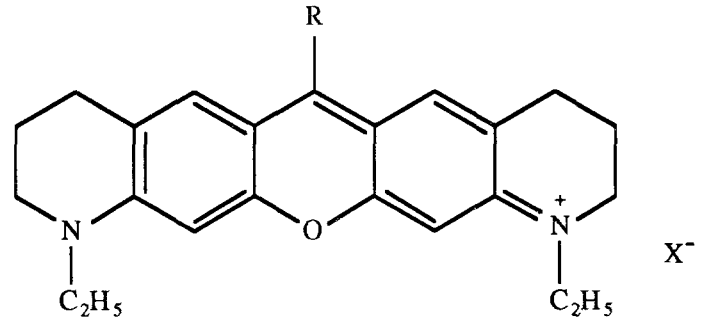
EXPERIMENTAL

All solvents—not degassed—were spectroscopic grade (Aldrich) and used without further purification. Measurements in aqueous systems were performed using the following buffers: 100 m*M* citrate buffer pH 3.0, 100 m*M* phosphate buffer, pH 7.0, 100 m*M* Tris buffer, pH 9.0, 100 m*M* Tris–borate buffer containing 7 *M* urea, pH 8.4 (Beckman). To ensure complete protonation of rhodamines with carboxy groups, a drop of trifluoroacetic acid was added to the alcoholic dye solutions. The 4% polyacrylamide gel was obtained from Dr. A. Paulus (Ciba Geigy). This polyacrylamide gel is only partially cross-linked, allowing measurements in standard cuvettes. The temperature at all measurements was 25°C. The dye concentration was kept strictly below 10^{-6} *M* in all solutions. Reversed-phase HPLC with a gradient of 0–75% MeCN in 0.1 *M* aqueous triethyl ammonium acetate (TEAA) was used for the purification of the nitrophenyl-rhodamines. Polarity and viscosity parameters of the solvent were obtained from the literature.

Samples with lifetimes greater than 1.5 ns were measured with a hydrogen/argon filled flashlamp (1.7-ns FWHM) using the time-correlating single-photon counting technique with an instrument from Photon Technology International (Model LS 100). The samples were excited at the long-wavelength maximum. The fluorescence decay was monitored at the emission maximum. Lifetimes shorter than 1.5 ns were measured using the second harmonic (532 nm) of a flashlamp-pumped, actively and passively mode-locked Nd:YAG laser (Continuum PY61C-20) providing pulses of 40-ps length with an energy of 25 μJ. The fluorescence signal was monitored by a streak camera (Hamamatsu C 1587). An Omega bandpass filter (CWL, 585 nm; FWHM, 12 nm) and a Schott cutoff filter (cutoff, 570 nm) were used to discriminate scattered light. The quality of the decay fits were controlled by the reduced chi-squared statistical parameter. Most of the decays could be described satisfactorily by a monoexponential model ($\chi^2 < 1.2$).

RESULTS AND DISCUSSION

In the first class of "multiplex dyes" we have substituted a rhodamine chromophore with nitrophenyl groups (Fig. 1). Absorption and emission spectra of these derivatives differ only slightly (Table I). The fluorescence efficiency of our reference dye rhodamine 630 is close to unity, independent of the solvent polarity [8,11]. However, all rhodamines with nitrophenyl substituents show a reduced fluorescence lifetime, depending on the polarity of the solvent. Optical excitation of the rhodamine moiety induces an intramolecular electron transfer from the rhodamine subunit to the ground state of the nitrophenyl substituent, thereby quenching the rhodamine fluorescence and forming a radical ion pair. The free-energy change of the reaction in acetonitrile was estimated from the redox properties of rhodamine 630,



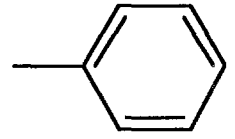
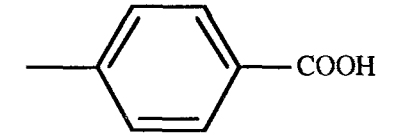
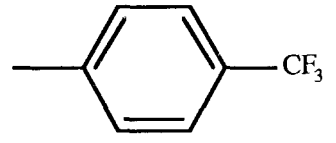
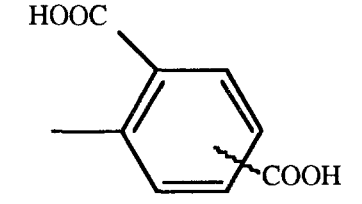
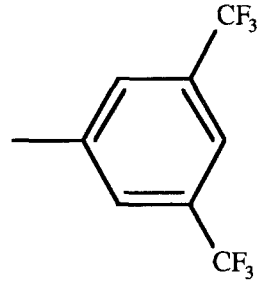
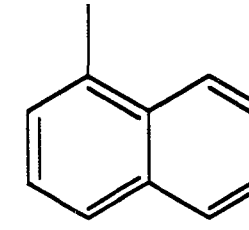
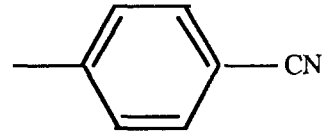
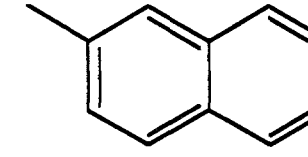
	R		R
Ros 1		Ros 5	
Ros 2		Ros 6	
Ros 3		Ros 7	
Ros 4		Ros 8	

Fig. 3. New rhodamines with modified phenyl groups at the central carbon atom.

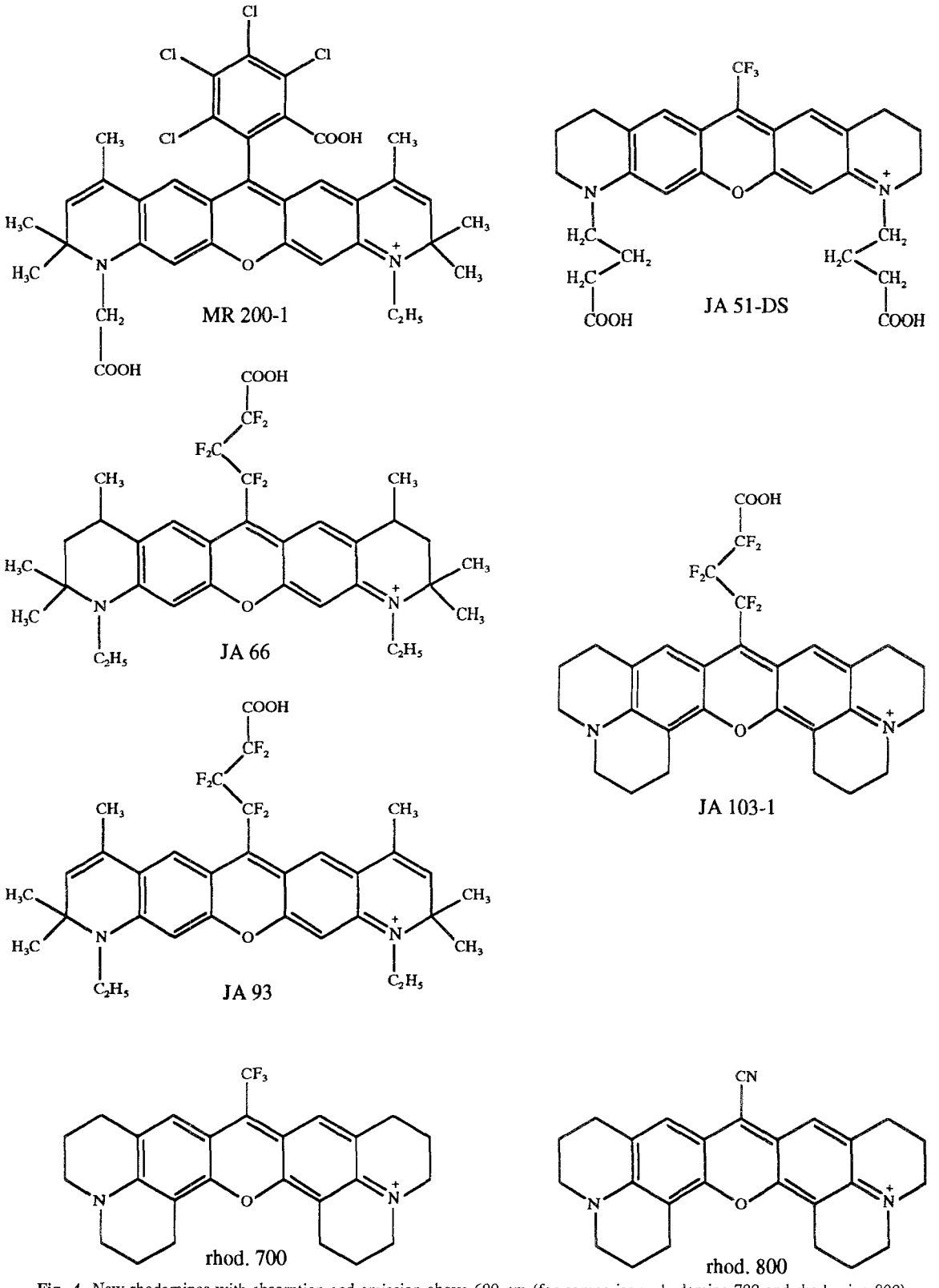


Fig. 4. New rhodamines with absorption and emission above 600 nm (for comparison, rhodamine 700 and rhodamine 800).

Table I. Fluorescence Lifetimes of Nitrophenyl-Substituted Rhodamines in Solvents of Different Polarities[a]

		τ (ns)			
		pn-rhod.	mn-rhod.	on-rhod.	dn-rhod.
		$\lambda_{abs}/\lambda_{em}$ (nm) in ethanol			
Solvent	$E_T(30)$ (kcal/mol)	555/581	556/582	553/580	552/578
1-Decanol	46.60	3.97	4.02	3.89	2.02[b] 3.22 (0.58)
1-Butanol	50.20	3.98	3.84	3.42	1.13 2.18 (0.45)
1-Propanol	50.70	3.92	3.76	3.10	0.94 2.57 (0.22)
Ethanol	51.90	3.89	3.72	2.70	0.66 1.78 (0.18)
Methanol	55.50	3.54	3.32	1.48 2.33 (0.33)	0.39 1.88 (0.05)
Glycerol	57.00	1.21 2.43 (0.12)	0.77 1.67 (0.39)	0.70 2.35 (0.06)	0.21 1.85 (0.04)

[a] The lifetime of rhodamine 630 in ethanol, $\tau = 4.05$ ns.

[b] Where three values appear, a double-exponential fit was necessary to describe the decay. The shorter lifetime and the longer lifetime (and its relative weight) are given.

nitrobenzene, and *m*-dinitrobenzene to approximately −0.03 eV for the electron transfer in on-rhod., mn-rhod., and pn-rhod. and −0.28 eV in dn-rhod. [8]. In dn-rhod., where the acceptor character of the substituent is more pronounced, quenching by photoinduced electron transfer is expected to be most efficient. In the case of on-rhod., mn-rhod., and pn-rhod. the distance between the donor (rhodamine) and the acceptor part of the substituent (nitro group) determines the rate of electron transfer as has been found in other systems [21]. The fluorescence decay of rhodamine 630 is monoexponential in all solvents investigated, whereas in the nitrophenyl-substituted rhodamines the decay is generally biexponential, in particular in solvents of a high polarity. To test the applicability of nitrophenyl-rhodamines also under biological conditions, we measured the fluorescence lifetime in various aqueous systems (Fig. 2 and Table II). Here all dyes show a dominant fast- and a weak slow-decaying fluorescence component. The data show that these multiplex dyes are applicable also in aqueous environments. They should be useful especially for DNA sequencing in gel-filled capillaries under denaturating conditions.

In the second class of multiplex dyes we have substituted the carboxyphenyl group by phenyl groups with different steric requirements and varying acceptor properties (Fig. 3). The maxima of absorption and emission of the new rhodamine derivatives differ only slightly ($\lambda_{abs} = 570 \pm 5$ nm, $\lambda_{em} = 594 \pm 5$ nm). In Table III the fluorescence lifetimes of the new rhodamine derivatives in solvents of different polarity and viscosity are given. The fluorescence decays were found to be monoexponential in all solvents. As shown in Table III, the new rhodamines can be roughly divided into those

Table II. Fluorescence Lifetimes of Nitrophenyl-Substituted Rhodamines in Aqueous Systems

	τ_1 (ns)	τ_2 (ns)	a_1	a_2
on-rhod.				
Aqueous buffer, pH 7.0	0.11	2.97	0.98	0.02
7 *M* urea, pH 8.4	0.15	2.48	0.98	0.02
4% polyacrylamide gel	0.18	2.72	0.93	0.07
mn-rhod.				
Aqueous buffer, pH 7.0	0.21	1.92	0.98	0.02
7 *M* urea, pH 8.4	0.23	1.93	0.98	0.02
4% polyacrylamide gel	0.19	1.73	0.95	0.05
pn-rhod.				
Aqueous buffer, pH 7.0	0.29	1.46	0.85	0.15
7 *M* urea, pH 8.4	0.32	1.42	0.87	0.13
4% polyacrylamide gel	0.33	2.06	0.81	0.19

Table III. Fluorescence Lifetimes τ (ns) and Arrhenius Activation Energies E_a of Rhodamines with Different Substituents at the Central Carbon

	Ethanol	Methanol	Water	Glycerol	
Viscosity (cP)	1.2	0.6	1.0	1480	E_a (kJ mol^{-1})
$E_T(30)$ (kcal/mol)	51.9	55.5	63.1	57.0	in ethanol[a]
	τ [ns]				
Ros 1	3.76	3.52	2.68	3.90	20.0
Ros 2	3.08	2.71	2.17	3.86	19.8
Ros 3	3.92	3.99	3.69	3.85	32.0[a]
Ros 4	3.37	2.81	2.23	3.82	22.1
Ros 5	3.11	2.60	2.07	3.80	20.1
Ros 6	3.99	3.90	3.82	3.90	—
Ros 7	4.03	4.04	3.93	4.18	—
Ros 8	3.75	3.30	2.98	4.16	24.5
Ros 9	3.10	2.58	2.31	3.78	20.9
Rhodamine 630	4.05	4.11	3.98	3.87	—

[a] Activation energies were calculated in the temperature range 5–60°C and found to be constant, except in the case of Ros 3. The activation energy for Ros 3 was calculated in the temperature range 20–60°C.

with long lifetimes unaffected by the solvent polarity and viscosity (Ros 6, Ros 7) and those with lifetimes influenced by the viscosity and polarity of the environment (Ros 1, Ros 2, Ros 3, Ros 4, Ros 5, Ros 8, and Ros 9). In highly viscous glycerol, however, all derivatives show a long lifetime. It has been postulated [12] that the structural mobility of the phenyl group in rosamine 4 (rhodamine 110 without a carboxyl group) decreases the fluorescence quantum yield. Comparison of the structures and fluorescence lifetimes of the new rhodamines confirms the idea that nonradiative deactivation is linked with the rotation of the phenyl substituent. This behavior can be clearly demonstrated by comparison of the dyes Ros 7 and Ros 8. Through the steric hindrance caused by the hydrogen in the 8- position of the naphthalene group, the rotation of this group is forbidden in Ros 7, and therefore lifetime and quantum yield have maximum values independent of viscosity. On the other hand, in Ros 8 the steric hindrance is lowered, resulting in a shorter fluorescence lifetime in fluid solvents such as ethanol, methanol, and water. Similar behavior has been observed also with triphenylmethane and related dyes [22].

The activation energies for the observed process in ethanol were calculated in the temperature range 5–60°C and found to be constant, except in the case of Ros 3. The rate constants k_{nr} for nonradiative deactivation were calculated, assuming that all derivatives have a lifetime of 4.0 ns in ethanol if the rotation of the phenyl substituents is forbidden as in rhod. 630, Ros 6, or Ros 7: $k_{nr} = \tau^{-1} - 4.0\ ns^{-1}$. The Arrhenius plots of k_{nr} for Ros 3 in polar solvents show curvature at temperatures below 20°C, indicating temperature-independent contributions to the nonradiative rate constant. The calculated activation energies of approximately 20 kJ/mol (Table III) correspond well to values found for rhodamines, which show nonradiative deactivation due to the flexibility of amino groups, e.g., rhodamine B [23–25]. In our new dyes, however, quenching by rotation of the amino groups can be excluded, because the amino groups are incorporated in rigid ring systems. The viscosity of many solvents can be empirically fit to $\eta = \eta_0 \exp(E_\eta/RT)$, where E_η is the viscosity activation energy. With temperature-dependent viscosity data we obtain a viscosity activation energy of $E_\eta = 12.9$ kJ/mol for ethanol. The calculated activation barriers E_a are greater than E_η. However, one has to take into account that the viscosity around a solvated dye molecule may be different from bulk ethanol. To distinguish between viscosity and polarity effects we have measured the fluorescence lifetime of Ros 5 with protonated and deprotonated carboxyl groups. The fluorescence lifetime of Ros 5 in aqueous buffer at pH 9.0 was found to be approximately 700 ps longer than at pH 3.0, indicating also a strong electronic influence of the phenyl group on the nonradiative rate constant: By deprotonation of the *p*-carboxy group, the electron-accepting property of the carboxyphenyl group is reduced. Comparison of the fluorescence lifetimes of the dyes Ros 1 and Ros 9 (hydrogen atoms instead of ethyl groups at the amino position) provides evidence

that also the electronic property of the xanthene chromophore influences the fluorescence quenching. This together with the reduced lifetimes in water, which has the same viscosity as, but a dielectric constant three times higher than, ethanol, suggests that the rotation is connected with a charge transfer between the excited xanthene moiety and the phenyl substituent. It follows from AM1 calculations that in the ground state S_0 the plane of the phenyl substituent is held nearly perpendicular to the xanthene moiety in Ros 6, Ros 7, or any other rhodamines with bulky phenyl substituents. In the rhodamine derivatives with sterically less pretentious phenyl groups, the potential energy curve for torsion is shallower. We assume that in the excited state S_1 rotation of the phenyl substituent can lead to a configuration in which the planes of the phenyl substituent and the xanthene ring system approach coplanarity. Such a rotation is not possible in rhod. 630, Ros 6, and Ros 7 due to the sterical hindrance by the *o*-carboxy group or the hydrogen atom in the 8 position of the naphthalene system, respectively. Thermal activation of the initial excited state over a potential barrier leads to a final state with a charge transfer character and a smaller S_1–S_0 energy gap. From this charge transfer state internal conversion to the ground state occurs, reducing the fluorescence efficiency and decay time. In this double-potential minimum model the height of the potential barrier and the energy difference between the initial and the final states are controlled by solvent viscosity and polarity as well as by steric and electronic properties of the phenyl substituent and the xanthene chromophore.

In practice, for the excitation of rhodamines mostly gas lasers such as argon ion or green helium–neon lasers are used [26]. However, these laser systems are power consuming, are rather expensive, and have a limited lifetime. In view of these limitations, it would be attractive to utilize diode lasers. These are compact, reliable, and highly stable devices that are easy to handle. Unfortunately, laser diodes are available in the red to near-IR region. The limited number of fluorescent dyes in this spectral region seriously hampers the application of diode lasers in biodiagnostics. For this reason we have sought new dyes that have a high quantum efficiency, even in aqueous systems, and show absorption and emission beyond 600 nm (Fig. 4). We have synthesized such dyes with carboxy functions that can be activated via succinimidyl esters and coupled to the analytes, e.g., antibodies or DNA bases. Besides the capability of covalent coupling to analytes, the new rhodamines exhibit higher quantum yields and lifetimes then the commercially available rhodamines (rhod 700, rhod 800) in this wavelength region (Table IV). On the other hand, all rhodamines show a strong decrease in lifetime by changing from ethanol to aqueous environment, except MR 200-1. The fluorescence lifetime of this dye is only slightly shortened in aqueous systems. All triphenylmethane-related dyes show a tendency to react at the central carbon with nucleophiles, in particular with hydroxide ions, if this position is sterically available. Thus a dye such as pyronin B becomes colorless on the addition of a base to its aqueous solution, owing to the formation of so-called pseudobase. Although this is reversible, subsequent reactions may lead to an irreversible destruction of the dye. In normal rhodamine dyes this reaction does not occur, because the central carbon of the chromophore is protected by the carboxyphenyl substituent. Among our new dyes the pseudobase formation is not possible in case of MR 200-1. This dye is completely stable even in strongly basic solutions. On the other hand, those dyes that carry a perfluoroalkyl substituent will form a pseudobase depending on the pH of the solution. Not only

Table IV. Spectroscopic Characteristics of Red to Near-Infrared Rhodamines[a]

	λ_{abs} (nm)	λ_{em} (nm)	τ (ns)
MR 200-1			
Ethanol (H^+)	621	642	4.06
Aqueous buffer, pH 3.0	618	638	3.62
7 *M* urea, pH 8.4	622	642	3.65
JA 51-DS			
Ethanol (H^+)	630	657	3.41
Aqueous buffer, pH 3.0	628	654	2.16
7 *M* urea, pH 8.4	631	657	2.33
JA 66			
Ethanol (H^+)	635	659	3.90
Aqueous buffer, pH 3.0	632	655	2.35
7 *M* urea, pH 8.4	638	660	2.78
JA 103-1			
Ethanol (H^+)	644	664	3.37
Aqueous buffer, pH 3.0	645	667	1.50
7 *M* urea, pH 8.4	650	674	2.26
JA 93			
Ethanol (H^+)	667	691	3.43
Aqueous buffer, pH 3.0	666	687	2.45
7 *M* urea, pH 8.4	669	694	2.13
Rhodamine 700			
Ethanol (H^+)	644	664	2.65
Aqueous buffer, pH 3.0	640	661	1.40
7 *M* urea, pH 8.4	648	670	1.79
Rhodamine 800			
Ethanol (H^+)	682	698	2.07
Aqueous buffer, pH 3.0	685	705	0.97
7 *M* urea, pH 8.4	694	718	0.92

[a] To ensure protonation a drop of trifluoroacetic acid was added to 1 ml of the alcoholic dye solution.

the equilibrium constant, but also the rate constant for the nucleophilic attack of the hydroxide ion depends on the proton concentration as well as on the solvent environment. These dyes are already destroyed slowly in aqueous systems of pH 7. However, the nucleophilic attack can be prevented by a denaturing buffer of pH 8.4 containing 7 *M* urea, as is used in DNA sequencing techniques. Interestingly, also, no formation of dimers could be observed in this solvent. Apparently urea replaces water molecules around the dye molecule, causing a change in the solvation of the latter, also demonstrated by a bathochromic shift in absorption and emission (Table IV). It is also shown in Table IV that the new dyes show a remarkably long fluorescence lifetime in 7 *M* urea. These results clearly demonstrate the suitability of the new dyes for labeling analytes also in basic systems with denaturing agents such as urea.

CONCLUSIONS

To enhance the number of discernible fluorescent labels we have developed a new class of dyes ("multiplex dyes") whose absorption and emission spectra are nearly identical but that differ in fluorescence lifetime. Combination of these dyes with laser diodes constitutes a new concept in biodiagnostics. Unfortunately laser diodes are available at present only in the red to near-IR region. Hence we had to develop new dyes with a high quantum efficiency, even in aqueous systems, showing absorption and emission beyond 600 nm. We are now engaged in studies to extend the multiplex principle to the near-IR region. Through the attachment of electron-accepting or donating groups at the amino position of rhodamine dyes, we are able to control the lifetime of the excited state without changing other spectroscopic characteristics. It is to be expected that the wavelength range of diode lasers will be extended toward the blue region of the spectrum in the near-future. With pulsed lasers that cover the spectral range from the blue to the near-IR, it will be possible to make full use of the multiplex principle: In addition to the spectral information (the "color"), a range of decay times at each color is utilized to identify a multitude of analytes in a complex mixture.

ACKNOWLEDGMENTS

We would like to thank Photon Technology International for the generous disposal of the LS 100 spectrofluorimeter and the *Bundesministerium für Forschung und Technologie* for financial support under Grants 0310183 A and 0310158 A. The financial support by the *Deutsche Forschungsgemeinschaft* is also gratefully acknowledged.

REFERENCES

1. I. A. Hemmilä (1989) *Appl. Fluoresc. Technol.* **1,** 1–16.
2. J. A. Whitaker, R. P. Haugland, D. Ryan, P. C. Hewitt, R. P. Haugland, and F. G. Prendergast (1992) *Anal. Biochem.* **207,** 267–279.
3. J.-M. Zen and G. Patonay (1991) *Anal. Chem.* **63,** 2934–2938.
4. R. P. Haugland (1992) in K. D. Larison (Ed.), *Handbook of Fluorescent Probes and Research Chemicals*, 5th ed. Molecular Probes Inc.
5. L. M. Smith (1991) *Nature* **349,** 812.
6. W. Ansorge, B. Sproat, J. Stegemann, C. Schwager, and M. Zenke (1987) *Nucleic Acid Res.* **15,** 4593.
7. J. M. Prober, G. L. Trainer, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen, and K. Baumeister (1987) *Science* **238,** 336.
8. M. Sauer, J. Arden-Jacob, G. Deltau, K. H. Drexhage, A. Schulz, S. Seeger, and J. Wolfrum (1993) *Ber. Bunsenges. Phys. Chem.* **97,** 1734–1737.
9. G. Bachteler, J. Arden-Jacob, K. H. Drexhage, K.-T. Han, M. Köllner, R. Müller, M. Sauer, S. Seeger, and J. Wolfrum (1993) *J. Luminesc.* (in press).
10. S. Seeger, G. Bachteler, K.-T. Han, M. Köllner, R. Müller, M. Sauer, A. Schulz, and J. Wolfrum (1993) *Ber. Bunsenges. Phys. Chem.* **97,** 1542–1548.
11. J. Arden, G. Deltau, V. Huth, U. Kringel, D. Peros, and K. H. Drexhage (1991) *J. Luminesc.* **48/49,** 352–358.
12. K. H. Drexhage (1973) in F. P. Schäfer (Ed.), *Topics in Applied Physics*, Springer-Verlag, Berlin, Chap. 4.
13. R. Marcus (1956) *J. Chem. Phys.* **24,** 966–971.
14. R. Marcus and N. Sutin (1985) *Biochim. Biophys. Acta* **811,** 265–312.
15. S. Seeger, K. Galla, K.-T. Han, M. Martin, M. Sauer, and J. Wolfrum (1993) *J. Fluoresc.* **3,** this issue.
16. S. A. Soper, Q. L. Mattingly, and P. Vegunta (1993) *Anal. Chem.* **65,** 740–747.
17. S. A. Soper, L. M. Davis, and E. B. Shera (1992) *J. Opt. Soc. Am. B* **9,** 1761–1769.
18. P. A. Johnson, T. E. Barber, B. W. Smith, and J. D. Winefordner (1989) *Anal. Chem.* **61,** 861–863.
19. S. J. Lehotay, P. A. Johnson, T. E. Barber, and J. D. Winefordner (1990) *Appl. Spectrosc.* **44,** 1577–1579.
20. G. Patonay, M. D. Antoine, S. Devanathan, and L. Strekowski (1991) *Appl. Spectrosc.* **45,** 457–461.
21. H. Oevering, M. N. Paddon-Row, M. Heppner, A. M. Oliver, E. Cotsaris, J. W. Verhoeven, and N. S. Hush (1987) *J. Am. Chem. Soc.* **109,** 3258–3269; G. McLendon (1988), *Acc. Chem. Res.* **21,** 160–167.
22. M. Vogel and W. Rettig (1985) *Ber. Bunsenges. Phys. Chem.* **89,** 962–968.
23. M. Vogel, W. Rettig, R. Sens, and K. H. Drexhage (1988) *Chem. Phys. Lett.* **147,** 452–460.
24. M. J. Snare, F. E. Treloar, K. P. Ghiggino, and P. J. Thistlethwaite (1982) *J. Photochem.* **18,** 335–346.
25. K. G. Casey and E. L. Quitevis (1988) *J. Phys. Chem.* **92,** 6590–6594.
26. H. Swerdlow, J. Z. Zhang, D. Y. Chen, H. R. Harke, R. Grey, S. Wu, and N. J. Dovichi (1991) *Anal. Chem.* **63,** 2835–2841.