1983

# On the Origin of Nonexponential Fluorescence Decay in Tryptophan and Its Derivatives

Jacob W. Petrich, *University of Chicago*
M. C. Chang, *University of Chicago*
D. B. McDonald, *University of Chicago*
G. R. Fleming, *University of Chicago*

protons. We believe that in the case of tryptophan $D_2O$ is reducing the rate of charge transfer from the indole moiety to the side chain.

Second, the activation energies obtained from the low-pH lifetime components of Trp and Trp-Gly are, within experimental error, the same as those obtained from the lifetime components of Gly-Trp, which does not produce the $T_1$ transient of Bent and Hayon.[28]

If the same primary nonradiative process is occurring in Gly-Trp and in Trp and Trp-Gly (which is implied by the Arrhenius parameters in Table VI), then $T_1$ must not be a primary product of the nonradiative decay. It could arise, for example, by a rapid proton transfer after the initial charge-transfer process and thus be only observable in those systems with labile protons close to the indole ring.

**(III) Self-Consistent Tryptophyl Photophysics.** If charge transfer is the nonradiative process involved in Trp, Trp-Gly, and Gly-Trp, then one must be able to invoke it when dealing with the following questions:

(1) Why does Gly-Trp have a lower quantum yield than Trp-Gly if the quenching mechanism in both cases is charge transfer from indole to peptide bond?

(2) Why does zwitterionic Gly-Trp have a lower quantum yield than anionic Gly-Trp? Are the nonradiative processes the same in the two species and one merely faster in the zwitterion, or is a new nonradiative process introduced in the zwitterion?

(3) What is the role of the protonated amino group in the fluorescence quenching of tryptophan?

(4) How does this charge-transfer interaction give rise to nonexponential decay?

Werner and Forster[36] have provided an answer to the first question through their examination of space-filling models. In Gly-Trp, the peptide bond which is the charge acceptor is able to make much better contact with the indole ring than in Trp-Gly, and thus charge transfer is enhanced. One might expect that such an orientational effect would decrease the observed activation energy. This is not necessarily so as Hopfield[45] has proposed models for electron transfer in which the orientation of the donor with respect to the acceptor affects the rate only through the frequency factor and not necessarily through the activation energy.

Ricci and Nesta[35] have argued that a given carbonyl group is able to accept electrons to the extent that there is an adjacent group capable of delocalizing the electron density in the carbonyl group. Thus we may argue, as do Werner and Forster,[36] that zwitterionic Gly-Trp has a lower quantum yield than anionic Gly-Trp because the protonated amino group is able to reduce the electron density in the peptide bond, making it a better charge acceptor. Such reasoning can be used to explain the fluorescence quantum yields of the following zwitterions studied by Weinryb and Steiner:[33] Gly-Trp < Gly-Gly-Trp < Gly-Gly-Gly-Trp. That is, the farther away the protonated amino group is from the peptide bond adjacent to the Trp the less able it is to delocalize the electron density in the peptide bond. In this way, the peptide bond becomes a less efficient quencher.

The protonated amino group plays a similar role in tryptophan. The $COO^-$ group may not be an efficient electron acceptor[26] unless an adjacent group such as $NH_3^+$ is present to decrease its electron density. Thus, at low pHs we observe the lower quantum yield in tryptophan and the associated nonexponential decay.

Finally, we must point out that while this charge-transfer mechanism seems to be quite adequate in explaining the quantum yields of these compounds, it does not *in itself* explain why they exhibit nonexponential fluorescence decay. A current explanation of the behavior has been provided by Szabo and Rayner's[11] application of Wahl and co-workers'[22,23] rotamer model. In this model, during the excited-state lifetime of the molecule there exist conformations around the $C^\alpha$–$C^\beta$ bond that do not interconvert. The different lifetimes of the rotamers arise from the different distances of the acceptor group from the indole ring in the charge-transfer interaction.

Although the rotamer model in tandem with the charge-transfer quenching mechanism seems to be adequate in explaining the fluorescence properties of the di- and tripeptides, it has not been given an exhaustive test by being applied to a wide range of tryptophan analogues that exhibit nonexponential decay and some of which do not. Such a test is the subject of our companion paper.[26]

**Acknowledgment.** This work was supported by a grant from the National Institutes of Health, Grant PHS-5-R01-GM 27825. We thank Professor N. C. Yang for generous access to the spectrofluorimeter and spectrophotometer and Professor E. T. Kaiser for the use of the HPLC. We also thank Professor Ware and co-workers for providing us with a preprint of their work.

**Registry No.** Trp-Ala, 24046-71-7; Gly-Trp, 2390-74-1; Trp-Gly, 7360-09-0; Ala-Trp, 16305-75-2; Gly-Trp-Gly, 23067-32-5; Trp, 73-22-3.

(44) Sadkowski, P. J.; Fleming, G. R. *Chem. Phys.* **1980**, *54*, 79-89.
(45) Hopfield, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3640-3644.

# On the Origin of Nonexponential Fluorescence Decay in Tryptophan and Its Derivatives

**J. W. Petrich, M. C. Chang, D. B. McDonald, and G. R. Fleming*†**

*Contribution from the Department of Chemistry and James Franck Institute, The University of Chicago, Chicago, Illinois 60637.* *Received October 21, 1982*

**Abstract:** The nonexponential fluorescence decay of tryptophan and its derivatives is discussed in terms of a simple model based on conformers about the $C^\alpha$–$C^\beta$ bond and the relative rates of charge transfer from indole to various electrophiles. Accurate predictions concerning the relative fluorescence lifetimes and the form of the fluorescence decay law are made for tryptophan and 17 of its derivatives, including three new derivatives synthesized specifically to test the model.

## Introduction

In our previous paper,[1] we discussed the quenching processes in the N- and C-terminal tryptophyl compounds and suggested that in both classes of compounds it is charge transfer that competes with fluorescence. As we noted, however, while such a proposal can explain the relative quantum yields of the N- and C-terminal tryptophyl compounds and the relative fluorescence quantum yields of anionic and zwitterionic N-terminal tryptophyl

† Alfred P. Sloan Foundation Fellow.

(1) Chang, M. C.; Petrich, J. W.; McDonald, D. B.; Fleming, G. R. *J. Am. Chem. Soc.*, preceding paper in this issue.

0002-7863/83/1505-3824$01.50/0 

compounds and of tryptophan itself, it is not *in itself* capable of explaining the nonexponentiality that is observed for the N-terminal tryptophyl compounds in the so-called pH-independent range and for the C-terminal compounds (and others such as NATE) over the entire pH range. In this paper, we present a model that is capable of rationalizing the photophysics of tryptophan and its simple analogues. The model accounts for both the magnitude of the fluorescence lifetimes and the presence or absence of nonexponential decay in a wide range of tryptophan derivatives.

Previously, Szabo and Rayner[2] have invoked the rotamer model of Wahl and co-workers[3,4] in order to explain this pH-independent nonexponentiality. In their model, different conformers of the indole ring are assumed to interconvert very slowly or not at all during the excited-state lifetime. If each conformer has a different lifetime, a given compound will exhibit nonexponential decay. Such a model is useful in that it provides a physical motivation for fitting the observed nonexponential decays to a sum of exponentials. Also, using this model, Szabo and Rayner[2,5,6] and Wahl and co-workers[3] have decomposed the steady-state fluorescence emission of various tryptophyl compounds into two spectra. In the case of tryptophan, Szabo and Rayner found that the short-lived 0.5-ns component corresponded to an emission spectrum that was blue shifted with respect to the 3-ns component. While this model is quite promising in explaining the nonexponentiality of tryptophan and its analogues, we feel that it must be investigated and developed more thoroughly before it is accepted as an explanation for the observed tryptophyl photophysics. The points requiring such scrutiny are as follows:

(1) Given that Szabo and Rayner assign different emission spectra to different rotamers, one might expect different rotamers to have different absorption spectra. Hence, the preexponential factors of the fluorescence decay should be a function of excitation wavelength. In an earlier investigation, Rayner and Szabo did not observe such a dependence.[7]

(2) While the rotamer model seems capable of describing the fluorescence decay of tryptophan, can it be extended to other compounds that exhibit nonexponential decay such as *N*-acetyltryptophan ethyl ester (NATE) and Gly-Trp and to compounds that exhibit single-exponential decay such as *N*-acetyltryptophanamide (NATA) and indole-3-propionic acid?

(3) A major assumption of the rotamer model is that certain rotamers about the $C^{\alpha}$–$C^{\beta}$ bond do not interconvert during the excited-state lifetime. Hence, the preexponential factors of the fluorescence decay are directly related to the excited-state rotamer populations. What is the barrier to rotameric interconversion and is it possible to detect a change in the preexponential factors as a function of temperature?

(4) Is a time-dependent quenching process such as that suggested by Robbins et al.[8] capable of explaining the observed nonexponential decay? What experiments are required to distinguish between the time-dependent and the static cases?

(5) In our previous paper,[1] we presented activation energies and frequency factors for Trp, Trp-Gly, and Gly-Trp in the intermediate pH range and suggested that the similarities in the activation energies and the frequency factors imply that the same nonradiative process is occurring in all three of these compounds. Do Arrhenius plots of other tryptophan analogues yield similar results? If so, how do we reconcile the similar activation energies with different fluorescence lifetimes?

(6) Finally, a self-consistent model of the tryptophyl photophysics not only must be able to address all of the points listed above but also must be able to explain the wide range of observed lifetimes: 10.5–0.1 ns.

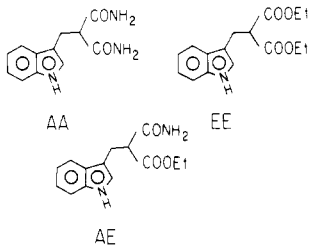



**Figure 1.** Structures of model compounds: AA, (3-indolylmethyl)-malonamide; EE, diethyl (3-indolylmethyl)malonate; AE, ethyl (3-indolylmethyl)malonamate.

The conclusions we have reached have been arrived at by: (1) following the reasoning presented in our previous paper[1] that charge transfer is the dominant mode of nonradiative decay competing with fluorescence in tryptophyl compounds, (2) using a model for charge transfer developed by Hopfield[9-11] in which the rate of charge transfer is a function of the distance between the donor and the acceptor and the ionization potential of the donor and the electron affinity of the acceptor, in tandem with the rotamer model, and (3) studying the time-resolved and steady-state emission spectra of three model compounds that we have synthesized: (3-indolylmethyl)malonamide (AA), diethyl (3-indolylmethyl)malonate (EE), ethyl (3-indolylmethyl)malonamate (AE); see Figure 1.

## Experimental Section

**(I) Materials and Data Analysis.** L-Tryptophan (Trp), *N*-acetyl-L-tryptophanamide (NATA), *N*-acetyl-L-tryptophan ethyl ester (NATE), *N*-acetyl-L-tryptophan (NAT), L-tryptophanamide, L-tryptophan ethyl ester, and glycyl-L-tryptophylglycine (Gly-Trp-Gly) were purchased from Sigma Chemical Co. The purity of Trp and Gly-Trp-Gly was checked by HPLC (Waters Associates 6000A). Glycyl-L-tryptophylglycylglycine (Gly-Trp-Gly-Gly) was obtained from Bachem. Preparation of buffer solutions is described in our previous paper.[1] Sample temperatures were controlled by a Neslab RTE4 (−20 to 80 °C) and a Neslab ULT80 (−70 to 15 °C) and were monitored by a digital thermometer (OMEGA 199) with a copper–constantan thermocouple.

Steady-state absorption and emission measurements were performed on the apparatus described earlier as were the time-resolved fluorescence measurements.[8] Unless otherwise indicated, the fluorescence decays were fit to single- or double-exponential decays by the method of iterative convolution. The quality of fit was judged by the $\chi^2$ criterion and by visual inspection of the weighted residuals. In no cases did a triple-exponential function give a significantly improved fit. All compounds reported as double-exponential give $\chi^2 \geq 2.0$ when fit to a single exponential. Double-exponential fits give $\chi^2 \leq 1.2$. We found that the fluorescence decays of tryptophanamide (pH 5) and NAT were well described by a double exponential whereas Szabo and Rayner report them to be single exponential.[2] For tryptophanamide (pH 9), NATA, AA, and EE, single-exponential fits yield $\chi^2 \leq 1.2$. The determination of the lifetime components of tryptophan ethyl ester at pH > 9 was complicated by hydrolysis of the ester group. Thus, the high pH measurements were made at pH 9 (~2 pH units above the $pK_a$ of the amino group[2]), and a fresh sample was prepared for each measurement.

In a few cases, another statistical test, the runs test,[12,13] was also employed to determine the quality of fit. The number of "runs" in an ordered set of residuals is the number of sequential groups of residuals with the same sign. Let $n_1$ = the number of positive residuals, $n_2$ = the number of negative residuals, and $u$ = the number of runs observed. If $n_1$ and $n_2 > 10$, the distribution of runs is approximately normal with

$$\mu = \frac{2n_1n_2}{n_1 + n_2} + 1/2 \qquad \sigma^2 = \frac{2n_1n_2(2n_1n_2 - n_1 - n_2)}{(n_1 + n_2)^2(n_1 + n_2 - 1)} \tag{1}$$

Then $Z$, the unit normal deviate, is defined to be

$$Z = (u - \mu)/\sigma \tag{2}$$

In other words, $Z$ is the number of standard deviations from which the

(2) Szabo, A. G.; Rayner, D. M. *J. Am. Chem. Soc.* **1980**, *102*, 554–563.
(3) Donzel, B.; Gauduchon, P.; Wahl, Ph. *J. Am. Chem. Soc.* **1974**, *96*, 801–808.
(4) Gauduchon, P.; Wahl, Ph. *Biophys. Chem.* **1978**, *8*, 87–104.
(5) Szabo, A. G.; Rayner, D. M. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 909–915.
(6) Cockle, S. A.; Szabo, A. G. *Photochem. Photobiol.* **1981**, *34*, 23–27.
(7) Rayner, D. M.; Szabo, A. G. *Can. J. Chem.* **1978**, *56*, 743–745.
(8) Robbins, R. J.; Fleming, G. R.; Beddard, G. S.; Robinson, G. W.; Thistlethwaite, P. J.; Woolfe, G. J. *J. Am. Chem. Soc.* **1980**, *102*, 6271–6279.
(9) Hopfield, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3640–3644.
(10) Hopfield, J. J. *Biophys. J.* **1977**, *81*, 311–321.
(11) Blankenship, R. E.; Parson, W. W. In "Photosynthesis in Relation to Model Systems"; Barber, J. Ed.; Elsevier: New York, 1979; Vol. 3, Chapter 3.
(12) Draper, N.; Smith, H. "Applied Regression Analysis", 2nd ed.; Wiley-Interscience: New York, 1981; pp 157–162.
(13) Swed, F. S.; Eisenhart, C. *Ann. Math. Stat.* **1943**, *14*, 66–87.

experimentally observed number of runs departs from the predicted mean. Good fits had $|Z| < 3.0$. When tryptophanamide (pH 5) or NAT were fit to single exponentials, $|Z| > 8.0$.

**(II) Synthesis of Model Compounds.** Indole-3-carboxaldehyde (97%), ethyl malonate (99%), and ethyl cyanoacetate (98.4%) were purchased from Aldrich. Indole-3-carboxaldehyde was recrystallized twice in ethanol, and ethyl cyanoacetate was distilled under vacuum and dried with magnesium sulfate. All other chemicals were reagent grade and were obtained from commercial sources. The nuclear magnetic resonance (NMR) spectra were obtained with a Bruker HS-270 spectrometer or The University of Chicago 500-MHz spectrometer. Chemical shifts ($\delta$) are reported as parts per million downfield from tetramethylsilane as internal standard. All melting points were taken on a Thomas Hoover capillary melting apparatus and are reported uncorrected. A Parr pressure reaction apparatus was used for hydrogenations. Elemental analyses of the model compounds were performed at Micro-Tech laboratories, Inc., Skokie, IL.

**(A) Diethyl (3-Indolylmethyl)malonate (EE).** EE was prepared by condensing indole-3-carboxaldehyde with diethyl malonate and then hydrogenating the intermediate, ethyl 2-carbethoxy-3-(3′-indolyl)acrylate, over $PtO_2$. The synthesis of Perron and Minor[14] was followed with few modifications. Recrystallization of the intermediate from ethanol and $H_2O$ yielded pale yellow crystals: mp 98 °C (lit. mp 99–100 °C[14]); $^1$H NMR ($CDCl_3$) $\delta$ 1.33–1.40 (m, 6 H), 4.25–4.45 (m, 4 H), 7.26 (m, 2 H), 7.44 (m, 1 H), 7.81 (m, 2 H), 8.15 (s, 1 H), 8.66 (br s, 1 H). Because of the extended conjugation, the intermediate had a maximum absorbance at 350 nm, which is approximately 70 nm red shifted from that of tryptophan. The intermediate was found to be nonfluorescent. Since a major interest in this work is the rotamer model, it would have been interesting to measure the fluorescence lifetime of this compound, which possesses only one rotamer about $C^\alpha$–$C^\beta$ due to the presence of the double bond. Hydrogenation to the product was monitored by the ultraviolet/visible absorbance spectrum. As the hydrogenation progressed, a peak at 280 nm increased and the peak at 350 nm decreased. After 1.5 h, the absorbance at 350 nm was virtually zero, and the hydrogenation was considered to be complete. The amount of catalyst used was approximately 5% of the weight of reactant. The diester derivative of Trp, after being recrystallized from ethanol and $H_2O$, was white and crystalline: mp 64 °C (lit. mp 62 °C,[15] 63–65 °C[14]); $^1$H NMR (methyl sulfoxide-$d_6$) $\delta$ 1.20 (t, 6 H), 3.37 (d, 2 H), 3.75 (t, 1 H), 4.14 (m, 4 H), 7.07 (s, 1 H), 7.16 (t, 1 H), 7.24 (t, 1 H), 7.38 (d, 1 H), 7.68 (d, 1 H), 8.10 (br s, 1 H). EE has a fluorescence maximum at 336 nm. Anal. Calcd for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62. Found: C, 66.58; H, 6.63.

**(B) (3-Indolylmethyl)malonamide (AA).** AA was prepared by exchanging the esters of the diester derivative of Trp in concentrated aq. ammonia.[15] The diester and 10 times its weight of ammonia were left stirring for 2 weeks. The diamide formed was recrystallized in ethanol and $H_2O$; mp 207 °C (lit. mp 207 °C[15]); $^1$H NMR (methyl sulfoxide-$d_6$) $\delta$ 3.07 (d, 2 H), 3.28 (s, 4 H), 3.35 (t, 1 H), 6.96 (t, 1 H), 7.03 (m, 2 H), 7.28 (d, 1 H), 7.55 (d, 1 H), 10.74 (br s, 1 H). AA has a fluorescence maximum at 340 nm and absorbance maximum at 280 nm. The purity of the diester and diamide derivatives was checked by HPLC; no impurities absorbing at 280 nm were detected. Anal. Calcd for $C_{12}H_{13}N_3O_2$: C, 62.32; H, 5.67. Found: C, 62.06; H, 5.60.

**(C) Ethyl (3-Indolylmethyl)malonamate (AE).** AE was prepared by condensing indole-3-carboxaldehyde with ethyl malonamate and again hydrogenating the intermediate. Ethyl malonamate was prepared by Pinner's method.[16,17] Reacting equimolar absolute ethyl alcohol, ethyl cyanoacetate, and gaseous HCl in ice for 4 h yielded the ethyl imidomalonate hydrochloride salt: mp 107 °C (lit. mp 102 °C[16]). HCl gas was bubbled slowly into the reaction flask through a glass frit, and excess HCl gas was trapped in a NaOH bath. The solution crystallized slowly. Once crystalline, the salt was decomposed to ethyl malonamate and ethyl chloride upon heating to 110 °C. The gaseous ethyl chloride evolved was trapped in ice. The remaining solution of ethyl malonamate was recrystallized by freezing for 12 h. The product, long colorless needles, was filtered and dired in a desiccator: mp 42 °C (lit. mp 45–46 °C,[18] 50 °C[19]); $^1$H NMR ($CDCl_3$) $\delta$ 1.15 (t, 3 H), 3.25 (s, 2 H), 4.11–4.20 (q, 2 H), 5.50–5.90 (br s, 1 H), 6.90–7.30 (br s, 1 H). The condensation of indole-3-carboxaldehyde and ethyl malonamate was done under the same conditions as the first step of the EE synthesis. Recrystallization of the intermediate olefin, 2-carbethoxy-3-(3′-indolyl)acrylamide, in ethanol and $H_2O$ and decolorization with charcoal yielded light yellow needlelike crystals: mp 167–168 °C; $^1$H NMR ($CDCl_3$) $\delta$ 1.30 (t, 3 H), 4.25 (q, 2 H), 6.53 (br s, 1 H), 7.23 (m, 2 H), 7.30 (br s, 1 H), 7.51 (d, 1 H), 7.84 (d, 1 H), 8.10 (s, 1 H), 8.40 (s, 1 H). The maximum absorbance was at 350 nm. The fluorescence emission was very weak but had a maximum at 440 nm. Hydrogenation proceeded under 52 psi for 105 min. The maximum absorbance was then at 280 nm. $PtO_2$ catalyst was filtered off, and ethanol solvent was evaporated under reduced pressure. Oil remained in the flask. Recrystallization by prolonged freezing in chloroform and *n*-pentane yielded a crude white solid. Final recrystallization in benzene and *n*-pentane resulted in white crystals: mp 93 °C; $^1$H NMR (methyl sulfoxide-$d_6$) $\delta$ 1.12 (t, 3 H), 3.13 (m, 2 H), 3.28 (s, 2 H), 3.61 (t, 1 H), 4.04 (q, 2 H), 6.95 (t, 1 H), 7.06 (m, 2 H), 7.32 (d, 1 H), 7.52 (d, 1 H), 10.76 (br s, 1 H). Maximum absorbance and fluorescence was at 280 and 340 nm, respectively. Anal. Calcd for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.76. Found: C, 64.81; H, 6.18; N, 10.82.

Table I. Excitation Wavelength Study of Trp and NATE[a]

| $\lambda_{em}$, nm | $A_1$ ($\lambda_{ex}$ 280 nm) | $A_1$ ($\lambda_{ex}$ 295 nm) | $A_1$ ($\lambda_{ex}$ 305 nm) |
|---|---|---|---|
| | | Trp | |
| 334 | 0.34 | 0.34 | 0.29 |
| 350 | | 0.26 ± 0.04 | |
| 360 | | 0.25 | 0.20 |
| 370 | 0.18 | 0.25 | 0.20 |
| 380 | 0.19 | 0.15 ± 0.02 | 0.16 |
| 390 | | 0.12 | 0.11 |
| 400 | | 0.14 | 0.08 |
| 420 | 0.0 | 0.0 | 0.0 |
| | | NATE | |
| 334 | 0.32 | 0.34 ± 0.01 | 0.29 ± 0.01 |
| 350 | | 0.34 | |
| 360 | | 0.31 | |
| 370 | 0.27 | 0.32 ± 0.03 | 0.27 ± 0.01 |
| 380 | 0.27 | 0.29 | |
| 390 | | 0.28 | |
| 400 | | 0.27 | |
| 420 | 0.27 | 0.24 | 0.24 |

[a] pH 7, 20 °C; data fit to a double exponential, $A_1 + A_2 = 1.00$. A value of 0.0 for $A_1$ implies that the decay was single exponential.

## Results and Discussion

**(I) Dependence of Decay Parameters on Excitation Wavelength.** If one assumes that different conformers have different lifetimes, it is not unreasonable to expect that different conformers have different absorption spectra and that the preexponential factors in the fluorescence decays will be a function of excitation wavelength. Rayner and Szabo investigated this possibility[7] for tryptophan and did not detect any excitation wavelength dependence, and we performed such a study for Trp and NATE (Table I) at excitation wavelenghs of 280, 295, and 305 nm, which agrees with their observation.

This result is disconcerting in light of the fact that we can observe a red-shifted absorption spectrum for anionic Trp or Trp-Gly with respect to their zwitterions.[1] In order to show, however, that a shift in fluorescence spectra can be accompanied by a very small, or no, shift in absorption spectra, we refer to the spectroscopy of the ANS derivatives which are believed to fluoresce from charge-transfer states.[20–23] Sadkowski and Fleming[24] have observed that for 1,8-ANS upon changing solvent from $H_2O$, $D_2O$, to MeOH, the maximum of the fluorescence emission shifted from 515, 510, to 484 nm while the change in the maximum of the absorption spectrum was much less severe, 355, 355, to 359 nm.

We note here that unlike the fluorescence decay of Trp, which becomes single exponential at the red edge of the fluorescence

(14) Perron, Y. G.; Minor, W. *J. Org. Chem.* **1959**, *24*, 1165–1167.
(15) Snyder, H. R.; Smith, C. W.; Stewart, J. M. *J. Am. Chem. Soc.* **1944**, *66*, 200–204.
(16) Pinner, A. *Ber. Chem. Ges.* **1895**, *28*, 479.
(17) Bailey, K. C. *Proc. R. Ir. Acad., Sect. B* **1933**, *41B*, 161–167.
(18) Wald, D. K.; Joullié, M. M. *J. Org. Chem.* **1966**, *31*, 3369–3374.
(19) Rising, M. M.; Hicks, J. S.; Moerke, G. A. *J. Biol. Chem.* **1930**, *89*, 1–25.
(20) Seliskar, C. J.; Brand, L. *J. Am. Chem. Soc.* **1971**, *93*, 5405–5414.
(21) Seliskar, C. J.; Brand, L. *J. Am. Chem. Soc.* **1971**, *93*, 5414–5420.
(22) Robinson, G. W.; Robbins, R. J.; Fleming, G. R.; Morris, J. M.; Knight, A. E. W.; Morrison, R. J. S. *J. Am. Chem. Soc.* **1978**, *100*, 7145–7150.
(23) Huppert, D.; Kanety, H.; Kosower, E. M. *Chem. Phys. Lett.* **1981**, *84*, 48–53.
(24) Sadowski, P. J.; Fleming, G. R. *Chem. Phys.* **1980**, *54*, 79–89.

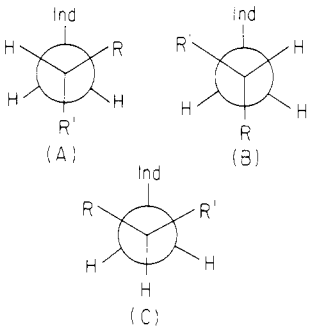



**Figure 2.** A, B, and C conformers about the $C^{\alpha}$–$C^{\beta}$ tryptophyl bond: Trp, R = $NH_3^+$, R′ = $CO_2^-$; EE, R = COOEt, R′ = COOEt; NATE, R = $NHCOCH_3$, R′ = COOEt; AE, R = $CONH_2$, R′ = COOEt; NATA, R = $NHCOCH_3$, R′ = $CONH_2$; AA, R = $CONH_2$, R′ = $CONH_2$.

**Table II.** Temperature Study of NATA[a]

| solvent | *T*, °C | $\tau$, ns |
|---|---|---|
| $H_2O$/pH7 | 5 | 3.97 |
| $H_2O$/pH7 | 20 | 2.95 ± 0.03 |
| EtOH | 20 | 3.65 ± 0.05 |
| EtOH | 0 | 4.74 ± 0.09 |
| EtOH | −20 | 6.05 ± 0.17 |
| EtOH | −40 | 6.85 |
| EtOH | −60 | 6.95 |
| EtOH | −65 | 7.23 |
| ethylene glycol | 20 | 4.58 |
| glycerol/$H_2O$ mixtures[32] | | |
| 100% glycerol, 1445 cp | 20 | 5.8 |
| 92% glycerol, 304 cp | 20 | 5.3 |
| 66% glycerol, 17 cp | 20 | 5.1 |
| 12% glycerol, 1.4 cp | 20 | 3.9 |

[a] $\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm.

emission, the decay of NATE remains nonexponential at 420 nm. Furthermore, the weight of the short component decreases much more slowly as a function of increasing emission wavelength in NATE than in Trp. Behavior similar to that of NATE was observed by us for Gly-Trp and by Szabo and Rayner for various tripeptides.[5]

**(II) Conformers and Nonexponentiality.** Szabo and Rayner[2] have implied that conformer C (Figure 2) does not form either A or B on the nanosecond time scale. If we ascribe the 0.5-ns decay component of zwitterionic Trp to C, we might attribute the 3-ns component to the average lifetimes of rapidly interconverting A and B. The same reasoning may be applied to NATE: The 0.4-ns component is due to C, and the 1.7-ns component is due to the weighted average of A and B. In the above assignments, the short lifetime component has, in both cases, been assigned to conformer C. Initially this seems quite reasonable; examination of space-filling models shows that it is very difficult for C to form either A or B. Furthermore, Cowgill[25,26] and several successive investigators have shown carbonyl to be an effective quencher of indole fluorescence;[27-31] the fact that two carbonyl groups are in close proximity to the indole moiety in C makes the assignment of the short-lived component to C even more likely.

Difficulties arise, however, when we find that NATA exhibits 3-ns single-exponential decay. On the basis of the discussion above, we would also expect NATA's C conformer to give rise to a shorter-lived decay component. A possible explanation for this result is that all of the NATA conformers interconvert rapidly at room temperature. Thus, we looked for nonexponential decay in NATA in EtOH at temperatures as low as −65 °C and in highly viscous solvents. The appearance of nonexponential fluorescence decay would be indicative of the "freezing out" of certain conformers and could give us information concerning the height of the barrier to rotation about the $C^{\alpha}$–$C^{\beta}$ bond. As Table II indicates, however, neither in EtOH at −65 °C nor in glycerol did NATA depart from single-exponential decay kinetics.

**Table III.** Temperature Study of Trp and NATE[a]

| *T*, °C | $A_1$ | $\tau_1$, ns | $\tau_2$, ns |
|---|---|---|---|
| | | Trp | |
| 5 | 0.26 ± 0.02 | 1.27 ± 0.15 | 5.05 ± 0.10 |
| 20 | 0.22 ± 0.01 | 0.62 ± 0.05 | 3.21 ± 0.12 |
| 35 | 0.21 ± 0.05 | 0.45 ± 0.21 | 2.16 ± 0.04 |
| 50 | 0.23 ± 0.05 | 0.23 ± 0.03 | 1.43 ± 0.02 |
| 65 | 0.27 | 0.22 | 0.95 |
| 75 | 0.37 | 0.18 | 0.72 |
| | | NATE | |
| 5 | 0.31 | 0.64 | 2.56 |
| 20 | 0.32 ± 0.03 | 0.42 ± 0.03 | 1.70 ± 0.03 |
| 35 | 0.28 | 0.41 | 1.28 |
| 50 | 0.33 ± 0.03 | 0.21 ± 0.03 | 0.89 ± 0.03 |
| 65 | 0.39 | 0.14 | 0.62 |
| 75 | 0.48 | 0.10 | 0.50 |

[a] $\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm, pH 7; data fit to a double exponential, $A_1 + A_2 = 1.00$.

We also found that from 5 to 50 °C in $H_2O$ at pH 7, the weights of the preexponential factors for Trp and NATE do not change (Table III). As the temperature is raised above 50 °C, the weight of the short-lifetime component in both Trp and NATE seems to increase. But such results are viewed skeptically as we are nearing the limit of the time resolution of our photon-counting apparatus.

Considering the size of the amide group in NATA, which is larger than the carboxylate group in Trp yet smaller than the ester group in NATE, it is difficult to rationalize the above results by postulating that the NATA conformers interconvert rapidly at −65 °C whereas the Trp and NATE conformers are still locked in place at 50 °C.

**(III) Fluorescence Properties of the Model Compounds: An Alternative Explanation of the Nonexponential Decay.** The above difficulties led us to consider the conformer explanation of nonexponential tryptophyl fluorescence decay from another viewpoint. We take as our initial assumption that the A, B, and C rotamers of Trp, NATE, and NATA do not interconvert quickly during the excited-state lifetime. Second, we assume that in tryptophan-containing compounds the dominant mode of nonradiative decay is charge transfer from the indole ring to an adjacent electrophile. For the compounds that we shall discuss, the electrophile is in most cases a carbonyl carbon and in some cases a protonated amino group. Third, we suggest that this charge transfer, in addition to being a temperature-dependent process,[1] has a rate that is dependent upon the proximity of the electrophile to the indole nitrogen and the electron affinity of the electrophile. Such a dependence of the rate of charge transfer has been considered by Hopfield, who derived a theoretical treatment for charge transfer in photosynthetic pigments[9-11] (vide infra).

Given the above, we suggest that the 0.4-ns component of the nonexponential decay of NATE is due to rotamters B and C, in which the more electrophilic ester groups are closer to the indole nitrogen than in rotamer A, where the less electrophilic peptide bond is closer to the indole nitrogen. (Relative electrophilicities are discussed in detail below.) An analogous situation exists in zwitterionic Trp. We suggest that the 0.5-ns component of its nonexponential decay is due to rotamers A and C, in which the protonated amino group is equidistanct from the indole nitrogen. Similarly, we assign the 3-ns component to rotamer B, in which the $NH_3^+$ is farther from the indole ring and hence less efficient in the charge-transfer interaction according to the Hopfield model.

(25) Cowgill, R. W. *Arch. Biochem. Biophys.* **1963**, *100*, 36–44.
(26) Cowgill, R. W. *Biochim. Biophys. Acta* **1967**, *133*, 6–18.
(27) Eftink, M. R.; Ghiron, C. A. *Biochemistry* **1976**, *15*, 672–680.
(28) Evans, R. F.; Ghiron, C. A.; Kuntz, R. R.; Volkert, W. A. *Chem. Phys. Lett.* **1976**, *42*, 415–418.
(29) Ricci, R. W.; Nesta, J. M. *J. Phys. Chem.* **1976**, *80*, 974–980.
(30) Werner, T. C.; Forster, L. S. *Photochem. Photobiol.* **1979**, *29*, 905–914.
(31) Fleming, G. R.; Morris, J. M.; Robbins, R. J.; Woolfe, G. J.; Thistlethwaite, P. J.; Robinson, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4652–4656.
(32) Munro, I.; Pecht, I.; Stryer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 56–60.

Table IV. Fluorescence Lifetimes of Tryptophyl Compounds[a]

| compound | $A_1$ | $\tau_1$, ns | $\tau_2$, ns |
|---|---|---|---|
| ethyl indole-3-propionate[2] | | | 3.36 ± 0.04 |
| tryptophan ethyl ester (pH 5)[b] | 0.59 ± 0.06 | 0.26 ± 0.02 | 0.87 ± 0.05 |
| tryptophan ethyl ester (pH 9) | 0.27 ± 0.05 | 0.93 ± 0.29 | 2.40 ± 0.22 |
| indole-3-propionamide[2] | | | 8.67 ± 0.05 |
| tryptophanamide[b] | 0.54 ± 0.04 | 1.14 ± 0.01 | 2.04 ± 0.04 |
| tryptophanamide (pH 9) | | | 7.01 ± 0.02 |
| indole-3-propionic acid[2] | | | 10.76 ± 0.08 |
| tryptophan (pH 7) | 0.22 ± 0.01 | 0.62 ± 0.05 | 3.21 ± 0.12 |
| tryptophan (pH 11)[8] | | | 8.18 ± 0.24 |
| Trp-Gly | 0.23 ± 0.01 | 0.51 ± 0.05 | 1.91 ± 0.06 |
| Trp-Gly (pH 11)[1] | | | 7.90 ± 0.24 |
| NATA | | | 2.95 ± 0.03 |
| NATE | 0.32 ± 0.03 | 0.42 ± 0.03 | 1.70 ± 0.03 |
| NAT | 0.33 ± 0.08 | 2.62 ± 0.13 | 5.18 ± 0.10 |
| Gly-Trp | 0.46 ± 0.03 | 0.29 ± 0.04 | 1.26 ± 0.01 |
| Gly-Trp-Gly | 0.60 ± 0.03 | 0.69 ± 0.06 | 1.69 ± 0.09 |
| Gly-Trp-Gly-Gly | 0.30 ± 0.03 | 0.34 ± 0.03 | 1.23 ± 0.01 |
| AA | | | 1.23 ± 0.04 |
| EE | | | 0.14 ± 0.01 |
| AE | 0.55 ± 0.06 | 0.11 ± 0.01 | 0.55 ± 0.02 |

[a] $\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm, $T$ = 20 °C, pH 5 unless otherwise specified. The absence of a value for $A_1$ implies the fluorescence decay was single exponential. $A_1 + A_2 = 1$ for double exponential decays. [b] $pK_a \sim 7$.[2]

It is difficult to determine whether or not $CO_2^-$ is a good electron acceptor in this case since we know from the fluorescence lifetime of indole-3-propionic acid (~10.5 ns) that when $CO_2^-$ is the lone substituent on the $\alpha$ carbon, it is a poor electrophile. Considering, however, the large decrease of the lifetime components of zwitterionic Trp with respect to protonated or unprotonated tryptamine and indole-3-propionic acid (Table IV), it is difficult to argue against any involvement of the $CO_2^-$ in the quenching process. If $CO_2^-$ is participating with the indole nitrogen in a charge-transfer interaction, it is necessary that something else, such as a protonated amino group, is enhancing its electrophilicity. Ricci and Nesta[29] have argued that the electrophilicity of a given carbonyl group is dependent upon the presence of an adjacent group capable of delocalizing the electron density of the carbonyl group. In our previous paper[1] we suggested this delocalization argument, as have Werner and Forster,[30] as the explanation for the lower quantum yield of Gly-Trp with respect to Trp-Gly. Considering zwitterionic Trp in the context of the above argument, we suggest that $CO_2^-$ is an efficient electrophile (relative to its role in indole-3-propionic acid) due to the presence of the $NH_3^+$, which is also attached to the $\alpha$ carbon and is hence capable of "activating" the $CO_2^-$.

In NATA, however, where both groups adjacent to the indole ring have peptide-like carbonyls, A, B, and C all have the carbonyl carbon equidistant from the indole nitrogen. Thus, according to our model, NATA would exhibit single-exponential fluorescence decay, which it does. Study of the fluorescence decay of AA, EE, and AE was carried out in order to test this explanation. These compounds are ideally suited for testing our hypothesis because in AA and EE the amide and ester groups are symmetrically placed about the indole ring—more so than in NATA. We would expect the fluorescence decay of AA and EE to be single exponential and to observe a shorter fluorescence lifetime in EE due to the higher electron affinity of the ester group. Furthermore, we would expect the fluorescence lifetime of AE to be double exponential. Measurement of the fluorescence decays completely confirmed these predictions (Table IV and Figure 3). In AE our model suggests the short-lifetime component is due to rotamers B and C, and the long lifetime component, to rotamer A.

**(IV) Temperature Dependence of the Fluorescence Lifetimes.** It has been well-noted that the fluorescence lifetimes and the fluorescence quantum yields of tryptophan-containing compounds decrease with increasing temperature.[8,29,31,33-36] In our previous paper,[1] we constructed Arrhenius plots from the lifetime components of zwitterionic Trp, Trp-Gly, and Gly-Trp. The similarity of the activation energies obtained led us to conclude that the same nonradiative process, charge transfer, was operative in all three of these molecules and that the presence of the protonated amino group, instead of being directly reponsible for the observed quenching through proton transfer, enhanced the electrophilicity of the adjacent carbonyl carbon.

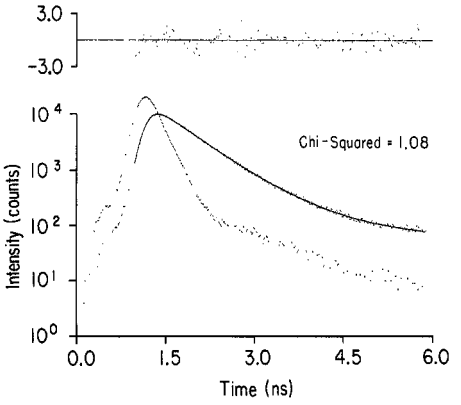



**Figure 3.** Fluorescence decay of AE fit to a double exponential: $A_1$ = 0.49, $\tau_1$ = 0.14 ns, $\tau_2$ = 0.59 ns, pH 5, 20 °C, $\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm. The weighted residuals are displayed at the top of the figure.

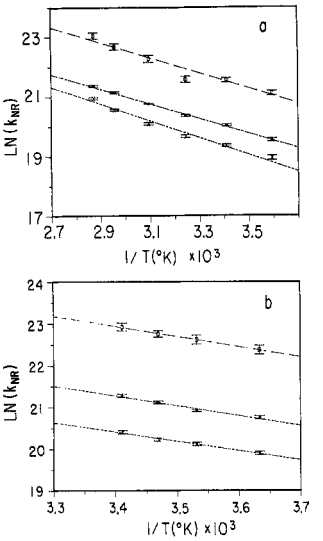



**Figure 4.** Arrhenius plots obtained from the lifetime components of NATA, NATE, AA, and AE ($\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm). The lifetime components decrease in the order $\tau_2 > \tau_1$. (a) NATE (---) $\tau_1$, (--) $\tau_2$; NATA (--). (b) AE (---) $\tau_1$, (--) $\tau_2$; AA (--). See text for a discussion of the calculation of the nonradiative rate, $k_{NR}$.

In order for our model to be consistent with the wide range of tryptophan-containing compounds, it is necessary that compounds such as NATA, NATE, AA, EE, and AE display activation energies and frequency factors similar to those obtained for the above zwitterions. The Arrhenius plots (Figure 4, a and b) were obtained following Robbins et al.[8] That is, we plotted ln $k_{NR}$ vs. $1/T$ ($K$), where $k_{NR} = k_F - k^\circ$. $k_F$ is the reciprocal of the fluorescence lifetime, and $k^\circ = k_R + k_{ISC}$, the sum of the radiative and intersystem crossing rates. We took $k_R = 5.0 \times 10^7$ s$^{-1}$ (or $6.3 \times 10^7$ s$^{-1}$)[1] and $k_{ISC} = 3.3 \times 10^7$ s$^{-1}$. Table V shows that NATA, NATE, and the model compounds exhibit $E_a$'s and $A$'s that are similar (but in general, lower) to those of the zwitterions of our previous study.[1]

(33) Kirby, E. P.; Steiner, R. F. *J. Phys. Chem.* **1970**, *74*, 4480–4490.
(34) Feitelson, J. *Isr. J. Chem.* **1970**, *8*, 241–252.
(35) Steen, H. B. *J. Chem. Phys.* **1974**, *61*, 3997–4002.
(36) Steen, H. B.; Bowman, M. K.; Kevan, L. *J. Phys. Chem.* **1976**, *80*, 482–486.

Table V. Summary of Data from Arrhenius Plots[a]

| compound | $E_a$, kcal/mol | $A$, $s^{-1}$ |
|---|---|---|
| tryptamine (pH 5.0)[33] | 8.9 | $3.2 \times 10^{14}$ |
| NAT[33] | 9.1 | $4.7 \times 10^{14}$ |
| ethyl indole-3-acetate[33] | 7.1 | $4.1 \times 10^{13}$ |
| NATE (pH 7.0, $\tau_1$) | 5.1 ± 0.3 | $(1.3 \pm 0.6) \times 10^{13}$ |
| NATE (pH 7.0, $\tau_2$) | 4.9 ± 0.1 | $(2.3 \pm 0.4) \times 10^{12}$ |
| NATA (pH 7.0) | 5.6 ± 0.2 | $(3.9 \pm 0.9) \times 10^{12}$ |
| AE (pH 6.6, $\tau_1$) | 4.9 ± 1.2 | $(3.8 \pm 8.3) \times 10^{13}$ |
| AE (pH 6.6, $\tau_2$) | 4.7 ± 0.5 | $(5.6 \pm 4.5) \times 10^{12}$ |
| AA (pH 6.6) | 4.5 ± 0.5 | $(1.5 \pm 1.3) \times 10^{12}$ |
| EE (pH 6.2) | 3.7 ± 1.3 | $(4.2 \pm 9.1) \times 10^{12}$ |

[a] The activation energies and frequency factors correspond to the nonradiative rates associated with the various lifetime components which decrease in the order $\tau_2 > \tau_1$.

We note from our previous work[1] that indole or indole compounds bound at the 3-position to a poor quencher or to a potential quencher that is not "activated" (vide supra) yield $E_a$'s and $A$'s of ~12.5 kcal/mol and $10^{17}$ $s^{-1}$. This suggests that there is a nonradiative process that is independent of the side chain and is a property of the indole chromophore. It seems likely that this process is also present when a good quencher is attached to the indole 3-position and that the observed $E_a$'s and $A$'s are an average of the two nonradiative processes: one that is intrinsic to the indole moiety and one that is due to the presence of a group that is a good acceptor in a charge-transfer interaction. We attribute the ~8 kcal/mol $E_a$'s of tryptamine (pH 5), NAT, and ethyl indole-3-acetate to the higher quenching efficiencies of $NH_3^+$, $CH_3CONH$, and COOEt with respect to $CH_3$, $COO^-$, and $CH_2CH_2OH$.

For Trp, Robbins et al.[8] suggested a method for calculating the rate of intramolecular quenching. We have generalized this method and applied it to the compounds in our study. The rate of charge transfer is given by

$$k_{CT} = k_{NR} - k_{NR,3MI} \tag{3}$$

where $k_{NR,3MI}$ is the nonradiative rate of 3-methylindole calculated as described above. Such a procedure introduces a large error in $k_{CT}$ because it involves the subtraction of two comparable quantities. Because of the large activation energy associated with $k_{NR,3MI}$, at higher temperatures the values of $k_{CT}$ are likely to be less accurate. We suggest that the curvature Robbins et al.[8] observed in their Arrhenius plot of the intramolecular quenching rate is a result of these difficulties.

We constructed Arrhenius plots of ln $k_{CT}$ vs. $1/T(K)$ and did not use data corresponding to temperatures greater than 50 °C. The data of Robbins et al. for the long-lived component of Trp at pH 7 then yield $E_{a,CT} = 4.2 \pm 1.7$ kcal/mol. Our data for Trp yield $E_{a,CT} = 5.8 \pm 1.9$ kcal/mol. The value of 4.2 kcal/mol is probably more accurate because Robbins et al. were able to obtain $k_{NR,3MI}$ directly from the 3-methylindole lifetimes, whereas we calculated $k_{NR,3MI}$ from $E_a$ and $A$ for 3-methylindole.[1] Nevertheless, *on the average*, we find that for our data the $E_{a,CT}$ are less than the $E_a$ (5.1 kcal/mol as opposed to 5.4 kcal/mol). The scatter in the value of $A_{CT}$ made a comparison with the $A$ values more difficult.

The value of $E_{a,CT}$ and in particular the low value of $E_{a,CT}$ of 3.7 ± 1.2 kcal/mol for EE, whose electrophilic ester groups effect a very efficient charge-transfer interaction can be usefully compared with the temperature studies of Ricci and Nesta.[29] In their study, Ricci and Nesta constructed Arrhenius plots of the Stern–Volmer quenching constant, $k_q$, for various quenchers of indole fluorescence: hydronium ion, cyanoacetic acid, acetic acid. Monitoring the temperature dependence of $k_q$ obviates the necessity of subtracting out $k_{NR,3MI}$; thus, if the above three quenchers are efficient charge acceptors, the activation energies should be similar to each other and less than or equal to 5 kcal/mol. This is indeed the case as Ricci and Nesta obtained an activation energy of ~3 kcal/mol for each of the three quenchers. The relative quenching efficiencies of indole fluorescence by various compounds is discussed below. It is well-known that hydronium ion is a good electron acceptor from its use as a scavanger of solvated electrons.[37]

**Nonradiative Pathways**

**(I) Parameters Affecting the Rate of Charge Transfer.** We have suggested,[1] following Werner and Forster,[30] that the reason Gly-Trp has a lower fluorescence quantum yield than Trp-Gly is that the peptide bond in Gly-Trp is able to make better contact with the indole ring than it is in Trp-Gly. A possible objection to this argument is that if better "contact" is made between the donor and the acceptor in the charge-transfer interaction, with all else being equal, why would one not see a decrease in $E_a$ for Gly-Trp with respect to Trp-Gly?

The model for charge transfer developed by Hopfield[9-11] presents an appealing answer to this question. In this model, the rate of charge transfer from donor (d) to acceptor (a) is given by

$$k_{da} = 2\pi/\hbar |T_{da}|^2 \int_{-\infty}^{\infty} D_d(E) D_a(E)\, dE \tag{4}$$

where $T_{da}$ is a matrix element containing information concerning the distance and orientation of the donor with respect to the acceptor. The $D_{d,a}(E)$ are the "electron removal" and "electron insertion" spectra of the donor and acceptor, respectively. The product $D_d(E)D_a(E)$ is an exponential whose argument contains the ionization potential of the donor and the electron affinity of the acceptor and is thus responsible for the observed activation energy. Variations in $T_{da}$ will only change the frequency factor. Thus, in the context of Hopfield's model, the decrease in the fluorescence lifetime components of Gly-Trp with respect to those of Trp-Gly and the constancy of the $E_a$'s derived from these lifetimes can be attributed solely to the preferred quenching orientation of the peptide carbonyl in Gly-Trp.

It is generally accepted that esters are more electrophilic than amides or peptide bonds, which are more electrophilic than carboxylate groups (see ref 38 and 39 and the discussion below). Hence, we would expect the $D_d(E)D_a(E)$ term to give rise to different $E_a$'s (and $E_{a,CT}$'s) for zwitterionic Trp and for NATE. Table V shows that this trend is present. Also, we would expect two different $E_a$'s from the NATE lifetime components. This is not observed, however, possibly due to the experimental uncertainties in $E_a$ (on the order of 0.5 kcal/mol), which may mask these expected changes. A change of $E_a$ from 4.5 to 5.0 kcal/mol decreases the calculated $k_{NR}$ by a factor of 2.3.

**(II) Possible Objections to the Modified Conformer Model. (A) Agreement between NMR and Fluorescence Data.** In their application of the conformer model to Trp, Szabo and Rayner made the assignment of the given conformers to the weights of the fluorescence lifetime components consistent with the rotameric populations determined via NMR.[2] Our assignment of conformers A and C to the short-lived component, however, does not give us such agreement. The NMR data of Skrabal et al.[40] predict A and C to comprise 64% and 20%, respectively, of the ground-state Trp population. This gives 84% compared with the 21% obtained from a consideration of the areas of the fluorescence spectra of the two components and their relative quantum yields.[2] Such a consideration is necessary for Trp because the two spectra are shifted with respect to one another and have different shapes. For compounds such as NATE, Gly-Trp, and Gly-Trp-Gly where the spectral shift is small (Table I and ref 5) the ratio of the preexponential factors may be used instead. For NATE, the extrapolated data of Kobayashi et al.[41] predict B and C to yield 30% and 13% of the ground-state population. This gives 43% compared with the 32% obtained for the short-lifetime component from our

(37) Feitelson, J. *Photochem. Photobiol.* **1971**, *13*, 87–96.
(38) March, J. "Advanced Organic Chemistry", McGraw-Hill: New York, 1977; pp 160–166.
(39) Cram, D. J. "Fundamentals of Carbanion Chemistry"; Academic Press: New York, 1965; pp 1–84.
(40) Skrabal, P.; Rizzo, V.; Baici, A.; Bangerter, F.; Luisi, P. L. *Biopolymers* **1979**, *18*, 995–1008.
(41) Kobayashi, J.; Higashijima, T.; Sekido, S.; Miyazawa, T. *Int. J. Pept. Protein Res.* 1981, *17*, 486-494.

Table VI. Quantum Yields and Average Lifetimes of Tryptophan and Its Analogues Relative to NATA[a]

| compound | $\phi_{rel}$ | $\tau_{rel}$ |
|---|---|---|
| NATA | 1.00 | 1.00 |
| NATE | 0.43 | 0.44 ± 0.01 |
| AE | 0.16 | 0.11 ± 0.01 |
| AA | 0.40 | 0.42 ± 0.01 |
| EE | 0.05 | 0.05 ± 0.01 |
| Trp | 1.0[30] | 0.89 ± 0.05 |
| Trp (pH 11) | 2.5[46] | 2.75 ± 0.08 |
| Trp-Gly | 0.62[30] | 0.54 ± 0.01 |
| Trp-Gly (pH 11) | 2.50[30] | 2.69 ± 0.08 |
| Gly-Trp | 0.30[30] | 0.28 ± 0.02 |
| Gly-Trp-Gly | 0.34[30] | 0.37 ± 0.02 |
| Gly-Trp-Gly-Gly | 0.32[30] | 0.33 ± 0.03 |

[a] The error in relative quantum yields is ~10%. All quantum yields in this work were measured at 21 °C. Unless otherwise indicated measurements were made on the zwitterionic forms of Trp and the tryptophyl peptides. See text for definitions of $\phi_{rel}$ and $\tau_{rel}$.

fluorescence decay measurements.

The discrepancy between the NMR and the fluorescence data and the fact that this discrepancy is larger for Trp than for NATE may be related to the observed blue shift of the short-lifetime component emission for Trp and the decreased sensitivity of our XP2020Q photomultiplier at 300 nm as opposed to 400 nm. In addition, in order to eliminate scattered light, we monitor fluorescence at $\lambda \geq 320$ nm, and this neglects a significant portion of the blue-shifted emission. It is also possible that while the absorption spectra of the rotamers have identical shape, their oscillator strength may differ slightly. In general, however, comparison of data obtained from NMR with that obtained from fluorescence measurements must be done carefully because of the different experimental conditions involved. For example, Skrabal et al. and most of the other investigators calculating the conformer populations of amino acids use samples at least 2 orders of magnitude more concentrated than those used in our fluorescence measurements.[40-45] These relatively high concentrations may be responsible for the interesting phenomena observed by Cavanaugh:[43] with increasing temperature, the rotamer populations diverge from those predicted by a Boltzmann distribution, and the temperature dependence of the rotamer populations is strongly dependent on concentration. Such behavior has led Cavanaugh and co-workers[43,44] and Kobayashi et al.[41] to consider the affect of solute–solute and solute–solvent interactions on the rotamer populations. These results suggest that the solvent plays an important role in determining the amino acid conformations and may be useful in understanding the relative spectral shifts of the lifetime components of Trp and NATE, for example. We feel, however, that due to the different experimental conditions and the fact that different NMR techniques predict different populations,[45] it is not possible to make more than qualitative comparisons of NMR data with fluorescence data.

**(B) Unresolved Decay Components.** An alternative to the charge-transfer rotamer explanation is that A, B, and C each have different fluorescence lifetimes. Thus, the fluorescence decay law is actually a triple exponential, but one of the three lifetime components is so short (~140 ps) and has a sufficiently low weight (for example, conformer C from NMR data[40]) that only two exponentials can be resolved by our apparatus. This was investigated by measuring the fluorescence quantum yields of various tryptophan-containing compounds relative to that of NATA and comparing these relative quantum yields to the average fluorescence lifetimes relative to that of NATA (Table VI). Such a

Table VII. Comparison of Fitting Parameters for Double-Exponential Decay and Transient Diffusion-Controlled Decay[a]

| compound | $\chi^2_{DE}$ | $Z_{DE}$ | $\chi^2_{DIFF}$ | $Z_{DIFF}$ | $10^6 D$, cm²/s | [Q], M |
|---|---|---|---|---|---|---|
| Trp | 1.1 | −1.9 | 1.3 | −2.0 | 0.3 | 2.2 |
| NATE | 1.1 | 0.7 | 1.4 | −1.6 | 1.1 | 1.5 |

[a] DE and DIFF refer to double exponential and transient diffusion-controlled decay, respectively. See text for a discussion of $Z$. $D$ and [Q] were calculated by using $\tau_0 = 8.1$ ns (i.e., the lifetime of Trp at pH 11) and $R' = 3 \times 10^{-8}$ cm.[8] pH 7, 20 °C, $\lambda_{ex} = 295$ nm.

study is motivated by the definition of the fluorescence quantum yield, $\phi_F$:

$$\phi_F = \int_0^\infty F(t)\, dt \tag{5}$$

$$F(t) = \sum_i^n A_i e^{-t/\tau_i} \tag{6}$$

$A_i = k_{R,i}[S_1]a_i$ and $\sum_i^n a_i = 1$. We have assumed that for a given compound, $k_{R,i} = k_R$. Thus

$$\phi_F = k_R[S_1](a_i\tau_i + \ldots + a_n\tau_n) = k_R[S_1]\langle\tau\rangle \tag{7}$$

and

$$\phi_{rel} = \phi_F/\phi_{F,NATA} = k_R[S_1]\langle\tau\rangle/k_{R,NaTA}[S_1]\tau_{NATA} = \tau_{rel} = \langle\tau\rangle/\tau_{NATA} \tag{8}$$

Thus, if we have neglected a lifetime component in the analysis of the fluorescence decays, the values for $\phi_{rel}$ and $\tau_{rel}$ will not agree. Assuming ~10% error in the relative quantum yields, we find that for all the compounds listed in Table VI eq 8 holds. We conclude that, within the experimental uncertainties of the lifetimes and quantum yields, there is no undetected short-lifetime component in the fluorescence decays.

The deviations observed for zwitterionic Trp and Trp-Gly could be compensated for by arguing that the radiative rate for these compounds is larger than that for the corresponding anions[1] and for compounds with blocked N-terminal amino groups such as NATA. It is more likely, however, that these deviations are due to failure to monitor the entire fluorescence emission and to the sensitivity of the XP2020Q phototube (vide supra).

**(C) Transient Effects in Fluorescence Quenching.** Another explanation for the tryptophyl nonexponentiality has been suggested by Robbins et al.,[8] who considered the possibility of the short-lifetime component arising from a transient diffusion-controlled intramolecular quenching mechanism. In this model, the observed fluorescence would conform to the following decay law:[47]

$$F(t) = A \exp(-at - 2bt^{1/2}) \tag{9}$$

where

$$a = 1/\tau_0 + 4\pi R'DN'[Q] \tag{10}$$

and

$$b = 4R'^2(\pi D)^{1/2}N'[Q] \tag{11}$$

$D$ is the mutual diffusion coefficient between the indole ring and the quencher of concentration [Q], $R'$ is related to the "encounter distance" between the indole ring and quencher, and $N'$ is the number of molecules per millimole.

We fit some of our decay curves for zwitterionic Trp and for NATE to eq 9. In *both* cases the fits were not as good as those obtained from the double-exponential function. We used the values of $a$ and $b$ to calculate $D$ and [Q]. Reasonable values for $D$ are $10^{-6}$–$10^{-5}$ cm²/s.[47] Considering the fact that eq 9 is a description of inter- and not necessarily intramolecular quenching processes,[47,48] the parameters obtained from the fits yielded values for

(42) Baici, A.; Rizzo, V.; Skrabal, P.; Luisi, P. L. *J. Am. Chem. Soc.* **1979**, *101*, 5171–5178.
(43) Cavanaugh, J. R. *J. Am. Chem. Soc.* **1970**, *92*, 1488–1493.
(44) Purcell, J. M.; Ramirez, J. E.; Cavanaugh, J. R. *J. Phys. Chem.* **1973**, *77*, 1501–1505.
(45) Dezube, B.; Dobson, C. M.; Teague, C. E. *J. Chem. Soc., Perkin Trans. 2* **1981**, 730–735.
(46) Ricci, R. W. *Photochem. Photobiol.* **1970**, *12*, 67–75.
(47) Nemzek, T. L.; Ware, W. R. *J. Chem. Phys.* **1975**, *62*, 477–489.
(48) Noyes, R. M. *Prog. React. Kinet.* **1961**, *1*, 129–160.

**Table VIII.** Comparison of Carbanion Stability with Quenching Ability

| compound (H-R) | $pK_a$[49] | $k_1$, $min^{-1}$ [a] | $10^{-9}k_q$, $M^{-1}$ $s^{-1}$[29] | $\phi_F$ (indole-3-$CH_2$R) | $\tau_F$, ns[c] (indole-3-$CH_2$R) |
|---|---|---|---|---|---|
| H-$CH_2COCH_3$ | 20 | $2.8 \times 10^{-8}$ | 10.0 | | |
| H-$CH_2COOH$ | 24 | $2 \times 10^{-11}$ | 0.40 | | |
| H-$CH_2COOEt$ | 24.5 | | 0.27 | 0.15[2] | 3.36 |
| H-$CH_2NH_3^+$ | | | | 0.34,[2] 0.30[33] | 6.14 |
| H-$CH_2CONH_2$ | 25 | $2 \times 10^{-12}$ | <0.01 | 0.39,[2] 0.42[4] | 8.67 |
| H-$CH_2NH_2$ | | | | 0.42[4] | 9.00 ± 0.25[d] |
| H-$CH_2CN$ | 25 | $4 \times 10^{-12}$ | | 0.44[b] | |
| H-$CH_2COO^-$ | | $2 \times 10^{-15}$ [50] | <0.01 | 0.44[2] | 10.76 |

[a] Unless otherwise specified, from ref 49. [b] From ref 26 scaled to the values in ref 33. [c] Unless otherwise specified, from ref 2. [d] The long-lived tryptamine lifetime component obtained at pH 11. $\lambda_{ex}$ = 295 nm, $\lambda_{em} \geq 320$ nm, 20 °C. This work.

*D* and [Q] that are not inconceivable (Table VII). Since, however, it was possible to fit both NATE and Trp equally well with eq 9, the possibility of intramolecular diffusional proton transfer as a quenching mechanism is ruled out.[1] Furthermore, the improved fits to double exponentials, in general, imply that a transient diffusion-controlled process is not occurring, and the fact that the fits to this equation are as good as they are point rather to the need for high-quality data.

### Self-Consistent Tryptophyl Photophysics

As we mentioned in the Introduction, any satisfactory description of the tryptophyl photophysics not only must be able to explain the presence (or absence) of nonexponential fluorescence decay but must also predict the relative fluorescence lifetimes of the tryptophyl compounds. We believe that it is possible to predict the relative lifetime and the form of the decay law for a given tryptophyl compound by assuming that charge transfer is the dominant mode of nonradiative decay and that the rate of charge transfer is dependent on (1) the proximity and the orientation of the donor with respect to the acceptor and (2) the ionization potential of the donor and the electron affinity of the acceptor. Since in this work the indole moiety is the donor in all cases, we need only concern ourselves with the relative electron affinities of the acceptors.

Because of the lack of electron affinities in the literature for ethyl ester, amide, and un-ionized and ionized carboxyl, we searched for another parameter that is proportional to the electron affinity. A suitable parameter is the acidity of hydrogens $\alpha$ to the carbonyl carbon since it is well-known that the acidity of such carbon acids is proportional to the stability of the carbanions that are their conjugate bases.[38,39,49,50] Table VIII presents the $pK_a$ of various carbon acids and the rate of dissociation, $k_1$, and compares these parameters with the Stern–Volmer quenching constants, the quantum yields, and the lifetimes of indole compounds that are bound to these groups. It is clear that increasing acidity is well correlated with ability to quench indole fluorescence. Note that the correlation between acidity and quenching efficiency made in this paper can be applied to a greater range of compounds (for example, acetone) than that proposed by Ricci and Nesta,[29] who correlated the acidity of the oxyacids of carbonyl compounds with quenching efficiency.

On the basis of the evidence presented above, we can make the following statements concerning the nonexponentiality of the tryptophan-containing compounds in the light of our model:

(1) The presence of two acceptors of different electron affinity attached to the $\alpha$-tryptophyl carbon will give rise to double-exponential decay. This can be seen in NATE, NAT, and AE as compared with NATA, AA, and EE. Furthermore, two quenching groups attached to the $\alpha$ carbon enhance the quenching efficiency of each other. For example, both of the lifetime components of zwitterionic Trp are shorter than either the lifetime of protonated tryptamine or of indole-3-propionic acid. Similarly both of the lifetime components of NATE are shorter than the lifetime either of tryptophanamide or of ethyl indole-3-propionate (Table IV).

That two species of differing electrophilicity attached to the $\alpha$ carbon give rise to double-exponential decay can also be observed in the high-pH species of trypotophan and tryptophanamide as compared with the low-pH species of tryptophan and tryptophanamide and tryptophan ethyl ester at all pHs. For example, at pH 7 the fluorescence lifetime of tryptamine ($pK_a$ = 10.2[51]) is ~6 ns, but when the pH is raised to 11, a 9-ns component appears. This is near the lifetime of indole-3-propionic acid, ~10.5 ns. Thus at high pH, $CO_2^-$ and $NH_2$ are equally poor electrophiles, and anionic tryptophan exhibits single-exponential decay. A similar argument may be made for tryptophanamide. Tryptophan ethyl ester, however, is different in that at all pHs the electrophilicity of the amino group is lower than that of the ester group. Thus, tryptophyl ethyl ester is double exponential over the entire pH range.

(2) Tryptophyl compounds that have only one substituent attached to the $\alpha$ carbon are single exponential because the conformers obtained by rotation about the $C^\alpha$–$C^\beta$ bond also possess values of $\phi$, $\psi$, and the angle formed by rotation about $C^\beta$–$C^\gamma$ that maintain equivalent quencher–indole nitrogen distances. Similar considerations must be made for tryptophyl compounds with two carbonyl groups attached to the $\alpha$ carbon. Examination of space-filling models for NATA or NATE, for example, shows that it is rotation about these bonds that prevents both carbonyl carbons from being equidistant from the indole nitrogen in conformer C. Such rotation prevents the lifetime of the NATA C conformer from being shorter than that of the A and B conformers.

### Conclusion

The qualitative features of the fluorescence decay of tryptophan and 17 of its derivatives are rationalized by our simple model. Our model makes definite predictions, is easily tested, and seems consistent with a large number of experimental observations. In particular, the form of the decay law is accurately predicted in all cases, and predictions of relative lifetimes based on the charge-transfer rate for different conformers about the $C^\alpha$–$C^\beta$ bond are quite good.

Quantitative explanation of tryptophyl photophysics will require information on conformations not only about the $C^\alpha$–$C^\beta$ bond but also about the $C^\beta$–$C^\gamma$ bond and the angles $\phi$ and $\psi$. Also sorely needed is an extension of the excellent study of Bent and Hayon[52] into the subnanosecond region with sufficiently low intensity excitation that biphotonic effects can be ruled out.

Despite these potential complications, we believe that we have made significant progress in understanding and predicting the fluorescence properties of small tryptophyl compounds and, what is ultimately more important, the small tryptophan polypeptides. On the basis of these studies, we suggest that peptides of the form $(X)_n$-Trp-$(Y)_m$ will all exhibit photophysics similar to that of Gly-Trp when $n$ and $m$ are small enough such that the effects of secondary and tertiary structure are minimal and X and Y do not possess side chains of high electron affinity.

**Acknowledgment.** This work was supported by NIH Grant PHS-5-R01-GM 27825. We are indebted to Professor N. C. Yang for suggesting and designing the synthesis of AA, EE, and AE

(49) Pearson, R. G.; Dillon, R. L. *J. Am. Chem. Soc.* **1953**, *75*, 2439–2443.
(50) Bonhoeffer, K. F.; Geib, K. H.; Reitz, O. *J. Chem. Phys.* **1939**, *7*, 664–670.
(51) Bridges, J. W.; Williams, R. T. *Biochem. J.* **1968**, *107*, 225–237.
(52) Bent, D. V.; Hayon, E. *J. Am. Chem. Soc.* **1975**, *97*, 2612–2619.

and for providing laboratory facilities for the synthesis. We also thank Peter Chen for help with the synthetic work and Albert Cross for many helpful discussions.

**Registry No.** AA, 53215-63-7; EE, 10184-98-2; AE, 85533-76-2; Trp-Gly, 7360-09-0; NATA, 2382-79-8; NATE, 2382-80-1; NAT, 1218-34-4; Gly-Trp, 2390-74-1; Gly-Trp-Gly, 23067-32-5; Gly-Trp-Lgy-Gly, 24591-52-4; ethyl indole-3-propionic acid, 40641-03-0; tryptophan ethyl ester, 7479-05-2; indole-3-propionamide, 5814-93-7; tryptophanamide, 20696-57-5; indole-3-propionic acid, 830-96-6; tryptophan, 73-22-3; indole-3-carboxaldehyde, 487-89-8; diethyl malonate, 105-53-3; ethyl 2-carbethoxy-3-(3'-indoyl)acrylate, 10184-96-0; ammonia, 7664-41-7; ethyl malonamate, 7597-56-0; 2-carbethoxy-3-(3'-indolyl)acrylamide, 85506-91-8.

# Novel Example of Simultaneous Double N-Inversion in the Solid State

**M. Kaftory**

*Contribution from the Department of Chemistry, Technion—Israel Institute of Technology, 32000 Haifa, Israel.* *Received July 26, 1982*

**Abstract:** Two configurational isomers (endo and exo) of the mono-adduct and one isomer (endo, endo) of the bis-adduct of 11-cyano-1,6-methano[10]annulene with 4-methyl-1,2,4-triazoline-3,5-dione have been isolated and their crystal structures determined by X-ray diffraction methods. The thermodynamically less stable isomer (exo) is transformed to the endo isomer upon heating a solid sample to ca. 175 °C. This process was detected by high-temperature diffraction methods. The change of configuration is explained by a simultaneous double N-inversion with a doubly planar transition state.

Atomic inversion is one of the most subtle of molecular processes, since only a reversal of configuration results, no bonds are broken, and no other chemical reactant is required.

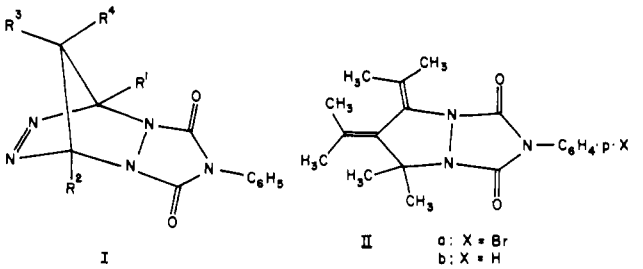
The subject of atomic inversion was extensively studied in the last 6 decades since the early work of Meisenheimer and co-workers[1] who suggested that an inversion process was responsible for the inability of trivalent nitrogen to sustain optical activity.

Thermodynamic data, mainly height of the energy barrier, were determined from experimental methods such as infrared, microwave, and NMR spectroscopy, from studies of the kinetics of decay of optical activity that accompanies racemization, or from theoretical calculations. These various approaches yielded thermodynamic data for over 200 compounds involving inversion of C, N, O, S, or P atoms. However, most of the experiments were carried out on solutions of the compounds. The results have been reviewed by Lambert,[2] Lehn,[3] and others.

Nitrogen is the most thoroughly studied central atom in the field of atomic inversion. Thorough studies have been made with molecules containing two nitrogen atoms that are singly bonded to each other.[4] Although conformational processes have been observed in some of these molecules, in most of them the process is a rapid consecutive inversion about two nitrogen atoms. A simultaneous inversion about both atoms has been rejected. In cyclic hydrazides in general or in the 1,2,4-triazolidinedione ring in particular, it is generally believed that conjugation of the N-lone pair with a carbonyl group stabilizes planarity at the N atoms, lowering the inversion barrier.[5]

Arnold and co-workers[6] have studied the stereochemistry of the urazole moiety in I by NMR spectroscopy.

The spectra were unchanged over the temperature range +60 to −60 °C. The observations were consistent either with a rigid urazole moiety, where the hydrazine nitrogens are planar or pyramidal, or with one that is rapidly inverting even at −60 °C. variable-temperature NMR studies of related compounds were carried out, leading to the conclusion that the hydrazine nitrogens in the urazole ring do not have rigid, pyramidal structures. The absence of temperature dependence in the spectra also argues against inverting pyramidal conformations for the hydrazine nitrogens. The conclusion was, therefore, that the hydrazine nitrogens in the urazole ring of I must be planar or very close to planar.




I II a: X = Br b: X = H

Pasto and co-workers[7] have reported results of dynamic nuclear magnetic resonance (DNMR) studies on IIb and the crystal structure of IIa. The molecular structure indicated that the hydrazine nitrogens in the urazole ring are not planar (the interplanar angle is 160.9°). The DNMR studies showed temperature-dependent spectra with coalescence temperature for the dynamic process at −28 °C, which afforded $\Delta G^{\ddagger} = 11.9$ kcal/mol. comparison of the two possible explanations for the change of configuration, enantiomerization of the skewed dienes chromophore or N–N bridge inversion, led to the conclusion that N–N bridge inversion is the phenomenon giving rise to the observed results.

In the course of a study of the configuration of Diels–Alder adducts of propellanes and of bridged[10]annulenes with *N*-methyl- and *N*-phenyltriazolinedione,[8a] the Alder *exo*-III- and *endo*-IV-type isomers of the adducts of 11-cyano-1,6-methano-[10]annulene with 4-methyl-1,2,4-triazoline-3,5-dione[8b] have been isolated and their structures determined by X-ray diffraction

(1) Meisenheimer, J.; Angerman, C.; Finn, O.; Vieweg, E. *Ber. Dtsch. Chem. Ges.* **1924**, *57*, 1744–1759.
(2) Lambert, J. B. *Top. Stereochem.* **1971**, *6*, 19–105.
(3) Lehn, J. M. *Fortschr. Chem. Forsch.* **1970**, *15*, 311–377.
(4) (a) Anderson, J. E.; Lehn, J. M. *J. Am. Chem. Soc.* **1967**, *89*, 81–87. (b) Allred, E. L.; Anderson, C. L.; Miller, R. L.; Johnson, A. L. *Tetrahedron Lett.* **1967**, 525–531. (c) Lehn, J. M.; Wagner, J. *Tetrahedron* **1969**, *25*, 677–689. (d) Wagner, J.; Wojnarowski, W.; Anderson, J. E.; Lehn, J. M. *Ibid.* **1969**, *25*, 657–676. (e) Nelsen, S. F.; Hollinsed, W. C.; Grezzo, L. A.; Parmelee, W. P. *J. Am. Chem. Soc.* **1979**, *101*, 7347–7352.
(5) The calculations were done by MNDO and ab initio STO-3G for suitable models. Arad, D.; Apeloig, Y., to be submitted for publication.
(6) Evning, A. B.; Arnold, D. R.; Karnischky, L. A.; Strom, E. *J. Am. Chem. Soc.* **1970**, *92*, 6218–6231.
(7) Pasto, D. J.; Scheidt, W. R. *J. Org. Chem.* **1975**, *40*, 1444–1447.
(8) (a) Ashkenazi, P.; Kaftory, M.; Ginsburg, D., to be submitted for publication. (b) Ashkenazi, P.; Kaftory, M.; Arad, D.; Apeloig, Y.; Ginsburg, D. *Hel. Chim. Acta* **1981**, *64*, 579–581.

0002-7863/83/1505-3832$01.50/0 