tonation and/or the amount of dissociation into free ions appear to decrease since the solution conductance rapidly reaches a maximum and then decreases. Infrared measurements of the concentration of formate ion in solutions beyond the conductance maximum were presented in Table I. As can be seen, the formate ion concentration appears to increase as the concentration of DMA increases. This observation, coupled with the fact that the conductance is decreasing in this concentration range, indicates that dissociation into ions is incomplete. Thus, the spectral concentration of $HCOO^-$ will be higher than the concentration of free $HCOO^-$ as determined conductometrically. A reasonable explanation appears to be the formation of associated ions, or ion pairs. Such ionic association is difficult to reconcile with the generally accepted theory for the behavior of 1:1 electrolytes in solvents of high dielectric constant.[40] Nevertheless, this type of association is not without precedent. Recently, D'Aprano and Fuoss[41] showed that nonconducting solutions of picric acid in acetonitrile became highly conducting upon addition of solvents, such as water, methanol, and ethanol, which are basic relative to picric acid. The conductance increased sharply on first addition of the basic solvent and continued to increase with the content of basic solvent, regardless of whether the dielectric constant increased or decreased. Specific solvent interaction of the basic solvent with the picrate ion was cited as the reason for the apparent inapplicability of simple electrostatic effects.

Specific solvent–solute interaction has been demonstrated in the FA–amide systems. The competing hydrogen bonding equilibrium is thought to be responsible for the decrease in the dissociation constant for equilibrium 2.

From the conductance data it is clear that dissociation into ions is complete at $X_{FA} > 0.9$. Thus, the initial rise in specific conductivity with the addition of solute, as well as the value of the conductance maximum, generally at $X_{FA} = 0.9$, directly reflects the degree of protonation by FA, and, consequently, the basicity of the solutes. In particular, FA appears to be probing the electron density at the oxygen carbonyl, providing a convenient method for the comparison of amide and peptide bonds.

Acknowledgments. The authors are grateful to Dr. Philip J. Chenier of the Department of Chemistry, Loyola University, Chicago, Ill., for taking the nmr spectra and assisting in their interpretation.

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Basicity Differences among Peptide Bonds[1,2]

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Abstract: The basicities of peptide bonds formed from various amino acids were investigated using anhydrous formic acid as a solvent probe. Conductivity measurements of the degree of protonation by formic acid gave the order of decreasing carbonyl basicity as: proline peptide bonds > serine > glycine ~ alanine. The peculiar position occupied by proline was established and discussed in terms of recent conformational analysis and solvent–polymer interaction.

Recent theoretical treatments of polypeptide conformation have achieved some success in predicting the stability of structures[4-6] of various polypeptides. Calculations of the dimensions of stable, isolated polypeptide helices[7] as well as the mean square properties of random chain polymers[8] give results in reasonable agreement with experimental data. However, some of the subtle effects observed in the study of polypeptide conformation and structural interconversions in solution[9] seem beyond the scope of existing treatments. Examples can be easily found among investigations involving conformational titrations wherein differences in the conformational stability of various polypeptides are explained on the basis of "side chain effects." Fasman's review[9] effectively illustrates the disparity between current theory and experiment. A compilation is given of a series of homopolypeptides with regard to the stability of their α-helical form in various nonaqueous solutions. Hydrophobic interactions are absent in these systems. However, optical rotatory dispersion measurements on these polymers, generally

(1) A report of work done under Contract No. 12-14-100-8933(73) with the U. S. Dept. of Agriculture and authorized by the Research and Marketing Act of 1946. The contract is being supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service.
(2) Presented in part at the 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969.
(3) Submitted by C. F. N. in partial fulfillment of the requirements for the Ph.D. degree at Northwestern University, Chicago, Ill., Aug 1969.
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in trifluoroacetic acid–chloroform or dichloroacetic acid–chloroform, show marked differences in α-helical stability.

A more detailed study of such side chain effects appeared necessary. At the present time, intramolecular interactions in which the side chains participate *directly* include:[6] (1) nonbonded interactions, (2) dipole–dipole interactions, (3) hydrogen bonding, and (4) hydrophobic interaction. These forces act to modify the backbone directed conformation. Polypeptide backbones are envisioned as linear arrays of equivalent peptide units.

This paper reports an investigation into the effect which side chains have on the electronic character of their adjacent peptide bonds. The question asked was whether different side chains exert a characteristic and differentiable effect on the peptide group. Such an *indirect* effect, if present, should be of an inductive type.

The investigations of Chao, *et al.*,[10] and Higuchi, *et al.*,[11] demonstrated substantial differences in the basicities of variously substituted model amides. Relatively minor substitutions had a dramatic effect on base strength with respect to formic and perchloric acids. Thus, it was anticipated that such substitutional effects might also influence the basicity of peptide bonds.

In the preceeding paper[12] the interaction of formic acid with model amides was investigated. Formic acid was found to protonate amides when present in high concentration and under anhydrous conditions. Dissociation of the protonated amide into formate and amide cations was found to be nearly complete when formic acid was present in amounts greater than 0.9 mole fraction. The specific conductance of solutions in this concentration range was found to be a convenient measure of the degree of protonation. Consequently, anhydrous formic acid was used as a solvent probe of peptide bond basicity in the present study. Appropriately selected oligopeptides were compared.

Experimental Section

Materials. Anhydrous formic acid (FA) was prepared by the method given in the preceeding paper.[12]

N$^{\alpha}$-(N-Acetylglycyl)glycinemethylamide (GLY) was prepared from the corresponding methyl ester (Cyclo Chemical Corp., Grade II, Lot No. P-2257) using the procedure of Applewhite and Niemann.[13] The product was recrystallized three times from absolute methanol, passed through a Dowex-1 (50–100 mesh) anion exchange column, and recrystallized from methanol again. Thin layer chromatography using 60/40 chloroform–methanol and elemental analysis indicated a trace impurity remained. Further purification was not attempted in view of the small amount of material on hand.

Anal. Calcd for $C_7H_{13}N_3O_3$: C, 44.92; H, 6.95; N, 22.46. Found (Midwest Microlab Inc.): C, 44.88; H, 7.29; N, 22.39.

N$^{\alpha}$-(N-Acetylglycyl)glycinamide (AGGA) was obtained from Cyclo Chemical Corp. (Lot No. C-1363) in a Grade I purity.

The following compounds were obtained as custom preparations in Grade I purities from Cyclo Chemical Corp.: N$^{\alpha}$-(N-acetyl-L-alanyl)-L-alaninemethylamide, Lot No. P-2249 (ALA); N$^{\alpha}$-(N-acetyl-L-seryl)-L-serinemethylamide, Lot No. P-2287 (SER); and N$^{\alpha}$-(N-acetyl-L-prolyl)-L-prolinemethylamide, Lot No. P-2248 (PRO).

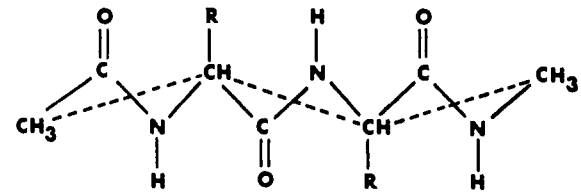

Figure 1. General structure of oligo-L-peptides used in this investigation.

Preparation of Solutions. Solutions were prepared in a drybox under dry nitrogen. Since most of the oligopeptides were very hygroscopic, they were dried *in vacuo* at 60° for 24 hr prior to use. Concentrations, determined by evaporation of volumetric aliquots, were calculated in terms of the mole fraction of formic acid, X_{FA}, according to the equation

$$X_{FA} = \frac{n_{FA}}{n_{FA} + n_{-CONH-}}$$

where n_{FA} = the number of moles of FA and n_{-CONH-} = the number of moles of peptide unit.[14] All of the oligopeptides had three peptide units per molecule.

Conductivity Measurements. Conductance measurements were performed as described in the preceeding article.[12] Two of the glycine-containing peptides (AGGA and GLY) were obtained in rather small amounts, so that the conductance cell was modified to permit measurements over a considerable concentration range with a minimum amount of material. Two 10-ml bulbs were adapted to fit into the two filling arms of the cell, and served as overflow vessels. The volume of the cell was extended in this way from 2.5 to about 23 ml, and dilutions could easily be performed inside the cell. Mixing was effected by displacing the solution into each overflow bulb several times with a slight air pressure produced and controlled with a syringe. In this way, only two stock solutions (2.5 ml each) were needed to cover the desired concentration range. A slight scattering of conductance values was observed using this dilution technique, probably a result of inefficient mixing. However, a comparison of these values with standards of known concentration showed a variation of only $\pm 1\%$.

Because of the trace impurity present in the sample of N$^{\alpha}$-(N-acetylglycyl)glycinemethylamide, a background conductance, obtained from aqueous solutions of the peptide, was subtracted from the conductance measured in formic acid. The appropriateness of this correction was demonstrated by comparing the results with those for a very similar pure compound, N$^{\alpha}$-(N-acetylglycyl)-glycinamide.

Viscosities and Densities. Experimental techniques were identical with those described in the preceeding paper.[12]

Results

The model oligopeptides used in this study were compounds having the general structure shown in Figure 1. These can be described as dipeptides blocked at the carboxyl and amino ends with methylamino and acetyl groups, respectively, or, alternatively, as chains of three peptide units.[14] Two "side chain" groups (R_1) are present and the center C–N bond in each compound, at least, can be considered to approximate a peptide bond. Model compounds of this type were chosen to ensure their solubility in anhydrous FA and because, with their similarity in size, they should have similar transference numbers if ionized. Furthermore, because of their small size, they could not attain regular intramolecular helical structures[9] and the results should be independent of conformation.

Amino acid residues of four types were used: (1) glycine, with no side chain, (2) L-alanine, containing apolar methyl groups, (3) L-serine, with its polar

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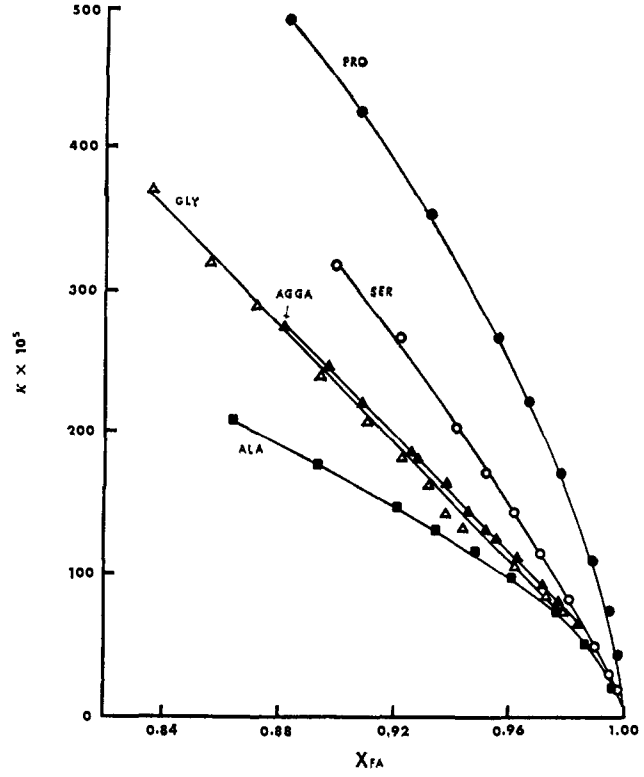

Figure 2. Specific conductivities (ohm^{-1} cm^{-1}) at 25° of solutions of oligopeptides in formic acid as a function of the mole fraction of formic acid. See text for explanation of abbreviations.

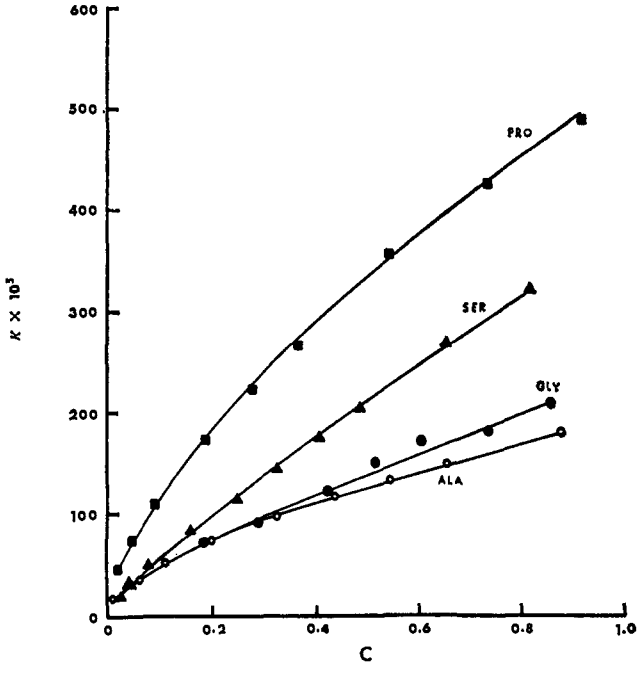

Figure 3. Specific conductivities (ohm^{-1} cm^{-1}) at 25° of solutions of oligopeptides in formic acid as a function of the molar concentration of solute (C). See text for abbreviations.

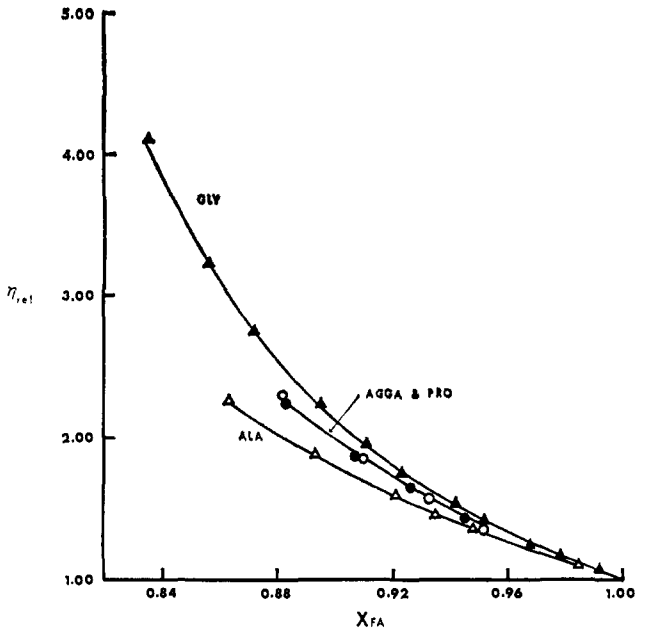

Figure 4. Relative viscosities at 25° of solutions of oligopeptides in formic acid.

hydroxymethyl groups, and (4) L-proline, containing rigid pyrrolidine rings and having no protons on the amide nitrogens. Charged, aromatic, and sulfur-containing residues were not considered. The individual compounds are referred to by their amino acid abbreviation, *viz.* (1) GLY, (2) ALA, (3) SER, and (4) PRO. In each case, $R_2 = CH_3$. Acetylglycylglycinamide (AGGA) in which $R_1 = R_2 = H$ was also studied.

The compound GLY could not be obtained free of some trace, highly conducting and presumably ionic materials. The conductance data for GLY in formic acid were therefore corrected by subtracting the conductance determined for GLY in water at the same mole fraction of solvent. The solubility of GLY in water, $0.97 < X_{H_2O} < 1.0$, was over a much more limited range than its solubility in formic acid. Hence, the correction factors for $X_{FA} < 0.97$ were obtained by extrapolation from the limited aqueous data and the corrections can be taken only as rough approximations. The corrected values, however, were very close to the values obtained with the pure compound AGGA in FA, lending credence to the corrected data.

Specific conductivities of formic acid solutions of all the oligopeptides are plotted in Figure 2 as a function of the mole fraction of FA. As noted above the two glycine derivatives give nearly identical conductance curves and their position with respect to other oligopeptides is validly established. The method of plotting used in Figure 2 creates the impression that each peptide unit is protonated, for which there is no evidence. However, a plot based on the assumption of one proton transferred per solute molecule, the opposite extreme, is given in Figure 3. In Figure 3 the molarity of solute is given on the abscissa. As can be seen, the order of the conductance curves is maintained and does not depend on the number of protons transferred per molecule of oligopeptide. The order of increasing degree of protonation is ALA ~ GLY < SER < PRO.

The relative ease of protonation can be expressed quantitatively in terms of the slopes of the specific conductance–concentration plots of Figure 3 either at high solute concentration or at infinite dilution. These values are presented in Table I, along with the ratios of the specific conductivities at $X_{FA} = 0.90$, $\kappa(90\%)$, to that of the ALA solution at $X_{FA} = 0.90$.

Table I. Limiting and High-Concentration Slopes for the Conductance–Concentration Plots for the Various Peptides in FA

Side chain	$\frac{\Delta\kappa}{\Delta C_{C\to 0}} \times 10^4$	$\frac{\Delta\kappa}{\Delta C_{C\to\infty}} \times 10^4$	$\frac{\kappa(90\%)}{\kappa(90\%)^{ALA}}$
$-CH_3$	43	15	1
$-H$		20	1.4
$-CH_2OH$	70	33	1.9
Pyrrolidine ring	124	37	2.6

Table II. Apparent Specific Volumes of the Peptides in FA Mixtures

FA–NMA		FA–AGMA		FA–ALA		FA–SER		FA–PRO	
$(M)^{1/2}$	ϕ_{v2}	$(M)^{1/2}$	ϕ_{v2}	$(M)^{1/2}$	ϕ_{v2}	$(M)^{1/2}$	ϕ_{v2}	$(M)^{1/2}$	ϕ_{v2}
0.71	0.98	0.28	0.87	0.11	0.82	0.13	0.66	0.14	0.87
1.11	1.00	0.44	0.83	0.22	1.29	0.20	0.73	0.22	0.84
1.56	1.01	0.60	0.89	0.31	1.07	0.29	0.74	0.30	0.83
1.79	1.02	0.85	0.84	0.38	1.10	0.41	0.74	0.43	0.84
1.96	1.02	1.03	0.84	0.44	1.12	0.50	0.74	0.53	0.84
2.10	1.02	1.19	0.84	0.54	1.04	0.57	0.74	0.61	0.84
2.47	1.03	1.46	0.84	0.62	1.05	0.64	0.75	0.68	0.85
2.72	1.03	1.68	0.86			0.70	0.75	0.74	0.85
		1.88	0.85			0.81	0.75	0.86	0.85
						0.91	0.75	0.96	0.84

Relative viscosities of these oligopeptide solutions are shown in Figure 4. All compounds exhibit very similar viscosity behavior, so that it is unlikely that the different conductance curves observed represent artifacts resulting from the viscous properties of these solutions.

The position of the serine peptide is tentative, since two additional functional groups, hydroxyl groups, are potential sites of protonation. Evidence for the possible involvement of these sites is indirect. Density data obtained on these solutions are shown in Figure 5. N-Methylacetamide (NMA) and N^{α}-acetylglycine-methylamide (AGMA) are included for comparison.[12] Apparent specific volumes calculated from these data are given in Table II. It is clear that the serine oligopeptide solution in FA exhibits the greatest degree of electrostriction. Since the specific conductance curve of PRO (see Figure 2 or 3) is higher than that of SER, it is probable that SER is protonated on its hydroxyls as well as the peptide bonds.

Discussion

The degree of protonation, as indicated by the conductivity results of Figure 2 and 3, of the oligopeptides studied decreases in the order PRO > SER > GLY ~ ALA. The data on the simple amide systems presented in the preceeding paper all point to the carbonyl oxygen of the amides as the predominant site of protonation by FA. It is concluded, therefore, that the series PRO > SER > GLY ~ ALA corresponds to the order of decreasing carbonyl basicity. The position occupied by proline peptide bonds as the most basic is firmly established. The observed differences must be consequences of side chain inductive effects, since steric and field effects should be unimportant in the systems selected for study.

The uniformity assigned to peptide dipole moments is clearly an oversimplification. Theoretical conformational analysis of polypeptides requires modification in this light. Special emphasis is placed on the examination of the role of proline peptide bonds, since these have been shown to possess the most basic carbonyl function.

Recently, Holtzwarth and Backman[15] estimated the intramolecular electrostatic interaction energy of the peptide dipoles in the forms I and II of poly-L-proline as well as in chains with I–II and II–I junctions. Based on their calculations, these electrostatic interactions were suggested to contribute significantly to the cooperativity of the I–II transition. The high basicity found here for the proline carbonyl indicates that the electrostatic energies calculated by Holtzwarth and Backman were probably minimum values and that dipole–dipole interactions are indeed a major source of the stability of poly-L-proline II in water and the cooperativity of the I–II interconversion.

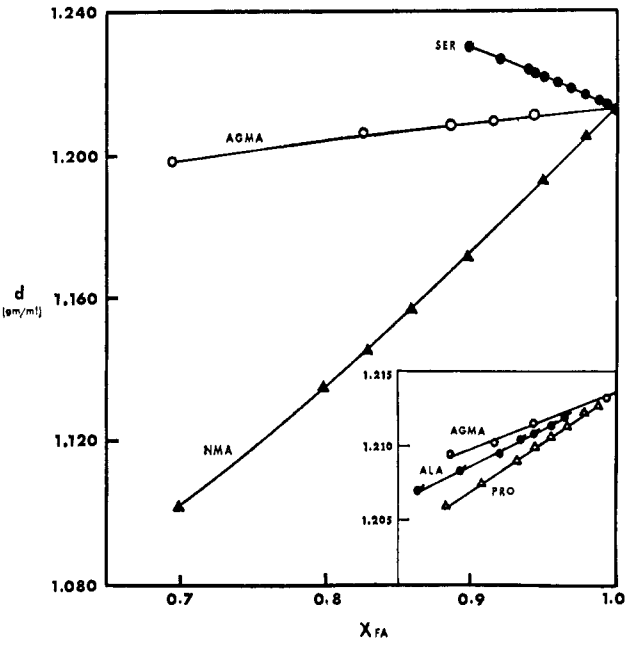

Figure 5. Densities at 25° of solutions of various amides and oligopeptides in formic acid. Note expanded ordinate scale of inset.

The results of this investigation are also applicable to polypeptide–solvent interaction studies. Peptide bonds with strongly basic carbonyl functions should be highly solvated in polar media. Protic solvents

(15) G. Holtzwarth and K. Backman, *Biochemistry*, 8, 883 (1969).

should readily hydrogen bond to these functions, making intramolecular hydrogen bonding unfavorable. Furthermore, specific solvent interaction may be expected to alter the potential energy for rotation about backbone C′–N, N–C$^{\alpha}$, and C$^{\alpha}$–C′ bonds.

One clear case of specific solvent binding and a rotational potential function which is sensitive to solvent is that of poly-L-proline. The compact *cis* helix (form I) is stable in poor solvents, but is converted to the extended *trans* helix (form II) in good solvents, such as water. From the transition kinetics in glacial acetic acid and acetic acid–propanol, the barrier to rotation about the C–N bond in this polymer has been estimated to be in excess of 20 kcal/mol.[16] However, this barrier must be significantly lowered in aqueous solution, since the I–II transition takes place readily.

(16) I. Z. Steinberg, W. F. Harrington, A. Berger, M. Sela, and E. Katchalski, *J Amer. Chem. Soc.*, **82**, 5263 (1960).

Upon heating an aqueous solution of poly-L-proline II to 55°, complete precipitation takes place to give solid form II.[17] Swenson and Formanek[18] have shown that hydrogen bonding of water to the proline carbonyl function is disrupted at temperatures prior to precipitation. Their infrared measurements indicated the appearance of a free and a bonded poly-L-prolinamide I band, the former increasing in proportion as the temperature is raised. Clearly, solvation of the carbonyl function is intimately related to the stability of the solution phase in water.

Acknowledgments. C. F. N. is grateful for the financial assistance of a NDEA Title IV Fellowship (No. 65-0684) and a Public Health Service grant (No. 5-SO1-FR-05370-08).

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Chemiluminescence of Decomposition of 1,4-Peroxy-1,4-dimethoxy-9,10-diphenylanthracene

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Contribution from the Battelle Memorial Institute, Columbus Laboratories, Columbus, Ohio. *Received November 29, 1969*

Abstract: A kinetic study is presented of the chemiluminescent decomposition of 1,4-dimethoxy-9,10-diphenylanthracene peroxide in 1,4-dioxane containing a carboxylic acid and a fluorescent hydrocarbon. It is shown that a nonreversible reaction, induced by the acid, is responsible for the generation of an excited product which in turn excites the fluorescent hydrocarbon by energy transfer.

In the course of their very extensive (and continuing) study of the photoperoxides of aromatic hydrocarbons Dufraisse and coworkers[1] noted that several of the *meso*-aryl substituted hydrocarbons formed peroxides which, on heating, regenerated the hydrocarbon and oxygen nearly quantitatively (in solid state) and at the same time produced chemiluminescence. Chemiluminescence was also observed when these peroxides were decomposed in solution.

Notable among the peroxides which they studied were the 1,4-dialkoxy-substituted anthracenes[1b,c] which decompose at relatively low temperatures as compared with the others, and produced a brighter light emission. Dufraisse and Velluz[1b] noted that the oxygen generated in this reaction was not in its normal state. It has recently been shown[2] that it is generated in an excited singlet state.

Because of the low energy of this reversible reaction[3] the singlet oxygen is probably in the $^1\Delta_g$ state. The dimer of this species would not have sufficient energy to excite the emission. We were not able to detect any emission from the hydrocarbon solution when singlet oxygen was generated photochemically (with Eosin as sensitizer). The reaction kinetics also argue against the participation of singlet oxygen in the excitation process, as shown below.

Since the energy released[3] per molecule in this reversible decomposition is much less than needed to excite the fluorescence of the hydrocarbon (which is the emission observed) the mechanism by which this excitation is produced has been an intriguing question. Since the chemiluminescence efficiencies seemed to vary symbatically with the extent of the reversible reaction, it has been thought that an irreversible side reaction was not likely to be the source of excitation.[4] This is contrary to our results as discussed below.

Recently Dufraisse and coworkers[1d] have shown that the structure of the 1,4-dialkoxy peroxides contain the oxygen bridge across the 1,4 positions rather than across the 9,10 positions as previously supposed. Further, Rigaudy[1e] has shown that in some solvents (*e.g.*, benzene) the peroxide decomposes to a very large extent *via* a nonreversible path. He found that only 20% of the hydrocarbon is regenerated when the peroxide of 1,4-dimethoxy-9,10-diphenylanthracene is decomposed in benzene at room temperature.

The reaction scheme proposed by Rigaudy[1e] is

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(4) E. J. Bowen, *Pure Appl. Chem.*, **9**, 473 (1964).