by visible light irradiation for 5 min at 25 °C[16] on the above liposome suspension brought about significant transfer of Phe from the interior of the liposomes to the exterior. Keeping the system in the dark at the same temperature did not lead to any spontaneous release of Phe (Table I). Additional transport of the amino acid took place by repeating the above procedures. This means that our present system functions repeatedly until an equilibrium concentration of Phe between the interior and the exterior of liposomes is attained. Decreasing the pH of the interior of the liposomes from 6.0 to 5.0, while keeping that of the exterior constant at pH 8.5, diminished the transport efficiency for Phe from 13.2 ± 1.2% to 6.2 ± 0.7%. This may be caused by protonation of the *p*-nitrophenolate moiety of the dye situated at the inner surface of liposomal bilayers, thereby impairing the formation of the ionic complex with zwitterionic Phe. When Phe-OMe was encapsulated in liposomes, substantial amounts of Phe-OMe were adsorbed on the outer surface of the liposomes even after gel filtration to eliminate free amino acid in the bulk aqueous solution. Under the circumstances, most interestingly, Phe-OMe was transported from the exterior to the interior of the liposomes against the concentration gradient (Table II). This was true also for the case when more Phe-OMe was added to the exterior. Though we cannot clarify the reason at the present time, it may be ascribed to the greater hydrophobicity of Phe-OMe compared with Phe and/or asymmetry in the distribution of **2** between the outer and inner leaflets of bilayers.[17] In any event, we have succeeded in the photocontrolled transfer of an amino acid across liposomal membranes accompanied by the photochromism of **1** embedded in the membranes, as schematically illustrated in Figure 1.

The fluidity change of the liposomal membranes caused by the photochromism of **1** was also investigated by use of the fluorescence depolarization technique.[18] 1,6-Diphenylhexatriene and *N*-dansylhexadecylamine[20] have been intercalated in liposomal bilayers as fluorescent probes in order to monitor the fluidity of membranes.[21] The former is localized in the hydrophobic domain of membranes,[19] while the latter binds close to the surface of membranes.[20] For both probes, the fluorescence polarization was increased with an increase in the concentration of the ring-opened species **2** upon UV irradiation. This suggests that the ring-opened species certainly immobilizes regions of both the surface and hydrophobic domains of bilayers, thereby possibly leading to a reduction in the diffusion of the complex and the photospiran **1** itself.

Several carrier-mediated transports of amino acids across liquid membranes (Pressman cells) have been reported.[22] However, none of them are photoresponsive, and the amino acids transported were not in the neutral form but were present as the ammonium or carboxylate salt. Thus, to our knowledge, the present system must be the first success in mimicking the transport of free amino acids across cell membranes. Most unfortunately, however, there still remain several disadvantages such as the restricted movement of the vehicle, with or without a passenger, in membranes, the competitive interaction of **2** between the amino acid and the phosphatidylcholine moiety of lecithins, and the photochemical instability of the photospiran **1** itself. Further investigation is in progress in our laboratories to enhance transport efficiency.

Registry No. 1, 1498-88-0; **2**, 18457-95-9; phe, 63-91-2; phe-OMe, 2577-90-4.

(16) In order to isolate ultraviolet light (250-350 nm) and visible light (>440 nm), we employed a liquid filter composed of 46% (w/v) $CoSO_4$-14% (w/v) $NiSO_4$ aqueous solution (20 mm thickness) and a sharp cutoff filter Y46 (Hoya Glass Works) were employed, respectively, with a Toshiba high-pressure Hg lamp SHL-100UV-2. The distance between the light source and sample cuvette was 10 cm.

(17) Nakamura, A.; Higuchi, R.; Hidaka, S.; Fujio, T. "Abstract of Papers"; 44th Annual Meeting of the Japan Chemical Society, Okayama, 1981, 2I13, p 1206.

(18) (a) Shinitzky, M.; Barenholz, Y. **1978**, *515*, 367. (b) Sunamoto, J.; Iwamoto, K.; Kondo, H.; Shinkai, S. *J. Biochem.* **1980**, *88*, 1219.

(19) (a) Shinitzky, M.; Inbar, M. *J. Mol. Biol.* **1974**, *85*, 603. (b) Sunamoto, J.; Iwamoto, K.; Inoue, K.; Endo, T.; Nojima, S. *Biochim. Biophys. Acta* **1982**, *685*, 283.

(20) Iwamoto, K.; Sunamoto, J. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 399.

(21) Fluorescence depolarization was measured by essentially the same method as that described in the literature (ref 19, 20). The amount of the open-ring form (**2**) was measured spectrophotometrically (absorbance at 500 nm).

(22) (a) Behr, J.-P.; Lehn, J.-M. *J. Am. Chem. Soc.* **1973**, *95*, 6108. (b) Newcomb, M.; Toner, J. L.; Helgeson, R. C.; Cram, D. J. *Ibid.* **1979**, *101*, 4941. (c) Maruyama, K.; Tsukube, H.; Araki, T. *J. Chem. Soc., Chem. Commun.* **1980**, 966.

^{15}N NMR Study of [^{15}N]Actinomycin D Complexed with d(pGpC) and DNA

Stephen C. Brown, Richard H. Shafer,* and Peter A. Mirau[1]

Department of Pharmaceutical Chemistry
School of Pharmacy
University of California, San Francisco, California 94143

Received June 7, 1982

^{15}N NMR has been used to characterize the solution conformation of several cyclic polypeptides such as gramicidin S[2] and viomycin[3] and others,[4] as well as linear peptides.[5-7] Those experiments have demonstrated that ^{15}N NMR is particularly well studied for probing hydrogen bonding and solvent exposure properties of peptide nitrogens and carbonyls. We have recently described the isolation and characterization of actinomycin D (Figure 2) isotopically labeled at each nitrogen with ^{15}N at greater than 90% enrichment.[8] In that report we assigned the ^{15}N NMR spectrum of [^{15}N]actinomycin in organic solvents as well as in water. We now present the first ^{15}N NMR spectrum of a drug in aqueous solution complexed with the dinucleotide d(pGpC) and with short segments of calf thymus DNA as part of our studies on the role of peptide conformation in the binding of actinomycin to nucleic acids.[9-11]

The effect of adding excess d(pGpC) is shown in Figure 1 and Table I. Actinomycin D is known to form a tight complex with d(pGpC) (one drug: two dinucleotides),[12,13] and this dinucleotide represents the preferred DNA binding site. The dimerization of actinomycin in water at millimolar concentrations is well documented.[14] Thus, for the spectrum of the free drug monomer, we refer to results obtained in a 93 mol% water/7 mol % methanol mixture.[8] The spectrum of the drug in aqueous buffer under conditions favoring dimer formation is included in Figure 1.

^{15}N NMR chemical shifts are sensitive to both solvent and conformation. While the relative importance of these effects has not been completely elucidated for actinomycin, our interpretation of the observed spectral changes is based on ^{15}N NMR results obtained on other peptide systems.[2-7] The large downfield shifts of the Pro resonance are consistent with the formation of the two

(1) Present address: Department of Chemistry, University of California, San Diego, La Jolla, CA 92093.

(2) Hawkes, G. E.; Randell, E. W.; Hull, W. E.; Convert, O. *Biopolymers* **1980**, *19*, 1815-1826.

(3) Hawkes, G. E.; Randell, E. W.; Hull, W. E.; Gattegno, D.; Conti, F. *Biochemistry* **1978**, *17*, 3986-3992.

(4) Williamson, K. L.; Pease, L. G.; Roberts, J. D. *J. Am. Chem. Soc.* **1979**, *101*, 714-716.

(5) Live, D. H.; Wyssbrod, H. R.; Fishman, A. J.; Agosta, W. G.; Bradley, C. H.; Cowburn, D. *J. Am. Chem. Soc.* **1979**, *101*, 474-479.

(6) Hull, W. E.; Kricheldorf, H. R. *Biopolymers* **1980**, *19*, 1103-1122.

(7) Kricheldorf, H. R.; Hull, W. E.; Formacek, V. *Biopolymers* **1977**, *16*, 1609-1616.

(8) Shafer, R. H.; Formica, J. V.; Delfini, C.; Brown, S. C.; Mirau, P. A. *Biochemistry*, in press.

(9) Shafer, R. H.; Burnette, R. R.; Mirau, P. A. *Nucleic Acids Res.* **1980**, *8*, 1121-1132.

(10) Mirau, P. A.; Shafer, R. H. *Biochemistry* **1982**, *21*, 2622-2626.

(11) Mirau, P. A.; Shafer, R. H. *Biochemistry* **1982**, *21*, 2626-2631.

(12) Krugh, T. R.; Neely, J. W. *Biochemistry* **1973**, *12*, 4418-4425.

(13) Patel, D. J. *Biochemistry* **1974**, *13*, 2388-2395.

(14) Angerman, N. S.; Victor, T. A.; Bell, C. A.; Banyluk, S. S. *Biochemistry* **1972**, *11*, 2402-2411.

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Table I. ^{15}N NMR Chemical Shifts of [^{15}N]Actinomycin D[a]

	Pro	Val	Thr	MeVal	Sar	NH_2
93 mol % water/7 mol % methanol[b]	30.4, 29.8	7.6, 6.5	1.9, 1.2	−2.5	−8.2	−30.5
aqueous buffer[b]	34.7, 34.3	8.9, 8.2	5.1, 4.5	3.8	−3.8	−33.2
aqueous buffer + d(pGpC)[c]	35.7	10.4, 9.8	3.6, 2.4	4.5	−3.3, −3.8	−33.3
aqueous buffer + calf thymus DNA[c,d]	36, 34.8	9.6, 7.5		3.8		

[a] Chemical shifts in ppm referenced to neat formamide at 15 °C, except the DNA spectrum is at 25 °C; downfield shifts are positive. See figure legends for experimental conditions. [b] Assignments based on ^1H decoupling, selective nuclear Overhauser effect measurements, and solvent titrations; see ref 8. [c] Assigned by reference to free drug spectra. [d] From ^1H-coupled spectrum.

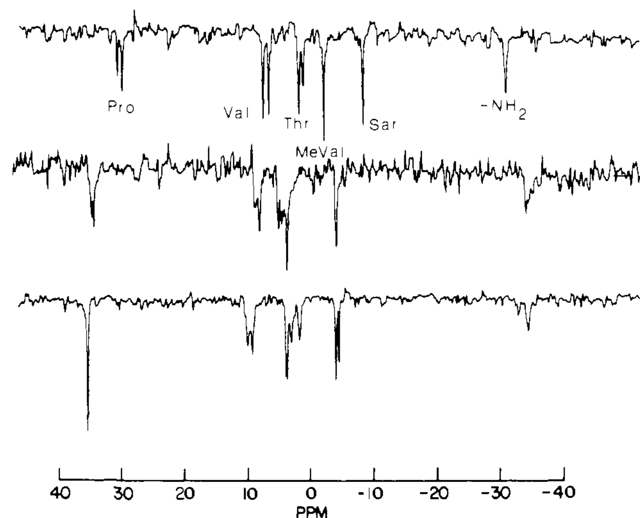

Figure 1. ^{15}N NMR spectra of [^{15}N]actinomycin D at 10.14 MHz at 15 °C: (top) 10 mM in 93 mol % H_2O/7 mol % CH_3OH, 600 transients; (middle) 1.5 mM in BPES buffer (0.1 M phosphate, 0.18 M NaCl, 0.01 M Na_2EDTA, pH 7.0) containing 10% D_2O, 16000 transients; (bottom) 5 mM in the presence of 20 mM d(pGpC) in BPES buffer, 15000 transients. Spectra were obtained on a Varian XL-100 in the FT mode with a 40° pulse, 1.4-s acquisition time, 2-s cycle time, ±1500-Hz spectral width, internal or external D_2O lock, and continuous broad band ^1H decoupling with resultant negative nuclear Overhauser effect factors. Assignments are shown for top spectrum.

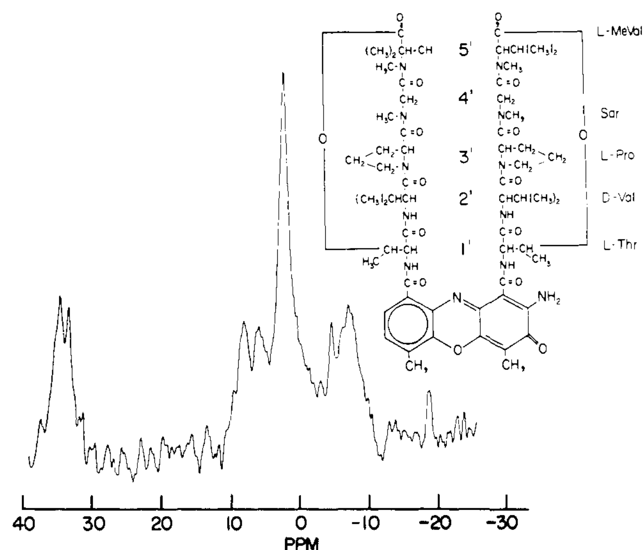

Figure 2. ^{15}N NMR spectrum at 50.66 MHz of [^{15}N]actinomycin D (1.4 mM) in the presence of calf thymus DNA (25 mM in nucleotides) in BPES buffer at 25 °C. DNA was digested to average length of 40 base pairs, determined by polyacrylamide gel electrophoresis, by DNAse II and S_1 nuclease. The spectrum was obtained on a Bruker WM 500 instrument without proton irradiation, 40° pulse, 0.58-s acquistion time, 2.1-s cycle time, spectral width ±7050 Hz, 14000 transients. Inset shows actinomycin D structure.

Val N–H to Val C=O interpeptide hydrogen bonds in both the aqueous dimer and the complex with d(pGpC). These bonds have been observed in the crystal structure of the actinomycin–deoxyguanosine complex[15] and more recently in the crystal structure of the drug complexed with d(GpC).[16] The downfield shifts observed for Sar and MeVal also reflect interactions at the shared carbonyl of the peptide bond.

The Val and Thr resonances may be affected by interactions at both the N–H and C=O groups of the peptide bond. Hydrogen bonding at the C=O group produces significantly larger downfield shifts of the peptide nitrogen than those resulting from hydrogen bonding at the N–H group.[2] The low-field position of the Val peaks in both the dimer and the dinucleotide complex are expected for the interpeptide hydrogen bonds discused above. These peaks occur further downfield in the dinucleotide complex than in the dimer, which is consistent with the presence of a hydrogen bond spanning the guanine amino group to the Thr carbonyl.[15,16] This is the first evidence in support of the participation of the Thr carbonyl in such an interaction in solution.

The Thr resonance is shifted downfield to a smaller extent in the dinucleotide complex than in the dimer. This may be due to differences in chromophore stacking and/or solvent interactions at the phenoxazone carbonyls. The weak hydrogen bond between the Thr N–H and the guanine N3 observed in the complexes with deoxyguanosine[15] and d(GpC)[16] would only shift the Thr peak slightly to lower field. This interaction, if present, is masked by other effects such as decreased solvent exposure of the chromophore carbonyls, which would induce large upfield shifts. The upfield shift of the amino group in Figure 1 is consistent with stacking interactions with the G-C base pairs.

Figure 2 shows the ^1H-coupled ^{15}N NMR spectrum at 50 MHz of actinomycin D in the presence of excess calf thymus DNA enzymatically digested to an average length of 40 base pairs. While the two Pro resonances appear superimposed in the dinucleotide complex, here they are split by 1.2 ppm (see Table I), twice the splitting observed in the other spectra. This enhanced magnetic nonequivalence in the Pro resonances supports the notion of asymmetry proposed by Sobell and co-workers with regard to the two peptide rings in the DNA complex,[17] which is not expected in the dinucleotide complex. We have recently proposed a molecular explanation for the slow rates found for the DNA dissociation kinetics of actinomycin in terms of cis–trans isomerization about the Val–Pro and possibly the Pro–Sar peptide bonds in one of the peptide rings.[9,11] Such a conformational change would entail differences in ^{15}N chemical shifts similar to our observations.[6]

As in the dinucleotide complex, the Val peaks appear to occur downfield from the MeVal Peak. The overlapping resonances upfield from MeVal in Figure 2 represent the Thr and Sar peaks, which appear further upfield than in the dinucleotide complex. Higher resolution spectra are needed, however, to provide firm assignments. Additional Val or Thr peaks may lie under the MeVal peak.

The experiments described above demonstrate that ^{15}N NMR has the potential for providing a great deal of information on the solution structure of actinomycin–nucleic acid complexes. In particular, the presence of a drug-specific label permits direct comparison of the drug bound to model nucleotide systems and

(15) Jain, S. C.; Sobell, H. M. *J. Mol. Biol.* **1972**, *68*, 1–20.
(16) Takusagawa, F.; Dabrow, M.; Neidle, S.; Berman, H. M. *Nature (London)* **1982**, *296*, 466–469.
(17) Sobell, H. M.; Tsai, C.-C.; Jain, S. C.; Gilbert, S. G. *J. Mol. Biol.* **1977**, *114*, 333.

to DNA. Studies currently underway with defined sequences of oligonucleotides should provide a more detailed picture of the peptide conformation in the bound drug. Other drugs should also be amenable to ^{15}N NMR, provided that isotopic enrichment is possible.

Acknowledgment. This work was supported by PHS Grant CA 27343 awarded by the National Cancer Institute, DHHS. P.A.M. was supported by Training Grant GM 07175 from the National Institute of Health. We thank Dr. W. Croasman and Professor S. I. Chan for assistance in obtaining the 50-MHz spectrum at the Southern California Regional High Field NMR Facility, supported by NSF Grant CHE79-16324 at the California Institute of Technology.

Registry No. Actinomycin D, 50-76-0; actinomycin D d(pGpC) complex, 52497-84-4.

Direct Evidence of Microscopic Homogeneity in Disordered Solids

P. Caravatti, J. A. Deli, G. Bodenhausen, and R. R. Ernst*

Laboratorium für Physikalische Chemie
Eidgenössische Technische Hochschule
8092 Zürich, Switzerland
Received June 15, 1982

In this communication, we describe an NMR technique that provides a direct measure for the homogeneity of solids on a molecular level. Most solid systems are to some degree disordered. Disorder may range from rare stacking faults in single crystals to heterogeneous mixtures of amorphous substances. For example, in a solid consisting of two or more molecular species, isomers, conformers, or ions, it is possible that phase separation has occurred on a microscopic scale.

Diffraction methods, which are most suitable for the study of periodic structures, also provide information on disorder.[1-5] Further insight can be obtained from thermal analysis[6] and from electron microscopy.[7] However, strongly disordered solids remain difficult to characterize.

The method proposed in the present communication is based on the phenomenon of nuclear spin diffusion that may occur between pairs of adjacent spins. Pairs of nuclear spins i and k in the solid are coupled by magnetic dipole–dipole interaction which can induce mutual spin flips with a rate proportional to $1/r_{ik}^3$ in the case of a rigid lattice and proportional to $1/r_{ik}^6$ in the presence of motional processes.[8-15] Due to the strong dependence on nuclear separation r_{ik}, spin diffusion is almost exclusively confined to neighboring molecules. If spin diffusion can be measured between two different species, then these species must be intimately mixed on a microscopic level.

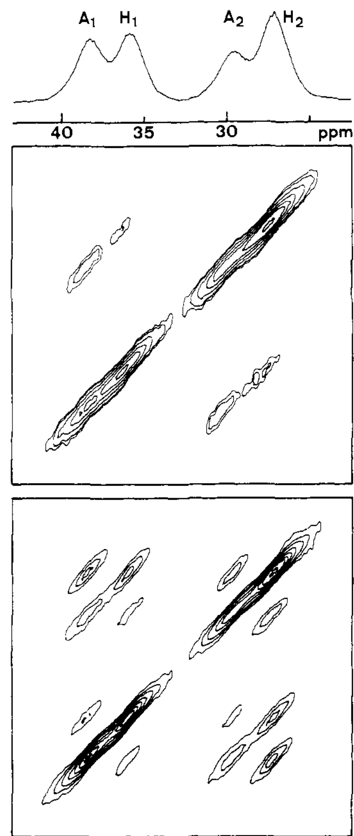

Figure 1. Two-dimensional carbon-13 spin diffusion spectra of mixtures of adamantane and 2,2,3,3-tetramethylbutane: (a) mixture of powders; (b) mixture by melt. Note the absence of cross-peaks between signals belonging to different species in the heterogeneous sample in Figure 1a. The spectra were recorded with static samples at room temperature with a Bruker CXP 300 spectrometer according to the procedure described in the text (τ_m = 5 s) and are presented in phase-sensitive mode. The phases of the last two 90° pulses were cycled together through $x,y,-x,-y$ phases while the signals were alternately added and subtracted.

A prerequisite for the measurement of spin diffusion rates is the ability to resolve NMR signals characteristic of the species under investigation. Carbon-13 solid-state NMR is particularly suitable because the dispersion of the chemical shifts is large compared to the line widths. The low natural abundance of carbon-13 reduces the spin diffusion rate due to large average nuclear separation. However, experiments have shown that in most solids with and without motional processes carbon-13 spin diffusion can be detected.[16,17]

Spin diffusion between individual sites in solids is formally analogous to cross relaxation and chemical exchange in liquids and can most conveniently be traced out by two-dimensional (2-D) exchange NMR spectroscopy, a method that has been used in liquids[18-21] and has recently been applied to solids by Szeverenyi et al.[16] and by Suter and Ernst.[22] After initial cross polarization,

(1) A. Guinier, "X-ray Diffraction in Crystals, Imperfect Crystals and Amorphous Bodies", Freeman, San Francisco, 1963.
(2) L. E. Alexander, "X-ray Diffraction Methods in Polymer Science", Wiley, New York, 1969.
(3) H. P. Klug and L. E. Alexander, "X-ray Diffraction Procedures of Polycrystalline and Amorphous Materials", Wiley, New York, 1974.
(4) J. E. Spruiell and E. S. Clark, in "Methods of Experimental Physics", R. A. Fava, Ed., Vol. 16, "Polymers", Part B, Academic Press, New York, 1980, p 1.
(5) Jing-I Wang and I. R. Harrison, in "Methods of Experimental Physics", R. A. Fava, Ed., Vol. 16, "Polymers", Part B, Academic Press, New York, 1980, p 128.
(6) A. Blazek, "Thermal Analysis", Van Nostrand-Reinhold, Princeton, NJ, 1973.
(7) R. G. Vadimsky, in "Methods of Experimental Physics", R. A. Fava, Ed., Vol. 16, "Polymers", Part B, Academic Press, New York, 1980, p 185.
(8) A. Abragam, "The Principles of Nuclear Magnetism", Oxford University Press, 1961.
(9) N. Bloembergen, S. Shapiro, P. S. Pershan, and J. O. Artman, *Phys. Rev.*, **114**, 445 (1959).
(10) I. J. Lowe and S. Gade, *Phys. Rev.*, **156**, 817 (1967).
(11) A. G. Redfield and W. N. Yu, *Phys. Rev.*, **169**, 443 (1968).
(12) A. C. Lind, *J. Chem. Phys.*, **66**, 3482 (1977).
(13) D. L. VanderHart and A. N. Garroway, *J. Chem. Phys.*, **71**, 2773 (1979).
(14) J. Virlet and D. Ghesquieres, *Chem. Phys. Lett.*, **73**, 323 (1980).
(15) J.-P. Korb and J. Maruani, *J. Chem. Phys.*, **74**, 1504 (1981).
(16) N. M. Szeverenyi, M. J. Sullivan, and G. E. Maciel, *J. Magn. Reson.*, **47**, 462 (1982).
(17) P. Caravatti and G. Bodenhausen, unpublished measurements.
(18) J. Jeener, B. H. Meier, P. Bachmann, and R. R. Ernst, *J. Chem. Phys.*, **71**, 4546 (1979).
(19) B. H. Meier and R. R. Ernst, *J. Am. Chem. Soc.*, **101**, 6441 (1979).
(20) S. Macura and R. R. Ernst, *Mol. Phys.*, **41**, 95 (1980).
(21) Anil Kumar, R. R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, **95**, 1 (1980).
(22) D. Suter and R. R. Ernst, *Phys. Rev. B*, **25**, 6038 (1982).
(23) N. M. Szeverenyi, private communication.
(24) M. Bee, J. P. Amoureux, and R. E. Lechner, *Mol. Phys.*, **40**, 617 (1980); J. P. Amoureux, M. Bee, and J. Virlet, *ibid.*, **41**, 313 (1980).
(25) A. R. Britcher and J. H. Strange, *Mol. Phys.* **37**, 181 (1979); A. R. Britcher and J. H. Strange, *J. Chem. Phys.*, **75**, 2029 (1981).

0002-7863/82/1504-5506$01.25/0 © 1982 American Chemical Society