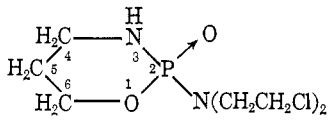
Cyclophosphamide Structure. Molecular Structure of 4-Ketocyclophosphamide

Norman Camerman*[1] and Arthur Camerman[2]

Contribution from the Department of Biochemistry, University of Toronto, Toronto M5S1A8, Ontario, Canada, and the Departments of Neurology and Pharmacology, University of Washington, Seattle, Washington 98195.
Received March 13, 1973

Abstract: The three-dimensional structure of 4-ketocyclophosphamide (KCPA), a metabolic product of the active form of the antineoplastic substance cyclophosphamide, has been determined by single-crystal X-ray diffraction. KCPA crystallizes in the monoclinic space group $P2_1/c$, with cell dimensions $a = 7.388$, $b = 9.286$, $c = 16.976$ Å, $\beta = 100.08°$; Z = four molecules per unit cell. The structure was solved by direct phasing procedures and refinement by anisotropic least squares converged at a discrepancy index $R = 0.051$. The structure is compared to that expected for the cytostatic precursor, 4-hydroxycyclophosphamide.

Cyclophosphamide (CPA) (I) is one of the most widely used agents in the treatment of many types of cancer (see, for example, ref 3 and 4), and is also being increasingly used as an immunolytic or anti-inflammatory agent in such diseases as systemic lupus erythematosus, the nephrotic syndrome, and rheumatoid arthritis.[5] Though it is an effective antineoplastic agent against a number of animal and human tumors,



I

CPA itself has virtually no cytotoxic activity against mammalian cell cultures;[6] it has been shown that *in vivo* pharmacological activity requires conversion of CPA to alkylating substances by the mixed function oxidase system of liver microsomes.[7] Since this has been recognized, widespread effort has been expended to isolate and identify the active metabolite of CPA, and evidence has been presented implicating diverse metabolic products ranging from minor structural modifications of CPA[3,8] to acrolein.[9] 4-Ketocyclophosphamide (KCPA) was isolated from the urine of animals treated with CPA,[3,10] and was the first metabolite recovered which retains the ring structure. Because it is a much more potent inhibitor of clone formation of H.Ep.2 cells than CPA, KCPA was postulated[3] to be an important step in the activation of CPA, but further study showed it to have no toxicity against L1210 leukemia cells in culture or against implanted tumor cells,[4,8,11] indicating that the activation of CPA occurs in an earlier phase of the oxidation. Recent results[8,12] have now very strongly suggested that the active antitumor metabolite of CPA is aldophosphamide (the aldehyde obtained by oxidation of the N(3)–C(4) bond), either as the free aldehyde or in cyclized form (4-hydroxycyclophosphamide). Bioassay results[13] have demonstrated that the active metabolite is highly unstable and very difficult to isolate; because of this and because of the very close structural resemblance of KCPA to the cyclized form of aldophosphamide, we undertook a three-dimensional structural elucidation of 4-ketocyclophosphamide. The results are of interest in showing geometrical and stereochemical relationships in this important class of antitumor agents, and possibly in revealing those features necessary for antineoplastic action in the active cyclophosphamide metabolite.

Experimental Section

Small crystals of 4-ketocyclophosphamide (crystallized from water) were supplied by Dr. Robert F. Struck; they are colorless prisms, with well-developed $(10\bar{2})$ faces. The unit cell dimensions were determined from diffractometer measurements, and the crystal data are as follows: 4-ketocyclophosphamide, $C_7H_{13}PO_3N_2Cl_2$, mol wt 275.07, monoclinic, $a = 7.388 \pm 0.003$, $b = 9.286 \pm 0.004$, $c = 16.976 \pm 0.007$ Å, $\beta = 100.08 \pm 0.04°$, space group $P2_1/c$. The density, calculated on the basis of four molecules of KCPA per unit cell, is 1.593 g cm^{-3}. Absorption coefficient, μ (for Cu Kα radiation), is 63.6 cm^{-1}.

Intensity data were collected on an automated four-circle diffractometer (Ni-filtered Cu radiation), and all independent reflections having 2θ (Cu Kα) $\leq 132°$ (corresponding to a minimum interplanar spacing of 0.84 Å) were measured. The θ–2θ scan method was employed, with stationary background radiation measurements taken on both sides of each reflection. Intensities of 1590 reflections (out or a total of 1993 in the range recorded) were greater than twice their standard deviation and these reflections were classified as observed and were later used in the structure refinement. The crystal used for the data collection was very small and of uniform dimensions, and absorption corrections were not carried out. The standard geometrical corrections to the intensities were applied,

(1) University of Toronto.
(2) University of Washington.
(3) D. L. Hill, M. C. Kirk, and R. F. Struck, *J. Amer. Chem. Soc.*, **92**, 3207 (1970).
(4) R. F. Struck, M. C. Kirk, L. B. Mellett, S. El Dareer, and D. L. Hill, *Mol. Pharmacol.*, 7, 519 (1971).
(5) See references in J. J. Miller III, G. F. Williams, and J. C. Leissring, *Amer. J. Med.*, **50**, 530 (1971).
(6) H. Arnold, F. Bourseaux, and N. Brock, *Nature (London)*, **181**, 931 (1958).
(7) N. Brock and H.-J. Hohorst, *Arzneim.-Forsch.*, **13**, 1021 (1963); J. L. Cohen and J. Y. Jao, *J. Pharmacol. Exp. Ther.*, **174**, 206 (1970), and references therein.
(8) H.-J. Hohorst, A. Ziemann, and N. Brock, *Arzneim.-Forsch.*, **21**, 1254 (1971).
(9) R. A. Alarcon and J. Meienhofer, *Nature (London)*, *New Biol.*, **233**, 250 (1971).
(10) J. E. Bakke, V. J. Feil, and R. G. Zaylskie, *J. Agr. Food Chem.*, **19**, 788 (1971).
(11) K. Norpoth, E. Golovsinky, and H. M. Rauen, *Hoppe-Seyler's Z. Physiol. Chem.*, **351**, 377 (1970); A. Takamizawa, Y. Tochino, Y. Hamashima, and T. Iwata, *Chem. Pharm. Bull.*, **20**, 1612 (1972).
(12) D. L. Hill, W. R. Laster, Jr., and R. F. Struck, *Cancer Res.*, **32**, 658 (1972).
(13) T. A. Connors, A. B. Foster, A. M. Gilsenan, M. Jarman, and M. J. Tisdale, *Biochem. Pharmacol.*, **21**, 1373 (1972).

Table I. Fractional Atomic Coordinates and Anisotropic Thermal Parameters

Atom	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
O(1)	0.8839	−0.0199	0.2007	3.60	2.89	2.07	0.01	0.36	0.10
P(2)	0.9069	−0.0644	0.1134	2.50	2.09	1.84	0.17	0.22	0.06
N(3)	0.8323	0.0827	0.0613	3.25	2.19	1.83	0.16	0.44	0.22
C(4)	0.8081	0.2186	0.0913	2.66	2.67	3.39	−0.02	0.23	0.11
C(5)	0.8165	0.2337	0.1779	5.24	2.97	3.69	0.55	−0.23	−1.07
C(6)	0.9389	0.1239	0.2283	4.36	3.83	2.66	−0.35	−0.22	−1.13
O(7)	0.7775	0.3214	0.0448	5.28	2.45	4.91	0.09	−0.20	0.77
O(8)	1.0928	−0.1064	0.1023	2.41	3.45	3.43	0.32	0.41	−0.15
N(9)	0.7583	−0.1939	0.0912	2.67	2.10	2.84	0.02	0.39	0.08
C(10)	0.8110	−0.3287	0.0565	4.18	2.34	2.77	0.06	0.37	−0.01
C(11)	0.7019	−0.3646	−0.0250	4.97	2.98	3.06	−0.44	0.38	−0.37
Cl(12)	0.7041	−0.2198	−0.0940	5.03	4.87	2.95	−0.36	0.19	0.93
C(13)	0.5664	−0.1698	0.1002	2.92	2.99	3.15	−0.01	0.39	−0.01
C(14)	0.5171	−0.2157	0.1792	3.82	3.02	4.06	−0.22	1.02	0.11
Cl(15)	0.5227	−0.4076	0.1913	6.34	3.38	4.64	−0.74	1.73	0.61

Atom	x	y	z	Atom	x	y	z
H(3)	0.848	0.093	0.012	H(11)	0.584	−0.390	−0.015
H(5)	0.668	0.190	0.185	H(11)	0.771	−0.461	−0.062
H(5)	0.842	0.345	0.198	H(13)	0.465	−0.228	0.062
H(6)	0.930	0.143	0.287	H(13)	0.542	−0.066	0.096
H(6)	1.078	0.140	0.219	H(14)	0.378	−0.188	0.188
H(10)	0.783	−0.041	0.105	H(14)	0.603	−0.167	0.234
H(10)	0.931	−0.331	0.043				

Approximate Standard Deviations

P, Cl	0.0002	0.0002	0.0001	0.07	0.07	0.06	0.05	0.05	0.05
N, O	0.0005	0.0004	0.0002	0.19	0.16	0.17	0.14	0.15	0.12
C	0.0008	0.0006	0.0003	0.28	0.24	0.22	0.22	0.19	0.19
H	0.008	0.006	0.003						

and structure amplitudes $|F|$ and normalized structure amplitudes $|E|$ were derived.

Structure Determination. The symbolic addition procedure[14] was used to determine the structure of KCPA. Three reflections having appropriate parities and high $|E|$ values were assigned phases of 0° to specify the unit cell origin, three more were assigned symbolic phases, a, b, and c, and the $\Sigma 2$ procedure was employed to determine additional phases. New phases were accepted only if the probability of being correct was greater than 0.98; after several cycles of the $\Sigma 2$ formula the phases of 108 reflections with $|E| \geq 1.8$ were determined. In addition, there were numerous indications that the symbols corresponded to $a = c = 180°$, $b = 0°$, and these values were accepted. Phase determination was extended to planes with $|E| \geq 1.4$, a total of 282 reflections were ultimately assigned phases and a three-dimensional E map was computed; 14 of the 15 nonhydrogen atoms in the molecule were clearly visible on this map (one carbon atom of a chloroethyl group was not found). A Fourier summation, using as coefficients the observed structure amplitudes with phases based on these 14 atoms, revealed the missing carbon atom.

Structure Refinement. The atomic positional and anisotropic thermal parameters were refined by full matrix least squares. The function minimized was $\Sigma w(|F_o| - |F_c|)^2$ with weights, w, inversely proportional to the square of the standard deviations of the measured structure amplitudes, and the atomic scattering factors were from the International Tables for X-Ray Crystallography.[15] Several cycles of refinement followed by computation of difference Fourier maps enabled coordinates to be assigned to all hydrogen atoms in the molecule. A final cycle of least squares, varying everything but the hydrogen atoms' thermal parameters (kept fixed at the values of the atoms to which the hydrogens are bonded), resulted in a discrepancy factor R for the observed reflections of 0.051. The atomic fractional coordinates and thermal parameters are given in Table I, in which the B_{ij} are coefficients in the expression: $\exp(-0.25[B_{11}h^2a^{*2} + \ldots\ldots 2B_{23}klb^*c^*])$. The observed and calculated structure factors may be found in the microfilm edition of this journal.[16]

Results and Discussion

Figure 1 is a stereoscopic diagram showing the three-dimensional molecular conformation of KCPA. The six-membered ring has a buckled conformation: the carbonyl group and attached atoms (N-3, C-5) lie on a plane, atom O-1 is 0.06 Å from coplanarity with them, and atoms P-2 and C-6 deviate considerably (0.25 and 0.65 Å, respectively) from this plane. (All three deviations from the plane are in the same direction, toward O-8.) The exocyclic nitrogen atom, N-9, and its three substituents P-2, C-10, and C-13, also form a planar system (maximum deviation = 0.03 Å, for N-9), with the phosphoryl oxygen, O-8, lying 0.06 Å from this plane. The acute angle between normals to these two planes is 84°.

Bond lengths and angles in KCPA are shown in Figures 2 and 3. The P–O-8 length of 1.471 Å is close to the average value of 1.46 Å found for phosphoryl bonds in a survey of phosphate structures;[17] similarly the P–O-1 distance of 1.576 Å does not differ significantly from the average value of 1.59 Å (±0.02 Å) for corresponding bonds in a number of P–O ring systems.[17] The two P–N bonds in KCPA differ considerably in length, the exocyclic one being 0.04 Å shorter than the ring bond. This is quite understandable: the trigonal exocyclic N-9 and the atoms to which it is bonded form a planar system, and the phosphoryl O-8 is close to being on the plane; thus a high degree of p_π–d_π bonding between the nitrogen and phosphorus atoms, and possibly a contribution from the phosphoryl oxygen, would be expected. The ring N-3, on the other hand, deviates somewhat from

(14) J. Karle and I. L. Karle, *Acta Crystallogr.*, **21**, 849 (1966).

(15) "International Tables for X-Ray Crystallography," Vol. III, Kynoch Press, Birmingham, England, 1962.

(16) The final observed and calculated structure factors will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N.W., Washington, D. C. 20036, by referring to code number JACS-73-5038. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche.

(17) D. E. C. Corrbridge, *Bull. Soc. Fr. Mineral. Cristallogr.*, **94**, 271 (1971).

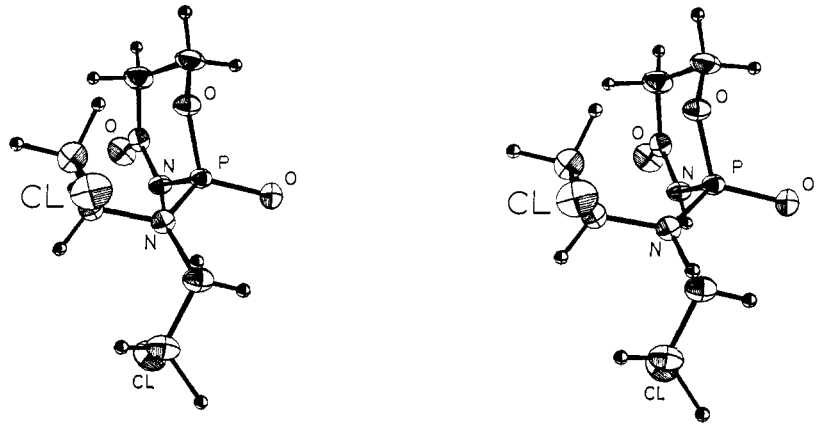


Figure 1. Stereoscopic drawing of 4-ketocyclophosphamide showing the molecular conformation and the relative sizes and shapes of the thermal motion ellipsoids. Carbon and hydrogen atoms are not labeled.

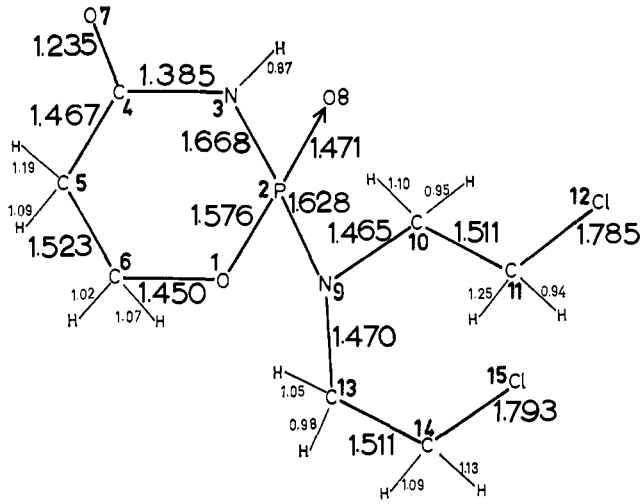


Figure 2. Bond lengths (Å) in 4-ketocyclophosphamide. Estimated standard deviations are 0.005 Å for "heavy" atom bonds and 0.06 Å for bonds involving hydrogen atoms.

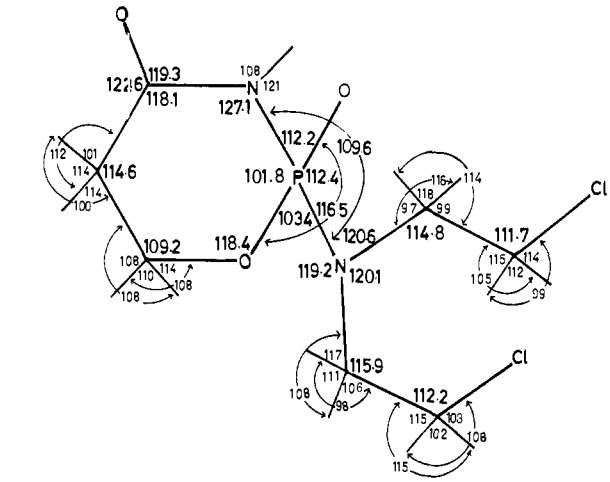


Figure 3. Bond angles (deg) in 4-ketocyclophosphamide. Estimated standard deviations are 0.4° for "heavy" atom angles and 3–5° for angles involving hydrogen atoms.

a trigonal configuration; it and attached substituents are less planar, and the degree of p_π–d_π N–P bonding is not as appreciable. In addition, the N-3–C-4 distance of 1.385 Å is considerably shorter than the Nsp^2–Csp^2 single bond distance of 1.47 A,[18] indicating that the N-3 lone-pair electrons interact to a great extent with the C-4 carbonyl system and are less available for π bonding with phsophorus. Theoretical concepts of bond order are difficult in these types of bonds because of the complex characteristics of d orbitals, but 30% π-bond character has been attributed to P–N distances of 1.66 Å in phosphoric triamide[19] by comparison with bond lengths in phosphazenes and with P–N single bond compounds; the two P–N bonds in KCPA would then, by comparison, have somewhat less (ring bond) and somewhat more (exocyclic) π-bond character than this.

The other bond distances in KCPA are normal. The lengths involving the chloroethyl groups appear a little shorter than expected, but this is undoubtedly due to high thermal motion in these terminal groups.

The configuration about the phosphorus atom is pseudotetrahedral. In $O{=}PX_3$ compounds the O=P—X angles are usually larger than the X—P—X angles, though π bonding in P—X reduces this difference. This is well illustrated in KCPA: the O=P—N angles are 112°, O=P—O is 116.5°, the N—P—O angles are 102 and 103°, and the N—P—N angle, influenced by the P–N π bonding, is 110°.

There is a relatively weak intermolecular hydrogen bond in the KCPA crystal structure between the N-3 hydrogen and the phosphoryl oxygen, O-8, on the molecule situated across the center of symmetry. The N—H···O=P distance is 2.07 Å (N···O distance = 2.93 Å) and the N—H···O angle is 175°. All other intermolecular contacts correspond to normal van der Waals distances.

During the writing of these results a note was published[20] reporting the synthesis of 4-hydroxycyclophosphamide (HCPA) and the finding that it is an active cytostatic agent both *in vitro* and *in vivo*, and that KCPA is a metabolite of *in vivo* administration of HCPA. Because of the very strong evidence[3,12,20] that HCPA (or the N(3)–C(4) ring-opened aldehyde derived from it) is indeed the active metabolite of

(18) A. Camerman, *Can. J. Chem.*, **48**, 179 (1970).
(19) G. J. Bullen, F. S. Stephens, and R. J. Wade, *J. Chem. Soc. A*, 1804 (1969).
(20) A. Takamizawa, S. Matsumoto, T. Iwata, K. Katagiri, Y. Tochino, and K. Yamaguchi, *J. Amer. Chem. Soc.*, **95**, 985 (1973).

CPA, it is of interest to compare the closely related structure of KCPA to that expected for HCPA. The structural differences will be in the configuration at C-4: (1) in KCPA this is planar, whereas in HCPA C-4 would have a tetrahedral configuration, and the six-membered ring would likely have a chair conformation; (2) both KCPA and HCPA have a potential electron-donor oxygen atom on C-4; how closely their positions in space match will depend largely on whether the hydroxyl in HCPA is in the equatorial or axial position; (3) HCPA has the potential of hydrogen-bond formation through its hydroxyl hydrogen. In all other respects the two molecules would be expected to be closely similar in structure.[21]

Acknowledgments. We thank Dr. Robert F. Struck for supplying the crystals of 4-ketocyclophosphamide. This work was supported in part by U. S. Public Health Service Research Grant No. 1 R01 NS 09839-02 BBCA from the National Institute of Neurological Diseases and Stroke.

(21) NOTE ADDED IN PROOF. We have recently become aware of a paper on the crystal structure of cyclophosphamide itself: S. Garcia-Blanco and A. Perales, *Acta Crystallogr., Sect. B*, **28**, 2647 (1972). The bond lengths and angles in cyclophosphamide are all very similar to the corresponding ones in KCPA with the exception of the P–N ring bond which is significantly longer in KCPA (1.668 *vs.* 1.625 Å), undoubtedly owing to withdrawal of electrons from this bond by the neighboring carbonyl group in KCPA. The ring angles at N-3 and C-5 are also larger in KCPA, reflecting the difference in configuration at C-4, to which they are bonded.

Communications to the Editor

Relaxation of Excited State Carbene to Ground State. An Internal Heavy Atom Effect

Sir:

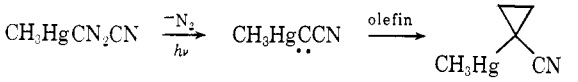
The effect of internal (directly affixed)[1] and external (solvent)[2] heavy atoms on the singlet–triplet interconversions of organic molecules has been the object of much theoretical and spectroscopic work.[3] Experimentally, the effect can be observed as an increase in the rates of both radiative and nonradiative "spin forbidden" electronic transitions as the atomic number of the perturbing atom is increased. The effect has been correlated with theory in terms of spin-orbital coupling.[4,5]

The limitation which this effect places on the kinetic criterion for spin state assignment to carbenes was recognized early.[6] Now the heavy atom effect is employed to accelerate relaxation of carbenes to the ground state to help define that state. The introduction of an α-methylmercuric substituent on the carbene is effective.

The stable esr spectrum observed at $-196°$ from photolyzed diazoacetonitrile has been interpreted as the signal from ground-state triplet cyanomethylene.[7]

Photolysis of diazoacetonitrile in *cis*-2-butene produces 1-cyano-2,3-dimethylcyclopropanes, 94% cis dimethyls, and only 6% trans. The yield of trans product (nonstereospecific addition) is decreased by addition of a radical scavenger such as 1,1-diphenylethylene, or increased by dilution of the reactants with methylene chloride or butane; it is increased to 62% by carrying out a thermal reaction in the gas phase. Thus, photolysis is characterized by decomposition of the diazo compound to the singlet carbene which then reacts stereospecifically with *cis*-2-butene as solvent, somewhat faster than intersystem crossing to the triplet state. Dilution favors the unimolecular intersystem crossing to triplet ground state over the bimolecular trapping.

Photolysis (>335 nm) of α-methylmercuridiazoacetonitrile[8] in olefins results in the formation of 1-methylmercuri-1-cyanocyclopropanes in yields of 65–90%. With *cis*-2-butene as the trapping agent either



direct photolysis or triplet benzophenone sensitized decomposition results in formation of *trans*- and *cis*-dimethylcyclopropanes in a 1.0 ratio (total yield of mercuricyclopropanes is 65–80%). 1,3-Butadiene is much more reactive than 2-butene in trapping the methylmercuricyanomethylene, a 1:1 mixture of olefins yielding only 4% product by reaction with 2-butene. In this latter experiment, the ratio of *trans*- to *cis*-dimethylcyclopropanes is 0.05. These results are rationalized exactly as with cyanomethylene, except that the presence of the α-mercuric group greatly accelerates the intersystem crossing to the triplet ground state.

The ready syntheses of α-mercuridiazo compounds[8] make it possible to apply this method to structures with a variety of other substituents on the diazo compound. For example, an esr signal has not been detected for any of the photodecomposition products of diazoacetic esters. Photo- or thermally initiated additions of the resulting carbalkoxycarbenes are highly stereospecific. On the other hand, triplet benzophenone sensitized decompositions result in nonstereospecific additions.[9] Thus, there is ambiguity regarding the ground state of this carbene. Photodecomposition of α-methylmercuridiazoacetate esters produces the α-methylmercuricarbene which adds stereospecifically to the 2-butenes. Thus, even with an increase of the rate of intersystem crossing the singlet state is the sole reactant. This ob-

(1) D. S. McClure, *J. Chem. Phys.*, **17**, 905 (1949).
(2) T. Medinger and F. Wilkinson, *Trans. Faraday Soc.*, **61**, 620 (1965).
(3) S. P. McGlynn, T. Azumi, and M. Kinoshita, "Molecular Spectroscopy of the Triplet State," Prentice-Hall, Englewood Cliffs, N. J., 1969, Chapters 5–8.
(4) M. Kasha, *J. Chem. Phys.*, **20**, 71 (1952).
(5) G. W. Robinson, *J. Chem. Phys.*, **46**, 572 (1967).
(6) R. C. Woodworth and P. S. Skell, *J. Amer. Chem. Soc.*, **78**, 4496 (1956); **81**, 3383 (1959).
(7) R. J. Bernheim, R. J. Kempf, J. V. Gramas, and P. S. Skell, *J. Chem. Phys.*, **41**, 1156 (1964).
(8) S. J. Valenty and P. S. Skell, submitted for publication to *J. Org. Chem.*
(9) See also T. DoMinh and O. P. Strausz, *J. Amer. Chem. Soc.*, **92**, 1766 (1970), footnote 3.