radicals. Such a reaction has recently been shown to give singlet oxygen by Howard and Ingold.[29]

The low-temperature nmr spectrum of ozonized isopropyl ether contains small peaks which can be assigned to isopropyl acetate and acetone as well as two new peaks at 8.53 and −2.98[30] with an area ratio of approximately 6:1. These latter absorptions decay and disappear at *ca.* −10° which is the same temperature at which gas evolution is observed. The absorption at 8.57 can be assigned to the methyl groups attached to the carbon bearing the hydrotrioxy group in the hydrotrioxide. The compounds 2-methoxyisopropyl-*t*-butyl peroxide and 2,2-bis(*t*-butylperoxy)propane have absorptions at 8.64 and 8.59, respectively, for the comparable methyl groups.[31] The absorption at −2.98 would then tentatively be assigned to the proton of the hydrotrioxy group. Taken together these observations would seem to favor the hydrotrioxide structure.

These results also suggest that our earlier proposal[3] that singlet oxygen may be produced in the atmosphere from a variety of reactions of ozone with organic substrates should now be extended to include substrates of even relatively low reactivity such as saturated hydrocarbons. The increasing concentrations of ozone in polluted atmospheres as well as the suggestion[32] that atmospheric singlet oxygen may be involved in the important NO to NO_2 conversion in such atmospheres makes these results of particular importance to the air pollution problem.

Acknowledgment. We thank the National Air Pollution Control Administration, Consumer Protection and Environmental Health Service, Public Health Service, for support of this work through Grant No. AP 00925-01.

(29) J. A. Howard and K. U. Ingold, *J. Amer. Chem. Soc.*, 90, 1056 (1968).

(30) The nmr spectra were taken on a Varian Associates HA-100 nmr spectrometer beginning at −41°. The chemical-shift values are τ values relative to internal TMS.

(31) R. D. Youssefyeh and R. W. Murray, *Chem. Ind.*, 1531 (1966).

(32) A. U. Khan, J. N. Pitts, Jr., and E. P. Smith, *Environ. Sci. Technol.*, 1, 656 (1967).

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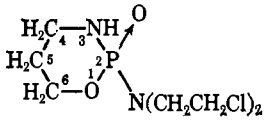
Isolation and Identification of 4-Ketocyclophosphamide, a Possible Active Form of the Antitumor Agent Cyclophosphamide

Sir:

The mechanism of activation of cyclophosphamide (I), an agent widely used in the treatment of many types of cancer,[1] has been a matter of significant interest for several years.[2] We wish to report our results on the isolation and identification of a cyclophosphamide metabolite from dog urine in which the tetrahydro-2*H*-1,3,2-oxazaphosphorine ring has not been opened.

(1) See, for example: R. Nissen-Meyer and H. Höst, *Cancer Chemother. Rep.*, 9, 51 (1960); M. P. Sullivan, *ibid.*, 51, 393 (1967); M. E. Haggard, *ibid.*, 51, 403 (1967); W. W. Sutow, *ibid.*, 51, 407 (1967).

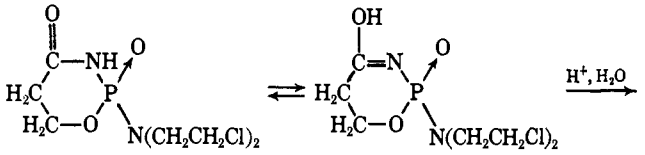
(2) See, for example: N. Brock and H.-J. Hohorst, *Arzneim. Forsch.*, 13, 1021 (1963); O. M. Friedman, *Cancer Chemother. Rep.*, 51, 327 (1967); J. L. Cohen and J. Y. Jao, *Proc. Amer. Ass. Cancer Res.*, 10, 14 (1969); H. M. Rauen and K. Norpoth, *Arzneim. Forsch.*, 17, 599 (1967); H. M. Rauen and K. Norpoth, *Naturwissenschaften*, 52, 477 (1965).



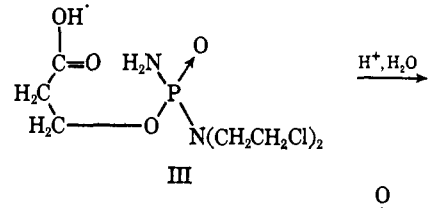
I

Urine from a dog injected intravenously with 20 mg/kg of side chain labeled ^{14}C-cyclophosphamide (0.02 μCi/mg) was collected for 6 hr. The sample contained 14% of the total dose. Several volumes of ethanol were added and the insoluble materials removed by filtration. Ethanol was removed at 30° under reduced pressure, and the remaining solution was passed through a DEAE-Sephadex A-25 column, previously equilibrated with 0.02 *M* NH_4HCO_3. The column was washed with this buffer, and a linear gradient of 0.02–0.2 *M* NH_4HCO_3 was applied. Three major radioactive materials were obtained. The first was cyclophosphamide, which was eluted in washing the column. The second was further purified by paper chromatography and Sephadex G-10 column chromatography.

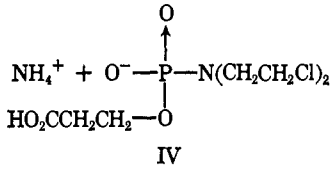
This metabolite upon mass spectral analysis gave a molecular ion at *m/e* 274, and infrared analysis showed a band at 1695 cm^{-1}, which is indicative of an amide carbonyl group. The metabolite (*ca.* 100 μg) was crystallized from ethanol, and several crystals were isolated and dried. The crystals, which melted sharply at 148–149°, were analyzed again by mass spectral and infrared methods. Mass spectral analysis gave a molecular ion at *m/e* 274 and a base peak at *m/e* 225, corresponding to loss of $-CH_2Cl$. A strong band appeared at 1695 cm^{-1} in the infrared spectrum of the crystalline metabolite along with the two strongest bands which appeared at 1630 and 1615 cm^{-1} and suggested the presence of –C=C– and/or –C=N– group(s). These differences in the infrared spectra of the total metabolite fraction and the crystalline metabolite suggested possible keto–enol tautomerism. The crystalline sample was dissolved in distilled water (pH *ca.* 6), allowed to stand at room temperature for 1 hr, frozen for 3 days, and lyophilized. Two strong bands appeared at 3130 (broad) and 1395 cm^{-1} (sharp) in the infrared spectrum of the residue and clearly indicated the presence of NH_4^+. Thus, the compound is subject to hydrolytic decomposition in weak acid solution.



II



III

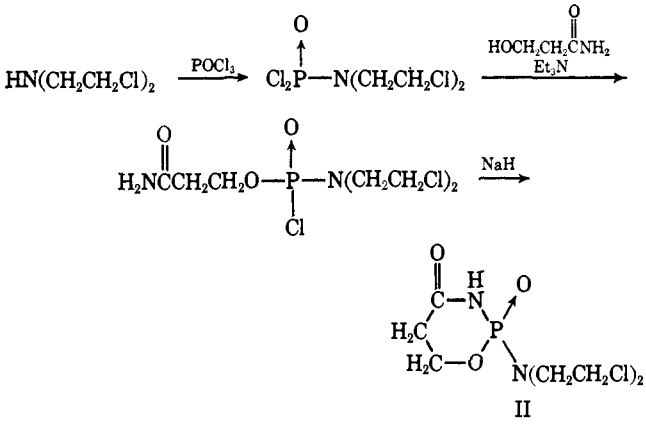


IV

These observations suggested 4-ketocyclophosphamide (II) as the structure of the metabolite. The infrared spectral behavior can be summarized as shown above.

The mass spectrum of the metabolite is clearly different from mass spectra of N^3-methylcyclophosphamide,[3] which we synthesized for comparison purposes, and 6-methylcyclophosphamide.[4] The melting point of the metabolite is significantly higher than that of either 6-methyl or N^3-methylcyclophosphamide and suggests increased polar character.

In order to confirm the structural assignment, 4-ketocyclophosphamide (II) was synthesized by the following route. The synthetic product's melting

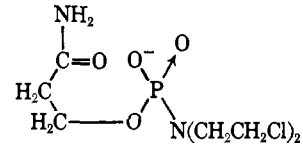


II

point, infrared spectrum, and mass spectrum were identical with those of the metabolite. In addition, a mixture melting point of the metabolite and the synthetic product gave no depression. The synthetic product gave satisfactory elemental analyses.

In subsequent experiments, 4-ketocyclophosphamide (II) was obtained consistently by ether extraction of fresh urine from catheterized dogs. Mass spectral analysis proved the extracts to be mixtures of II and cyclophosphamide (I) contaminated with other components of urine. From these extracts, the metabolite II was purified by DEAE-Sephadex column chromatography and crystallization.

Further evidence that C_4 of cyclophosphamide is oxidized by the dog comes from the fact that β-hydroxypropionamide was isolated as a fragment of the metabolite present in the third peak from the DEAE-Sephadex A-25 column. In this case, the urine came from a dog treated as before, except that the cyclophosphamide (0.03 μCi/mg) was labeled in position 6 of the ring. To obtain this fragment, the metabolite was treated with 0.1 *N* HCl at room temperature for 24 hr. After neutralization of this solution, alkaline phosphatase was added. The material was again placed on the DEAE-Sephadex A-25 column, and the radioactive compound washed from the column with 0.02 *M* NH_4HCO_3 was deionized by passage through a Sephadex G-10 column. The mass spectral pattern and the infrared spectrum of the fragment were identical with those of an authentic sample of β-hydroxypropionamide. This amide can arise from cyclophosphamide only by oxidation of C_4. We surmise that the third peak contained the ring-opened compound derived from II. Its structure is likely to be the following.



V

It seems probable that 4-ketocyclophosphamide is either the active form of cyclophosphamide or a precursor of the active form. 4-Ketocyclophosphamide (II) inhibits clone formation of H. Ep. 2 cells by 50% at 1 μg/ml. Under identical conditions, 50 μg/ml of cyclophosphamide (I) is required for comparable inhibition. Synthesis of the ring-opened compounds III, IV, and V, as well as C_4-hydroxy and C_5- and C_6-keto and -hydroxy analogs, is in progress.

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The Crucial Role of Dynamic Effects in the Hydrogen–Iodine Reactions[1]

Sir:

Mechanistic details of chemical reactions follow from the application of dynamic principles to nuclei and electrons of the reaction complex. This problem is usually separated into three parts: (1) motion of the electrons in the field of the nuclei clamped at successive positions[2] (this leads to the concept of a potential energy surface (PES)); (2) motion of the nuclei in regions of the PES corresponding to reactants, products, and transition state[3a] (this leads to the concept of internal energy levels for these species); (3) momentum-conserving motions of the nuclei[3b] required to pass from reactants to transition state to products (this leads to what we henceforth call *dynamic effects*[4]).

Powerful techniques for probing chemical mechanisms have generally concentrated upon parts 1 (*e.g.*, the Woodward–Hoffmann rules[5]) and 2 (*e.g.*, the transi-

(1) Supported by Oklahoma State University Research Foundation, PHS Grant No. GM 13253, and by Los Alamos Scientific Laboratory of the University of California.

(2) M. Born and J. R. Oppenheimer, *Ann. Phys.*, 84, 457 (1927).

(3) (a) H. Eyring, *J. Chem. Phys.*, 3, 107 (1935); H. Pelzer and E. Wigner, *Z. Phys. Chem., Abt. B*, 19, 445 (1932); S. Glasstone, K. J. Laidler, and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941; (b) explicit representation of such nuclear motions on a PES appears to have first been made by J. Hirschfelder, H. Eyring, and B. Topley, *J. Chem. Phys.*, 4, 170 (1936).

(4) As we use the term, dynamic effects do not include the "ponderal" effects discussed by P. B. D. de la Mare, L. Fowden, E. D. Hughes, C. K. Ingold, and J. D. H. Mackie, *J. Chem. Soc.*, 3200 (1955). The latter pertain to nuclear motions giving rise to internal energy levels of the transition state (part 2), and are thus included in a transition-state theory treatment.

(3) H. Arnold, F. Bourseaux, and N. Brock, *Arzneim. Forsch.*, 11, 143 (1961).

(4) Obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland.