Infrared Studies of Carbon Monoxide Bound to Hemocyanin and to Simple Copper Complexes

Sir:

Hemocyanin, the oxygen-transporting copper protein in the hemolymph of many molluscs and arthropods, has long been known to bind reversibly one oxygen molecule per two atoms of copper.[1] As a result, various types of structures have been suggested in which oxygen forms a bridge of some sort between the copper atoms.[2] No direct evidence has been available to test these hypotheses. Electron paramagnetic resonance is not observed for either oxy- or deoxyhemocyanin, but is observed after oxidation with H_2O_2.[3] When oxygen is excluded, hemocyanin binds carbon monoxide in the same ratio as oxygen.[4] A binding ratio of 2 (Cu/CO) was measured by Kubowitz[5] and recently confirmed by Rocca and Ghiretti[6] and by Vanneste and Mason.[7] Williams[8] discussed possible bridging structures for carbon monoxide bound between two copper atoms and indicated that no bridging structures are known for copper carbonyls and that they are unlikely to occur. We have been able to test for the possibility of a bridging copper carbonyl structure in hemocyanin by high-resolution infrared spectroscopy. The infrared absorption maximum of hemocyanin carbonyl complex (ν_{CO}) has been determined and compared with those of simple copper carbonyls in solution.

Hemocyanin, prepared from the giant key hole limpet (*Megathura crenulata*), was purchased from Mann Research Laboratories and used without further purification. Portions were dissolved in water at about 200 mg/ml and centrifuged to remove small amounts of insoluble material. Infrared cells with CaF_2 or BaF_2 windows were used with light paths of approximately 0.05 mm, which were carefully measured by interference fringes with the empty cells. Infrared difference spectra (measured with a Perkin-Elmer Model 102 double-beam spectrometer with a grating-prism double monochrometer) were generally obtained with water in the reference cell. The infrared spectrum contains no sharp bands due to protein in the region of interest. Visible and ultraviolet spectra of samples in the infrared cells (A_{280} = 1.0, A_{348}/A_{280} = 0.15) were obtained with a Spectracord Model 4000 A (Perkin-Elmer) split-beam spectrophotometer or manually with a Gilford Model 220 photometer attached to a Beckman Model DU monochrometer. The visible and ultraviolet spectra of limpet oxyhemocyanin are similar to those of oxyhemocyanin from cephalopods[9] and other gastropods.[10] The absorption band at 348 mμ disappears reversibly upon deoxygenation by equilibration with 100% CO, as reported by Kubowitz[5] for octopus hemocyanin. Carbon monoxide (Matheson) was reagent grade.

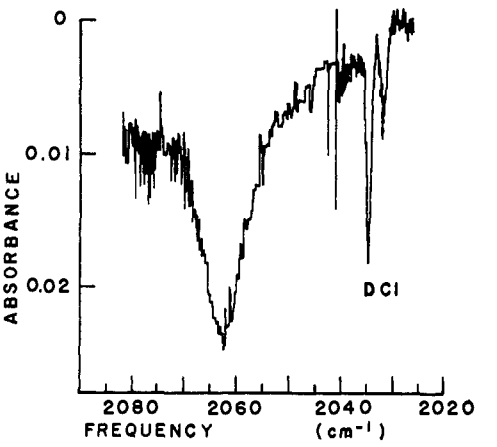
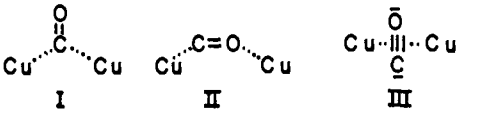


Figure 1. Infrared spectrum of carboxyhemocyanin (ν_{CO}) recorded at 4-cm^{-1} spectral resolution and 50 × absorbance, with a 0.054-mm sample cell path. Frequency calibration curves constructed with DCl and CO[11] were compared with DCl peaks recorded with the carboxyhemocyanin spectrum.

The infrared spectrum of carboxyhemocyanin contains an absorption band due to bound carbon monoxide at 2063 ± 1 cm^{-1} (Figure 1). No other bands were observed between 2250 and 1800 cm^{-1}. Similar infrared spectra were obtained for carboxyhemocyanin whether oxygen was removed by $Na_2S_2O_4$ or by extended flushing with CO. The narrow half band width (9 cm^{-1}) indicates that this CO is bound within the protein, where it is protected from solvent water, in analogous fashion to hemoglobin[12] and myoglobin.[13]

The frequency (ν_{CO}) of the carboxyhemocyanin absorption is in the region where mononuclear, nonbridging metal carbonyls are expected to absorb and 100–200 cm^{-1} higher than would be expected for a bridging carbonyl.[14] (Eischens, *et al.*, reported absorptions at 2120 and about 1830 cm^{-1} for CO chemisorbed to copper supported by carbosil silica powder and interpreted these as due to linear and bridging complexes, respectively.[15] We have observed single peaks at 2112 and 2069 cm^{-1}, respectively, for the CO complexes formed from cuprous chloride dissolved in water or pyridine. These complexes have been shown[16] to form with a stoichiometry of Cu/CO = 1.

Structures such as I or II, which were suggested



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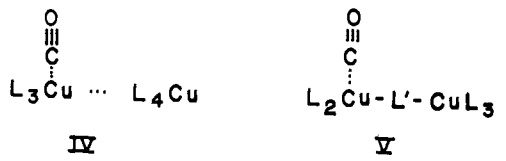
as unlikely possibilities by Williams,[8] in order to explain the stoichiometry of binding CO in carboxyhemocyanin, would require lower stretching frequencies and are inconsistent with the data. Structure III, in which carbonyl π_x and π_y bonds coordinate with copper, is not tested by these data but is unknown in copper chemistry and will not be considered further. The probable structures for carboxyhemocyanin are therefore IV or V. In structure IV the second copper



IV V

does not take part in binding CO but may stabilize the protein, whereas in structure V the second copper may influence CO binding through a bridging ligand or metal–metal bond. The infrared data indicate that CO is coordinated to only one copper per binding unit in hemocyanin. It is probable that oxygen is similarly coordinated to only one copper in oxyhemocyanin.

Acknowledgments. This work was supported in part by Grant GM-11067 from the National Institute of General Medical Sciences. The Perkin-Elmer Model 102 infrared spectrometer was made available under a contract with the Air Force Materials Laboratory, Air Force Systems Command, U. S. Air Force. This support is gratefully acknowledged.

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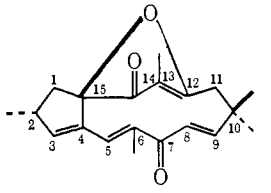
Jatrophone, a Novel Macrocyclic Diterpenoid Tumor Inhibitor from *Jatropha gossypiifolia*[1,2]

Sir:

Extracts of *Jatropha gossypiifolia* L. (*Euphorbiaceae*) and related species have been used for many years to treat cancerous growths.[3] In the course of a continuing search for tumor inhibitors of plant origin[4] we found that an alcoholic extract of *J. gossypiifolia*[5] showed significant inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (KB) and *in vivo* against four standard animal tumor systems.[6] We report herein the isolation and structural elucidation of jatrophone (**1**), a novel macrocyclic diterpenoid tumor inhibitor[7] from *J. gossypiifolia*.

Fractionation of the ethanol extract was guided by the KB assay.[6] Trituration of the alcoholic extract with benzene followed by trituration of the benzene solubles with hexane afforded a cytotoxic hexane-soluble fraction. Treatment with Darco G-60 followed successively by chromatography on silica gel and neutral alumina (activity III) yielded jatrophone (**1**): $C_{20}H_{24}O_3$;[8] mp 152–153°; $[\alpha]^{24}D$ +292° (*c* 1.23, C_2H_5OH); uv max (95% C_2H_5OH) 285 (ϵ 10,200), 225 mμ (sh); ir (KBr) 3.35, 3.43, 3.46, 5.90, 6.05, 6.20, 7.10, 7.35, 8.07, 8.15, 10.10 μ; nmr (C_6D_6) τ 3.25 (1 H, d, J = 15 Hz), 3.74 (1 H, d, J = 15 Hz), 3.96 (1 H, m), 4.14 (1 H, m), 7.09 (1 H, m), 3.12 (1 H, d, J = 14 Hz), 7.61 (1 H, d, J = 14 Hz), 8.17 (3 H, d, J = 2 Hz), 8.18 (3 H, s), 8.67 (3 H, s), 8.80 (3 H, s), 9.07 (3 H, d, J = 7 Hz).

Jatrophone was converted to intractable mixtures when treated under alkaline conditions, but was quite stable in acid media. Treatment of **1** with ethylene glycol and *p*-TsOH afforded an oily C-14 ketal, $C_{22}H_{28}O_4$ [uv max 285 mμ (ϵ 8800); ir ($CHCl_3$) 5.91, 6.20 μ; nmr spectrum similar to that of **1**, with additional signals for the ethylenedioxy group], and crystalline ketal **2**, $C_{22}H_{28}O_4$ [mp 140–141°; uv max 255 mμ (ϵ 20,000); ir (KBr) 5.70 μ; nmr ($CDCl_3$) τ 4.02 (1 H, d, J = 4 Hz), 4.15 (1 H, m), 4.22 (1 H, s), 6.25 (m, ethylenedioxy), 8.21 (3 H, br s), 8.73 (3 H, d, J = 7 Hz), 8.82 (3 H, s), 8.85 (3 H, s), 8.90 (3 H, d, J = 7 Hz)]. The nature of the two ketals indicated that jatrophone possesses two ketone groups, one of which is in a five-membered ring.



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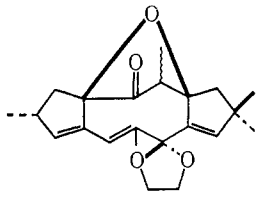

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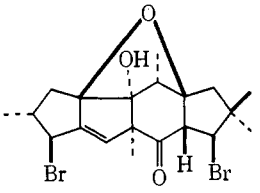

3

Treatment of **1** in glacial acetic acid with dry hydrogen bromide afforded the dihydrobromide **3**: $C_{20}H_{26}Br_2O_3$; mp 154–156° dec; uv max 300 (ϵ 1200), 232 mμ (ϵ 4700); ir (KBr) 2.75, 5.85 μ; nmr (C_6D_6) τ 5.20 (1 H, s), 5.75 (1 H, d, J = 11 Hz), 6.06 (1 H, d, J = 5 Hz), 6.28 (1 H, d, J = 11 Hz), 7.00 (1 H, m), 8.62 (3 H, s), 8.78 (3 H, s), 9.09 (3 H, s), 9.18 (3 H, d, J = 7 Hz), 9.72 (3 H, d, J = 7 Hz). Dehydrobromination of **3** to afford jatrophone in high yield was effected by stirring a chloroform solution of **3** with a suspension of neutral alumina. The reversible interrelation of jatro-

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(6) Significant inhibitory activity was noted against sarcoma 180, Lewis lung carcinoma, and P-388 lymphocytic leukemia in the mouse and the Walker 256 intramuscular carcinosarcoma in the rat. Cytotoxicity and *in vivo* activity were assayed under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, by the procedures described in *Cancer Chemother. Rep.*, **25**, 1 (1962). Cytotoxicity was also assayed by differential agar diffusion by Professor D. Perlman, University of Wisconsin; *cf. J. Pharm. Sci.*, **58**, 633 (1969).

(7) Jatrophone showed significant antileukemic activity against P-388 lymphocytic leukemia at 27 and 12 mg/kg, and cytotoxicity (ED_{50}) against KB cell culture at 0.17 μg/ml.[6]

(8) Elemental formula confirmed by high-resolution mass spectrometry. We cordially thank Dr. D. Rosenthal, Research Triangle Institute, and Drs. W. E. Baitinger and W. L. Budde, Purdue University, for the mass spectra. All crystalline compounds have also been characterized by concordant elemental analyses.