deuterium atom on the methylene carbon of the side chain is indicated by the collapse of the methyl triplet to a doublet at 1.7 and by a decrease in the integral for the broad multiplet at 3.1. Thus, a deuterium atom shifted from the tertiary carbon to the methylene carbon of the ethyl side chain in the course of formation of **2**.

The $Eu(DPM)_3$-shifted nmr spectra of the normal hydroformylation products **4**-*d* and the diastereomeric mixture **5**-*d* both contained singlets for the 4-methyl and the 3-methyl groups, respectively, and demonstrated that these products were formed without shift of deuterium.

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Inactivation and Reactivation of Hemocyanin by Radiolytic OH and e_{aq}^-

Sir:

When exposed to ionizing radiation the biochemical function of enzymes and proteins can be impaired or destroyed. In this communication we demonstrate that ionizing radiation can also restore the biochemical function of a previously radiation-damaged macromolecule.

In experiments with the oxygen-carrying copper protein, hemocyanin, we find that in neutral, *oxygen-free media*, radiolytically produced OH eliminates the oxygen-carrying capacity by oxidizing the protein-bound copper to the cupric state, while the primary reducing species e_{aq}^- fully restores the oxygen-carrying capacity by reducing cupric copper to the cuprous state. Similarly, the secondary radical[1,2] CO_2^-, produced when formate is used as an OH scavenger, is capable of restoring inactivated hemocyanin. The radiation doses employed (0–110 krads) do not cause any significant changes or damage to the protein moiety. It has been demonstrated previously[3,4] that the elimination and restoration of oxygen-carrying capacity of hemocyanins by irradiation in *oxygenated* media was solely due to H_2O_2 produced during irradiation. It has long been known that H_2O_2 and other oxidizing–reducing agents modify the oxygen-carrying capacity of hemocyanin.[5,6]

We also find that the reduction of Cu(II) to Cu(I) parallels the restoration of the oxygen-carrying capacity of hemocyanin derived from one species of Mollusca (*Busycon*, the channeled whelk). However, no restoration of oxygen-carrying capacity is effected in hemocyanin obtained from a species of Arthropoda (*Limulus*, the horseshoe or king crab), despite the fact that complete reduction of Cu(II) to Cu(I) occurs following irradiation in the presence of suitable scavengers. These observations, indicative of a difference in the nature of the binding of the copper in the hemocyanins from the two different species, appear to be borne out by certain distinctive features in their respective circular dichroism (CD) spectra as described below.

The hemocyanins employed in these experiments (obtained from the Marine Biological Laboratories, Woods Hole, Mass.) were usually used as naturally present in the hemolymph (serum) of the animals. However, before use, the hemocyanin hemolymph was conditioned[4] so as to remove extraneous clotting protein. The hemocyanin hemolymph was diluted with 0.05 *M*, pH 7.0, sodium dihydrogen phosphate buffer. The oxygen-carrying capacity was measured by the absorbance (A_0) at 345 nm and the activity of the hemocyanin was expressed as the per cent oxygen-carrying capacity, defined as the ratio of the A_0 of irradiated hemocyanin to that of a nonirradiated control sample. Deoxygenation of oxyhemocyanin was accomplished by passage of helium through the hemocyanin solution.[3,4] The total copper content of hemocyanin was determined by atomic absorption. The cuprous copper fraction in deoxyhemocyanin was measured by the 2,2′-biquinoline method.[7] The CD spectra were recorded with a Cary-60 spectropolarimeter on samples of hemocyanin isolated and purified by ultracentrifugation and dialysis. Irradiations were carried out at 25 ± 1° with a cobalt-60 γ source.

Hemocyanin from *Busycon* was first inactivated by irradiation in oxygenated media with 17 krads of cobalt-60 γ radiation followed by deoxygenation as described previously.[3,4] Immediately afterward, in an attempt to restore the oxygen-carrying capacity, the inactivated hemocyanin was irradiated with varying doses in oxygen-free media, in the presence and absence of 0.5 *M* sodium formate, an OH radical scavenger.[8] In the absence of formate, no restoration of the oxygen-carrying capacity was observed (Figure 1). However, in the presence of formate the oxygen-carrying capacity increased with increasing dose to the point that at 60 krads practically all of the original activity was restored. Concomitantly, the cuprous ion concentration increased in the same manner as the oxygen-carrying capacity (Figure 1). The restored or reactivated hemocyanin appeared to be identical with that of the nonirradiated hemocyanin, *e.g.*, the oxygenation–deoxygenation cycles, optical absorption spectra, and CD spectra.

Since the secondary CO_2^- radical is capable of reducing the cupric ion,[1] we investigated its restorative ability in the absence of e_{aq}^- by irradiating inactivated *Busycon* hemocyanin in the presence of both N_2O, an efficient e_{aq}^- scavenger, and sodium formate. We found that the CO_2^- radical did, indeed, reactivate *Busycon* hemocyanin. Finally, we investigated the restorative capability of e_{aq}^- in the absence of the CO_2^- radical by carrying out irradiations in the presence of 2-propanol. Again, we observed restoration of *Busycon* hemocyanin.

In related experiments with *Limulus* hemocyanin, we found that neither e_{aq}^- nor the CO_2^- radical restored the oxygen-carrying capacity. However, the reduction of Cu(II) to Cu(I) increased with increasing radiation

(1) D. M. Donaldson and N. Miller, *Radiat. Res.*, **9**, 487 (1958); N. Miller, *ibid.*, **9**, 633 (1958).
(2) A. Fojtik, G. Czapski, and A. Henglein, *J. Phys. Chem.*, 74, 3204 (1970).
(3) J. Schubert and E. R. White, *Science*, **155**, 1000 (1967).
(4) J. Schubert, E. R. White, and L. F. Becker, Jr., *Advan. Chem. Ser.*, **No. 81**, 480 (1968).
(5) G. Felsenfeld and M. P. Printz, *J. Amer. Chem. Soc.*, **81**, 6259 (1959).
(6) R. Lontie and R. Witters in "The Biochemistry of Copper," J. Peisach, P. Aisen, and W. E. Blumberg, Ed., Academic Press, New York, N. Y., 1966, pp 455–463.
(7) G. Felsenfeld, *Arch. Biochem. Biophys.*, **87**, 247 (1960).
(8) E. J. Hart, J. K. Thomas, and S. Gordon, *Rad. Res. Suppl.*, **4**, 74 (1964).

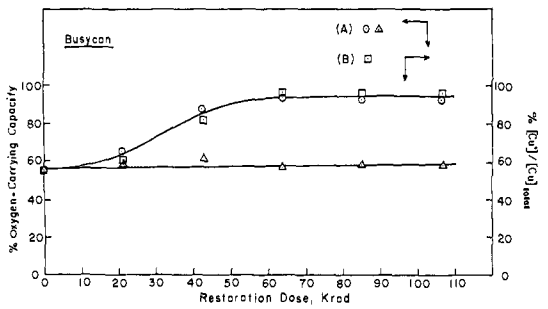

Figure 1. (A) Radiation-induced restoration of the oxygen-carrying capacity of *Busycon* hemocyanin: ⊙, with 0.5 *M* sodium formate; △, without sodium formate. (B) Radiation-induced reduction of Cu^{2+} to Cu^{+}: □, with 0.5 *M* sodium formate. The hemocyanin solution, pH 7.0 and 0.05 *M* in sodium dihydrogen phosphate buffer, containing 5×10^{-5} *M* protein-bound copper, was initially inactivated with a dose of 17 krads of cobalt-60 γ irradiation delivered to the hemocyanin in oxygenated media prior to deoxygenation.

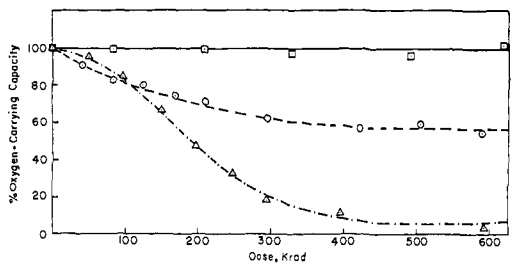

Figure 2. Diminution of the oxygen-carrying capacity of *Limulus* hemocyanin ([Cu] = 4.5×10^{-5} *M*) following γ irradiation in oxygen-free, pH 7.0, 0.05 *M* sodium dihydrogen phosphate buffered solution: □, with 0.5 *M* sodium formate; ○, no scavenger; △, with 0.1 *M* sodium chloroacetate.

dose to the same extent as found with *Busycon* hemocyanin. Obviously, the failure to reactivate *Limulus* hemocyanin cannot be due to an inability to reduce Cu(II) to Cu(I) as had been presumed in unsuccessful attempts to reactivate *Limulus* hemocyanin by chemical agents.[5] It would appear that the differences in restorability between *Busycon* and *Limulus* hemocyanins are due to the chemical and/or structural features of the copper at the active sites in the respective hemocyanins. This postulate is supported by differences in the CD spectra of *Busycon* and *Limulus* hemocyanins although their absorption spectra are very similar. The CD spectrum of *Busycon* hemocyanin consists of four positive peaks at ~700, 485, 285, and 253 nm and three negative peaks at 580, 350, and 215 nm. This pattern is similar to those of *Octopus vulgaris* and *Loligo pealei* (Atlantic squid) hemocyanins in which histidyl residues are considered to bind the copper.[9] However, we find that the CD spectrum of *Limulus* hemocyanin appears to be quite unique, consisting of two positive peaks with about equal intensity at 620 and 500 nm and three negative peaks at 340, 280, and 210 nm. The characteristic double peak (with opposite sign) observed for metal ions interacting with histidyl residues in small peptides[9,10] is not observed. For a variety of reasons, it appears that -SH groups derived from cysteine are involved in the binding of copper in *Limulus* but not in *Busycon* hemocyanin. For example, Felsenfeld[7] has shown that *Limulus* hemocyanin possesses several reducing sulfhydryl groups per copper which can be blocked by *p*-mercuribenzoate.

In the experiments described above, the initial inactivation of the hemocyanins is due to the H_2O_2 formed by irradiation in the oxygenated media.[3,4] In an irradiated oxygen-free system, in which the role of H_2O_2 is negligible, inactivation of hemocyanin is also produced (Figure 2). Note that only in the presence of chloroacetate, which is an e_{aq}^- scavenger,[11] were we able to obtain nearly complete elimination of the oxygen-carrying capacity. This result rules out any significant reduction competition by the secondary radical, $CH_2CO_2^-$, produced by the scavenging action of chloroacetate.[12]

The results of the investigation reported here lead to the conclusion that in neutral, oxygen-free media, the loss of oxygen-carrying capacity of hemocyanin by γ irradiation is due principally to the OH radical which oxidizes the copper at the active site, thus forming methemocyanin.[5] The restoration of the oxygen-carrying capacity of methemocyanin is due principally to the reduction of cupric copper to the cuprous state by the solvated electron, e_{aq}^-, and, in the presence of formate scavenger, to the secondary CO_2^- radical. Obviously, in the absence of a scavenging agent, the OH and e_{aq}^- both compete for the copper during irradiation and, therefore, complete inactivation or reactivation of hemocyanin cannot be attained unless an appropriate scavenging agent is added. We are able to use unusually low doses of radiation to reactivate hemocyanin because of the very high rate constant for the reaction between Cu(II) and e_{aq}^-. At the radiation doses and conditions employed, practically no apparent damage to the protein moiety occurred as indicated, for example, by minimal changes in the characteristic protein absorption peak region at 280 nm.

It would appear that the OH radical, the reducing species, e_{aq}^-, and appropriate secondary radicals could be used in many cases to restore the activity of metalloenzymes and proteins in which inactivation is due to a change in the valence state of the metal at the active site. It is also of interest to note that the apoproteins of many metalloenzymes and proteins can be reconstituted to give an active product only with a specific or a limited number of metal ions. However, it is conceivable that reconstitution of an apoprotein might be effected with abnormal valence states of metal ions. For example, the redox potential of e_{aq}^- is so high (E° = 2.6 V) that we are investigating the possibility that the apoproteins of hemocyanins which normally can only be reconstituted as an oxygen carrier with Cu^+ might be reconstituted with ions such as Zn^+, Pb^+, Cd^+, and Ni^+. Finally, it appears that e_{aq}^- can be used as a probe of the nature of the bonding of a metal at an active site as indicated by our observations on *Limulus* and *Busycon* hemocyanins.

Acknowledgments. This investigation was supported by U. S. Public Health Service Research Grant No.

(9) K. E. Van Holde, *Biochemistry*, **6**, 93 (1967).
(10) J. M. Tsangaris, J. W Chang, and R. B. Martin, *Arch. Biochem. Biophys.*, **103**, 53 (1969).
(11) M. Anbar and E. J. Hart, *J. Phys. Chem.*, **69**, 271 (1965).
(12) R. L. S. Willix and W. M. Garrison, *ibid.*, **69**, 1579 (1965).

EC00098-05. We wish to thank Dr. James Franzen for his help with the CD measurements.

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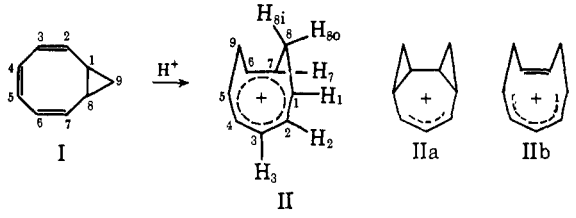
Protonated *cis*-Bicyclo[6.1.0]nona-2,4,6-triene, a Monocyclic 1,3-Bishomotropylium Ion

Sir:

We have recently reported[1] a series of bicyclic 1,4-bishomotropylium ions,[2] all of which possess considerable homoaromatic character. We now wish to communicate our results on a second type of bishomotropylium ion—a 1,3-bishomotropylium species, II,[2] which represents the first monocyclic bishomotropylium ion.



When a solution of I[3] in CD_2Cl_2 was extracted into a 1:4 (v/v) mixture of FSO_3H–SO_2ClF at *ca.* −125°, there resulted an orange-red solution which gave a quite clean nmr spectrum (Figure 1).[4] A priori, I

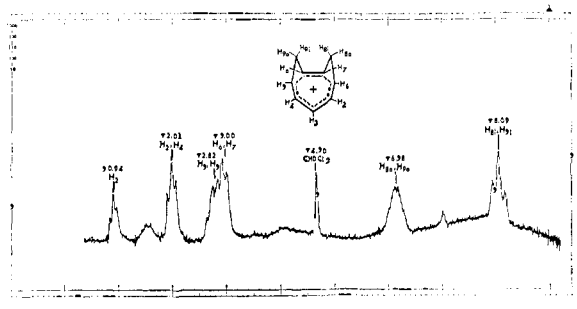


Figure 1. 100-MHz nmr spectrum of protonated *cis*-bicyclo-[6.1.0]nona-2,4,6-triene (II) at *ca.* −125° (sweep width = 1000Hz).

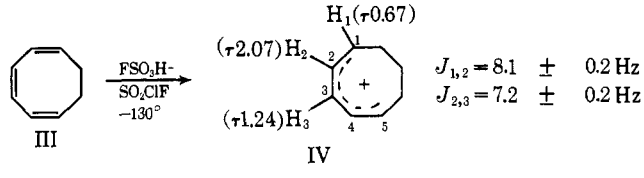
could have protonated at any one or more of five positions—C_1 (≡C_8), C_2 (≡C_7), C_3 (≡C_6), C_4 (≡C_5), or C_9. However, based on relative nucleophilicities, one would expect protonation at one or more of the double bond positions, only. Protonation at C_2, C_3, or C_4 would give a 1,2-, 1,3-, or 1,4-bishomotropylium ion, respectively. It is noteworthy that the nmr spectrum of II (Figure 1) is consistent only with C_3 protonation (*i.e.*, a 1,3-bishomotropylium ion), to give what should be the most stable of the three possible bishomotropylium ions.[5] All chemical shifts and coupling constants were unambiguously assigned *via* decoupling experiments; the nmr parameters are summarized in Table I.

Table I. Nuclear Magnetic Resonance Data for Protonated *cis*-Bicyclo[6.1.0]nona-2,4,6-triene (II)[a]

Proton	Chem shift,[b] τ	Coupling constants, Hz
H_3	0.94	$J_{2,3} \equiv J_{3,4} = 6.0 \pm 0.5$
H_2, H_4	2.02	$J_{1,2} \equiv J_{4,5} = 9.0 \pm 0.5$
H_1, H_5	2.82	$J_{1,8o} \equiv J_{5,9o} = 9.0 \pm 0.5$
H_6, H_7	3.00	$J_{7,8o} \equiv J_{8,9o} = 9.0 \pm 0.5$
H_{8o}, H_{9o}	6.18	$J_{1,8i} \equiv J_{5,9i} = 11.0 \pm 0.5$
H_{8i}, H_{9i}	8.09	$J_{8i,8o} \equiv J_{9i,9o} = 12.0 \pm 0.5$
		$J_{7,8i} \equiv J_{8,9i}$ = small

[a] In FSO_3H-SO_2ClF (1:4, v/v) at *ca.* −125°. [b] Chemical shifts measured relative to internal $CHDCl_2$ (τ 4.70).

The nmr data of Table I are compatible, we believe, only with the arrangement of carbons and hydrogens shown in II;[6] however, there remains the problem of the electronic structure of II. Besides the bishomotropylium representation, II, the tricyclic structure, IIa, and the noninteracting monocyclic structure, IIb, are conceivable. It is immediately possible to eliminate IIa as an adequate representation for II, since the $|J_{gem}|$ = 12 Hz is far too large for a cyclopropane ring.[7] Also, the charge distribution at C_2, C_3, and C_4 observed for II is the opposite of what one would expect for IIa.[8,9] Structure IIb, with a simple pentadienyl unit and an isolated double bond, is not easily dismissed without recourse to models. Therefore, 1,3,5-cyclooctatriene (III) was protonated to yield the cyclooctadienyl cation IV.



A comparison of the nmr data for II and IV immediately reveals that IIb is not an adequate representation for II. In particular, H_1 and H_5 in II are each shifted some 2 ppm to higher field relative to their counterparts in IV. If this upfield shift is really due

(1) P. Ahlberg, D. L. Harris, and S. Winstein, (a) *J. Amer. Chem. Soc.*, **92**, 2146 (1970); (b) *ibid.*, **92**, 4454 (1970); (c) M. Roberts, H. Hamberger, and S. Winstein, *ibid.*, **92**, 6346 (1970).

(2) The two segments of a 1,4-bishomotropylium ion consist of three and four carbons, while those of a 1,3-bishomotropylium ion consist of five and two carbons; for a further discussion of nomenclature, see S. Winstein, *Quart. Rev., Chem. Soc.*, **23**, 141 (1969).

(3) R. Rieke, M. Ogliaruso, R. McClung, and S. Winstein, *J. Amer. Chem. Soc.*, **88**, 4729 (1966).

(4) When the probe temperature was raised to *ca.* −90°, decomposition took place with a half-life of *ca.* 15 min.

(5) This is true because, in the 1,3-bishomotropylium ion, the charge resides on the *longest* linearly conjugated carbon segment (neglecting homoconjugation). This is the overriding factor when one considers overall energy (neglecting strain contributions, which probably change less than the electronic factors in the isomeric ions in question).

(6) The possibility that the methylene bridges are trans to one another, rather than cis as shown, is deemed unlikely on the basis of the charge distribution observed for II, as well as the chemical-shift difference between inner and outer protons; this will be discussed in a full paper. In order to further confirm this interpretation, a bicyclic 1,3-bishomotropylium ion is being studied in these laboratories.

(7) See the discussion in P. Warner, D. L. Harris, C. H. Bradley, and S. Winstein, *Tetrahedron Lett.*, 4013 (1970).

(8) For studies of allylic ions, see (a) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, New York, N. Y., 1969, p 252; (b) G. A. Olah and J. M. Bollinger, *J. Amer. Chem. Soc.*, **90**, 6082 (1968); (c) P. Warner, Ph.D. Dissertation, U.C.L.A., 1970, p 153; (d) A. F. Diaz, D. L. Harris, M. Sakai, and S. Winstein, submitted for publication.

(9) For studies of allylic ions conjugated with cyclopropane rings, see (a) N. C. Deno, H. G. Richey, Jr., J. S. Liu, D. N. Lincoln, and J. O. Turner, *J. Amer. Chem. Soc.*, **87**, 4533 (1965); (b) M. Roberts, H. Hamberger, and S. Winstein, *ibid.*, **92**, 6346 (1970); (c) P. Warner and S. Winstein, submitted for publication.