Table 1. Potassium and argon data. Abbreviations: *r*, radiogenic; *p*, primordial.

Weekeroo Station sample		Nuclide			
		Content ($\times 10^{12}$ atom/g)		Ratio	Age
No.	Weight (g)	Ar^{40}	K^{41}	($Ar^{40}_r : K^{40}_p$)	($\times 10^9$ yr)
K-1	1.4032	30.3 ± 1.9	1540 ± 70	9.6 ± 1.7	8.5 + 0.2, − 0.4
K-2	1.3260	8.0 ± 0.8	215 ± 20	15.4 ± 3.1	9.3 ± 0.4
B-1	1.1880	17.2 ± 2.3	2370 ± 200	3.4 ± 0.7	6.5 ± 0.4
G-1	1.1653	22.8 ± 1.7	410 ± 30	26.8 ± 4.5	10.4 + 0.3, − 0.4

Table 2. Mass-spectrometric data.

Weekeroo Station sample	Data [$\times 10^{-8}$ (standard) cm^3/g]					
	He^3	He^4	Ne^{21}	Ne^{22}	Ar^{36}	Ar^{38}
K-92	480	1610	5.24	5.73	17.2	27.2
K-94	482	1560	5.25	5.71	17.8	28.4
K-98	462	1560	5.13	5.40	17.1	26.8
B-94	498	1660	5.79	6.12	19.1	30.4

exception that the meteorite samples were dissolved in HNO_3 rather than melted. Helium gas was bubbled through the dissolving solution and, together with carrier and meteoritic argon, was swept through a vacuum line. The argon was trapped on charcoal at liquid-nitrogen temperatures, purified over hot titanium, and pumped directly into a Geiger counter. The 1.8-hour Ar^{41} activity was followed down to background. Potassium was precipitated from the final solution, together with carrier, chemically purified, and counted as the tetraphenyl boron. The 12.4-hour K^{42} activity was followed down to background. Typical counting rates for both K^{42} and Ar^{41} were tens of counts per minute; background was 7 to 15 count/min. Before irradiation the samples were lightly etched to remove surface contamination; loss of potassium during this etching, shown to have been less than 15 percent, will be discussed in detail in a later publication. The data appear in Table 1.

Small samples were cut from those on which these measurements were made and were analyzed separately, for the cosmogenic (*c*) rare gases, by mass spectrometry according to well-established procedures. Table 2 shows no apparent anomalies; thus from these data we can calculate the amounts of cosmogenic Ar_c^{40}, K_c^{40}, and K_c^{41}, utilizing the production ratios determined earlier (*6*): Ar_c^{40}, 1.5×10^{12} atom/g; K_c^{40}, 11×10^{12}, and K_c^{41}, 6.4×10^{12}. Then, by correcting the raw data of Table 1 for cosmogenic Ar^{40} and K^{41} and for cosmoradiogenic Ar^{40} (from the decay of cosmogenic K^{40}), and by assuming the terrestrial K^{40}:K^{41} ratio for primordial potassium (*7*), potassium:argon ages can be calculated (Table 1).

The ages found by us are typical of the great ages found for most iron meteorites. From these, in conjunction with the strontium:rubidium data of Wasserburg *et al.* (*4*) on silicate inclusions in this meteorite, we conclude that the potassium:argon dating technique as applied to iron meteorites gives unreliable results. One may derive *ad hoc* possible explanations of the discord between the silicate and iron-phase ages, such as shock emplacement of these inclusions within the metal matrix without disturbing the potassium:argon ratios in the metal, but we feel that such mechanisms are unlikely.

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12 December 1966

Radiation of Hemocyanin: Inactivation and Reactivation of Oxygen-Carrying Capacity

Abstract. *Oxygen-carrying capacity of hemocyanin from* Limulus *and* Busycotypus (Busycon) *decreases with increasing radiation, giving initial yield values for* $G(-O_2)$ *of 1.1 and 1.0, respectively. High radiation doses regenerate this capacity of* Busycotypus *hemocyanin. These effects are attributed largely to the dual nature of hydrogen peroxide, which, at low concentrations, oxidizes protein-bound copper and at high concentrations, that is, at high doses, reduces oxidized copper. The ability of hemocyanin to decompose hydrogen peroxide is relatively unaffected by irradiation, which suggests that copper atoms at the active sites are not all equivalent. The catalase-like activity of* Busycotypus *hemocyanin can be simulated by amino acid chelates of copper, including arginine, histidine, and glycine.*

Hemocyanin is a nonheme oxygen-carrying copper protein found in the hemolymph of terrestrial and marine invertebrates (*1, 2*). It reacts reversibly with molecular oxygen at relatively high partial pressures and releases oxygen to tissues where the partial pressure is low. One molecule of oxygen combines stoichiometrically with two atoms of copper in hemocyanin, and it appears that the active site involves a pair of copper ions bridged by an oxygen molecule (*3*). Deoxygenated hemocyanin contains copper only in the cuprous state, while oxygenated hemocyanin contains cupric ions in what might be described as a state favoring rapid electron transfer (*1, 3*). Because of the very important and special role of oxygen in radiobiological damage and protection (*4, 5*), hemocyanin serves as a useful model for studying the effects of ionizing radiation on oxygenation reactions (*6*), particularly in view of the suggestion (*7*) that a contributory mechanism of radiobiological damage involves interference with the ability of copper-containing oxidases to interact with molecular oxygen. Presumably, radiation-induced oxidation of protein-bound copper to the cupric state leads to inhibition of oxygenation reactions and energy utilization.

Early investigators (*8–10*), who studied the actions of ionizing radiation on hemocyanin used, as criteria of radiation damage, rupture of the copper-protein bond or dissociation of pro-

tein molecules or aggregates. A more physiological, sensitive, and quantitative criterion is the capacity of irradiated hemocyanin to combine reversibly with molecular oxygen, as measured by changes in the optical absorption peak, at 340 mμ, of oxygenated hemocyanin. The peak disappears upon deoxygenation or destruction of oxygen-carrying ability, as for example, by hydrogen peroxide (*11*), organic peroxides (*6*), and ionizing radiation (*6*).

We made a more detailed study of the effects of ionizing radiation on hemocyanin derived from two different species, *Limulus* (the horseshoe or king crab) and *Busycotypus* (*Busycon*, the channeled whelk). A significant observation reported here is that oxygenation capacity lost by irradiation is regained by additional increments of radiation in the case of hemocyanin from *Busycotypus* but not of that from *Limulus*.

We used hemocyanin as it exists naturally in serum (*12*). Dilutions were made with 0.05*M* potassium dihydrogen phosphate buffer, *p*H 7.00, and the hemocyanin solutions were centrifuged to remove clotted material. Copper analyses were made by atomic absorption spectrophotometry; the radiation source was cobalt-60 gamma rays that delivered about 15,000 rad/min. Radiation doses were measured with a ferrous sulfate dosimeter, based on a value of 15.5 for $G(Fe^{3+})$ (*13*).

Oxygen-saturated solutions of hemocyanin were irradiated at 25°C in 4-ml glass vials sealed with Teflon-lined screw caps. After irradiation oxygenated solutions were transferred to silica cell cuvettes (path length, 1 cm) capped with sleeve-type rubber stoppers with an indented area in the center so that syringe needles for passage of oxygen or oxygen-free helium [$O_2 < 0.001$ percent by volume (< 0.3 molar part per million] could be inserted. To check occurrence of oxygen depletion during irradiation, optical densities were measured shortly after irradiation and again after oxygenation. If none occurred, these two measurements should agree, as they invariably did. Hemocyanin was subsequently deoxygenated by bubbling from 350 to 600 cm^3 of helium, at rates of 60 to 100 cm^3 per minute, through the solution until complete deoxygenation occurred as measured by the deoxygenated optical density, $(OD)_d$, a period of less than 6 minutes. The solution was kept in the deoxygenated state for a given time, then reoxygenated, and the oxygenated optical density, $(OD)_o$, was measured. Subsequent measurements were made until the $(OD)_o$ reached a constant value. The $(OD)_o$ of hemocyanin from *Limulus* attained a constant value immediately after oxygenation. With irradiated hemocyanin from *Busycotypus*, the time required for the $(OD)_o$ to reach a stable maximum value increased with increasing radiation, for example, a dose of less than 8000 rads required less than 1 minute; approximately 13,000 to 20,000, 1 hour; 20,000 to 27,000, 1 to 2 hours; and 3 to 4 hours for doses above 34,000 rads.

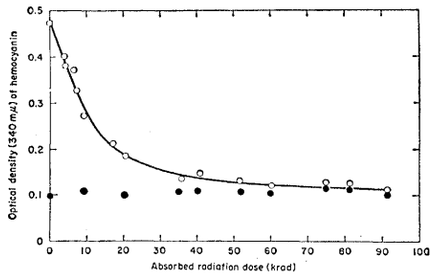

Fig. 1. Changes in optical densities of hemocyanin from *Limulus* after irradiation at 25°C with cobalt-60 gamma rays at a dose rate of 15,000 rad/min. Solutions consisted of *Limulus* hemolymph diluted with 0.05*M* potassium dihydrogen phosphate buffer (*p*H 7.00) and saturated with oxygen; a $3.92 \times 10^{-5}M$ concentration of copper was present. Hemocyanin was deoxygenated 10 minutes after irradiation and was allowed to remain in the deoxygenated state for 1 hour before reoxygenation, at which time the optical densities were measured at 340 mμ. Subsequent deoxygenation provided the final deoxygenated optical densities. Open circles, oxygenated state; closed circles, deoxygenated state.

Oxygenated hemocyanin in *Limulus* hemolymph was diluted with phosphate buffer to give an $(OD)_o$ of about 0.5 and was subjected to different doses of cobalt-60 gamma irradiation. No effect of ionizing irradiation was detected as long as the hemocyanin remained in the oxygenated state (*6*). Upon deoxygenation of the irradiated hemocyanin, followed by oxygenation, radiolytic products, primarily in the medium, attack the deoxygenated state of the hemocyanin, as shown by a decrease in $(OD)_o$. The amount of radiation damage depends on the time the irradiated hemocyanin remains in the oxygenated state (designated as oxygenated reaction time, o.r.t.) before a deoxygenation and reoxygenation cycle, and in the deoxygenated state (designated as deoxygenated reaction time, d.r.t.) before reoxygenation. However, for the dilute solutions that we used, the radiation effects were not influenced for o.r.t.'s in the time intervals employed. Data presented in this report were obtained at a constant 10-minute o.r.t., regardless of the length of the subsequent d.r.t.

In Fig. 1 data for a 1-hour d.r.t. (a period beyond which no further decreases in oxygen capacity occur) show that the $(OD)_o$ drops rapidly with increasing radiation and, in fact, more than 90 percent of the entire oxygen capacity is lost above 35,000 rads. From the slope of $\triangle(OD)_o$ as the radiation dose approaches zero, we obtained an initial yield value of 1.1 for $G(-O_2)$, that is, about 1.1 μmole of oxygen capacity are lost per 1000 rads (*14*). The $G(-O_2)$ values are not affected by changes in d.r.t. No significant variation in $(OD)_d$ occurs in the range of radiation doses that we used. Irradiation carried out with x-rays [280 kv (peak)] at a dose rate of 1600 rad/min and at total doses below 15,000 rads gives results in close agreement with those obtained with cobalt-60 gamma rays at a dose rate nearly ten times higher.

Experiments with *Busycotypus* were similar to those with *Limulus*. A remarkable response of hemocyanin from *Busycotypus* to irradiation is that, above radiation doses of 13,000 to 16,000 rads, the oxygen capacity begins to recover and above about 70,000 rads the oxygen capacity is nearly equivalent to the unirradiated hemocyanin. Results for d.r.t.'s of 15 minutes, 30 minutes, and 1 hour are shown in Fig. 2. Regardless of the d.r.t., the initial yield values for $G(-O_2)$ are 1.0, or nearly equal to that of *Limulus*. However, the maximum loss in oxygen capacity increases with increasing d.r.t. for the first hour, after which little change occurs. The $(OD)_o$ for the 2-hour d.r.t. is only 10 percent less than that of the 1-hour d.r.t. The $(OD)_d$ at doses of more than 15,000 rads tends to be slightly higher than that of unirradiated control samples and may be due partly to the oxidizing action of residual peroxides.

Qualitatively, and in the dilute solutions we used, the effects of irradiation on the oxygenation reactions of hemocyanin are due largely to the action of radiolytic H_2O_2. We carried out a series of experiments in which known and varying amounts of H_2O_2 were added to solutions of hemocyanin. This radiomimetic action, including the reactivation of *Busycotypus*

hemocyanin, parallel the reactions described by Felsenfeld and Printz (*11*).

We measured, by the triiodide method of Ghormley (*15*), the amount of H_2O_2 formed radiolytically in phosphate buffer saturated with oxygen. The results obtained are in good agreement with those reported by Baxendale (*16*) in neutral aerated water. Some typical values of H_2O_2, in micromoles per liter, produced radiolytically in our buffer solutions are: 18 at 10 krad, 26 at 15, 58 at 40, and 86 at 70 krad. In the deoxygenated buffer, H_2O_2 was less than 1 μmole/liter and could not be detected, even at doses as high 10^5 rads. The reactivation of hemocyanin from *Busycotypus* is not apparent until the molar concentration of radiolytic H_2O_2 exceeds that of the protein-bound copper, that is, the minimum in the curves shown in Fig. 2 occurs at about 13,000 rads where the molar ratio of H_2O_2 to copper is approximately unity. As expected, when the concentration of hemocyanin is doubled, the minimum occurs at about 25,000 rads. In these relatively dilute solutions of irradiated hemocyanin, the formation of H_2O_2 is, within 20 percent, the same as in irradiated buffer.

In the dilute solutions we used, the radiation-induced decrease in oxygen capacity is most readily explained as being caused largely by the oxidation of protein-bound copper by radiolytic H_2O_2. However, additional oxidizing action is probably produced by the hydroxyl radical and by hydroperoxides, ROOH, and organic peroxides, ROOR′, formed from the interaction of HOOH with small organic molecules in the serum (*17*), and by the usual radiolytic reactions occurring in the presence of organic solutes and oxygen (*7*, pp. 76–77; *16*).

The reactivation of *Busycotypus* hemocyanin illustrates a sometimes unappreciated dual nature of H_2O_2, namely, that it is also a reducing agent (*18*, p. 355). The energetics of the copper-H_2O_2 system, among others, have been described in some detail by Uri (*19*). In fact, at high concentrations of H_2O_2 and Cu^{++}, that is, high concentrations of oxidized or attacked hemocyanin, the reduction of Cu^{++} to Cu^{+} predominates over the oxidizing action of H_2O_2. Consequently, the radiation reactivation of *Busycotypus* hemocyanin, at the concentrations employed, is brought about by the reduction of Cu^{++} to Cu^{+}. There is chemical evidence

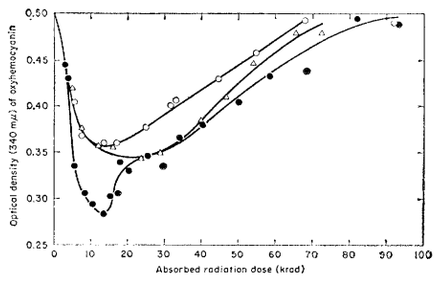

Fig. 2. Changes in optical densities of hemocyanin from *Busycotypus* with different deoxygenated reaction times after irradiation at 25°C with cobalt-60 gamma rays. All experimental conditions are the same as those described in Fig. 1; a $3.55 \times 10^{-5}M$ concentration of copper was present. In order to facilitate direct comparison, the optical densities for the oxygenated hemocyanin were normalized to 0.500 for the unirradiated hemocyanin and 0.200 for the deoxygenated hemocyanin. The actual optical densities were all within 10 percent of the normalized values. Deoxygenated reaction time: open circles, 15 minutes; open triangles, 30 minutes; and closed circles, 60 minutes.

for such a reducing action on hemocyanin from *Busycotypus* by H_2O_2 and by other reducing agents (*11*). The presence of cupric ion in H_2O_2-treated hemocyanin or in aged hemocyanin has been demonstrated by electron spin resonance (*20*, *21*); however, upon treatment with excess H_2O_2, cysteine, or hydroxylamine, the cupric ion signal disappears (*21*) as the hemocyanin is reactivated.

Some of the redox reactions that involve the protein-bound copper can be deduced, if we consider that we are dealing with a neutral, oxygenated, aqueous medium containing organic solutes. From both the radiation chemical (*16*) and metal-peroxide reactions (*19*, *22*), these redox reactions (where *P* is protein) include:

1) Oxidation (inactivation)

$$P\text{-}Cu^{+} + H_2O_2 = P\text{-}Cu^{++} + OH + OH^{-}$$
$$P\text{-}Cu^{+} + OH = P\text{-}Cu^{++} + OH^{-}$$

2) Reduction (reactivation)

$$P\text{-}Cu^{++} + H_2O_2 = P\text{-}Cu^{+} + HO_2 + H^{+}$$
$$P\text{-}Cu^{++} + HO_2 = P\text{-}Cu^{+} + H^{+} + O_2$$
$$P\text{-}Cu^{++} + e^{-}_{aq} = P\text{-}Cu^{+}$$

The reducing reactions take place more readily in neutral than in acid media. Reduction by HO_2 radical to yield O_2 is the result of the dissociation of HO_2 to yield H^+ and O_2^-. The latter species, also formed by the action of the hydrated electron (e^-_{aq}) on H_2O_2, reacts rapidly with *P*-Cu^{++} to yield O_2. Analogous redox reactions involving organic and hydroperoxides such as ROOR′ and ROOH also take place and appear to increase in importance relative to HOOH as the concentration of hemocyanin increases. Lack of reduction or reactivation of *Limulus* hemocyanin has been demonstrated by the use of H_2O_2 and reducing agents such as ferrocyanide or ascorbic acid (*11*). The differences probably lie in the location or accessibility of the active sites within the protein structure. If it is simply a question of conformational or activation energy considerations, then a radiation reactivation of *Limulus* might be effected at higher temperatures or in a different chemical environment, such as in the presence of urea which causes an unfolding of protein structure.

It is possible to differentiate the effects of H_2O_2 from ROOR′ and ROOH inasmuch as hemocyanin from *Busycotypus* possesses a far greater ability than that from *Limulus* to decompose H_2O_2 (*2*, *11*), but not organic peroxides. When relatively high concentrations of irradiated solutions of *Busycotypus* hemocyanin remain in the oxygenated, that is, radiation-resistant, state for protracted periods (more than 1 hour) before deoxygenation, the subsequent loss in oxygen capacity, observed upon deoxygenation and reoxygenation, decreases in proportion to the time elapsed before deoxygenation.

Irradiation, at least to 50,000 rads, while affecting the oxygenation reaction, does not seem to affect the catalase-like activity of *Busycotypus* hemocyanin, measured as the evolution of oxygen from added H_2O_2 by Warburg manometric techniques. Effects of irradiation on the catalase-like activity of *Limulus* hemocyanin at *p*H 7 were not investigated because its very low activity precludes accurate measurements. These observations suggest that not all of the copper atoms at the active sites are equivalent and that they may, in fact, be bound to entirely different amino acid residues.

It has been reported (*2*, *23*) that the ability of hemocyanin to decompose H_2O_2 may be due to its arginine content because it was claimed that, among all amino acids tested, only arginine gave a complex with copper that decomposes H_2O_2. We found that other copper complexes of amino acids, including histidine and glycine, and related compounds decompose H_2O_2. However, the catalytic function de-

pends, for example, on *p*H and on the molar ratio of the amino acid to copper. Thus, at *p*H 7 and a fixed copper concentration ($5 \times 10^{-4}M$), arginine, histidine, and histamine at molar ratios of 0.5:1, 1:1, 2:1, and 3:1 relative to copper, possess a marked catalytic action. However, at a molar ratio of 4:1, histidine no longer is catalytically active but histamine and arginine are.

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Fossil Actinomycetes in Middle Precambrian Glacial Varves

Abstract. *Fossil actinomycetes and other bacteria have been found in sulfide minerals from "varved" argillites in the middle Precambrian Cobalt Series of Canada. The fossils consist of branched and unbranched nonseptate hyphae and chains of rod-shaped cells. The presence of actinomycetes is consistent with the theory that the argillites are lacustrine desposits.*

The Gowganda Formation of the middle Precambrian Cobalt Series in southern Ontario and Quebec consists largely of tillites and "varved" argillites. The argillites are probably glacial lake deposits, as indicated by their numerous erratic pebbles and boulders and by their rhythmic stratification (*1*).

The dark gray to bluish coloration of the argillites suggests microbial activity under anaerobic conditions. Additional evidence for this was sought in argillite samples collected in Township 169 and Wells Township, Ontario, where the sediments are gently folded and practically unmetamorphosed. In these rocks were found (i) a lens of erratic sand grains intermingled with and encrusted by chalcopyrite crystals; (ii) a small rosette of pyrite crystals surrounded by concentric zones of pyrite; and (iii) a lens of sand-sized pyrite crystals surrounded by a yellow-brown zone containing smaller, disseminated pyrite crystals; the yellowish pigment is probably limonite formed by oxidation of very fine-grained pyrite. In the absence of evidence for hydrothermal deposition, such clusters of sulfide crystals in dark argillaceous sediments may

Fig. 1. Hyphae of actinomycete from sulfide crystal (germanium-shadowed carbon replica of cut-and-polished section) (× 25,300).