Reactions of Singlet Oxygen with Pine Pollen

Abstract. *Exposure of pine pollen to singlet oxygen, generated in an aqueous environment, resulted in a decrease in the relative quantities of unsaturated fatty acids that could be recovered by solvent extraction of surface and near surface pollen lipids. The involvement of excited oxygen was confirmed by substitution of deuterium oxide for water, which led to a twofold greater decrease in the unsaturated acids. The potential environmental and biomedical implications of these observations are discussed in terms of this model system.*

Singlet oxygen undergoes a number of reactions with olefins including unsaturated fatty acids (*1, 2*). It has been demonstrated that hydroperoxides, for example, can be formed from such substrates via the "ene" reaction (*2*), and fatty acid hydroperoxides are known to be toxic to mammals (*3*). Cortesi and Privett (*4*) showed that methyl linoleate hydroperoxide administered orally and by intravenous injection is lethal to adult male rats; the organs visibly affected were the lungs, which suffered hemorrhaging and edema. Our study was initiated to determine whether viable atmospheric particles such as plant pollens and fungal spores containing unsaturated lipids (*5*) can interact with singlet oxygen to give oxygenated products that are potentially toxic. To our knowledge, in vivo studies in this area are without precedent; however, Lamola, Yamane, and Trozzolo (*6*) independently observed that singlet oxygen is involved in the formation of cholesterol hydroperoxide from cholesterol on irradiation of red blood cell ghosts containing protoporphyrin, a known sensitizer. It has also been demonstrated in vitro that methyl linoleate is more susceptible to attack by singlet oxygen than by ground state oxygen (the rate ratio is 1450 : 1) (*2*).

While the degree to which singlet oxygen contributes to urban atmospheric pollution remains a subject of controversy (*7*), it has been recognized that energy transfer from NO_2 is efficient enough to make metastable oxygen a potential atmospheric contaminant (*8, 9*). Ambient steady-state concentrations of singlet oxygen as high as 3×10^6 cm^{-3} may be reached by this mechanism under typical midday solar exposure and NO_2 concentrations as low as 0.035 part per million (ppm) (*8*). In view of the widespread distribution of pollen and fungal spores in the atmosphere (*10*), the results obtained with this model system may have important implications in the areas of human respiratory physiology and dermatology.

The pollen (*Pinus echinata*) was collected during the 1971 growing season from mature short leaf pine trees grown in southern Mississippi and was refrigerated until needed. Samples (0.4 g) of the pollen suspended in water (or D_2O) were utilized in all experiments. A 500-watt tungsten iodine vapor lamp equipped with a Pyrex filter housed in a water-cooled immersion well was employed as a light source. In a typical case where singlet oxygen was desired, methylene blue (MB) ($5.0 \times 10^{-5} M$) was introduced as a sensitizer and the sample was irradiated for 2 hours at room temperature. Oxygen was simultaneously introduced through two fritted inlets at a rate of 120 ml/min, which provided sufficient agitation to maintain a fairly uniform dispersion. Controls were performed with nitrogen instead of oxygen, MB excluded, and light excluded. The treated pollen was separated by decantation and residual water was removed by desiccation. In all cases more than 90 percent of the treated pollen was recovered.

Table 1. Relative distribution of the free fatty acids isolated from pine pollen. Treated pollen was exposed to singlet oxygen. The relative concentrations (given here as percentages relative to total free fatty acid) were determined on the methyl esters of the fatty acids by gas chromatography and electronic digital integration. In the first column, the first number is the number of carbons in the molecule, the number after the colon gives the degree of unsaturation (number of double bonds), and the superscript gives the positions of the double bonds.

Free fatty acid (carbon skeleton)	Percentage in	
	Untreated pollen	Treated pollen
16 : 0	17.7	18.8
16 : 1	0.3	0.1
18 : 0	8.3	13.8
18 : 1	31.3	10.0
18 : 2 ($\Delta^{5,9}$)*	2.6	0.2
18 : 2 ($\Delta^{9,12}$)	10.9	4.6
18 : 3 ($\Delta^{5,9,12}$)	< 0.1	0.1
20 : 0	25.6	44.7
18 : 3 ($\Delta^{9,12,15}$)	0.9	1.2
21 : 0	< 0.1	2.0
22 : 0	2.6	3.5

* All evidence obtained to date suggests that the double bonds have a *cis* configuration.

Surface and near surface lipids were extracted from the intact, treated pollen by sequential washing in a fritted glass funnel with *n*-hexane (70 ml) and chloroform-methanol (3:1; 70 ml) in 5-ml portions. The volatile solvents were removed from the combined extracts under a nitrogen stream and fractionated by column chromatography on silica gel as described previously (*11*). Finally, esterification of the free fatty acids was achieved by using boron trifluoride in methanol (*12*). The resulting esters were separated on a stainless steel column (2.75 m by 3 mm) packed with 12 percent ethylene glycol succinate on Gas-Chrom-P (60/80 mesh) operated isothermally at 195°C (*12*). The free fatty acids present in pine pollen have been characterized by techniques involving a combined gas chromatograph, mass spectrometer, and computer (*13*), so that changes in the composition of these components could be monitored qualitatively and quantitatively with confidence.

The relative distributions of the fatty acids obtained from the untreated pollen and from pollen treated with singlet oxygen are compared in Table 1. In samples exposed to singlet oxygen the unsaturated fatty acids decrease markedly relative to their saturated counterparts, as would be expected if attack by singlet oxygen were occurring. It is informative to compare the ratios of saturated to unsaturated fatty acids before and after treatment with singlet oxygen, with the former normalized to unity. For example, the ratio of stearic acid to oleic acid (18:0/18:1; see Table 1) increased by a factor of 5 after treatment with singlet oxygen, and the ratio of total saturated acids to unsaturated acids increased by a factor of 4.3. This observation substantiates our contention that viable atmospheric particles such as pollen may be modified under environmental conditions in the presence of available atmospheric constituents such as NO_2 (*8, 9*), benzaldehyde (*14*), and polynuclear hydrocarbons (*1, 15*), which like MB are efficient sensitizers.

In control experiments pollen was treated in an oxygen-free environment in the presence of MB and light. No marked decrease in unsaturated fatty acids was observed, which indicates that oxygen is required to modify the free fatty acid distribution. In the absence of light, with oxygen and MB present, only minor variations in the

relative distribution of fatty acids were noted. That singlet oxygen is responsible for the observed effect was established by substituting D_2O for water as the fluid phase. It has been established experimentally that the lifetime of singlet oxygen is markedly extended (10 times) in D_2O (*16*). We detected a further twofold decrease in unsaturated free fatty acids when D_2O was employed as the medium under otherwise identical conditions. The less dramatic effect of the solvent on the rate of attack observed in this case may be due to the fact that the reactions noted occur on or near the surface of the pollen grain rather than in a homogeneous system.

These results confirm that surface and near surface components of common viable particulate matter in the atmosphere may be subject to rapid oxidation by singlet oxygen, leading to products which are probably allylic hydroperoxides (*2*). We have alluded to the known lethal properties of such compounds mimicking the effects of ozone in leading to pulmonary edema and lung congestion in rats and to our interest in the effects on pollen viability. The recent increase of atmospheric pollutants which may serve as sensitizers enhances the probability that materials toxic to mammalian lung tissue (and potentially biologically damaging to pollens and spores) may be oxidatively produced on the surfaces of viable particulate matter. Adsorption of appropriate sensitizers and substrates (polynuclear aromatics) on nonviable atmospheric particles might lead to similar interactions yielding toxic oxidative products.

BETTY DOWTY, JOHN L. LASETER
Department of Biological Sciences, Louisiana State University in New Orleans, New Orleans 70122
GARY W. GRIFFIN, IEVA R. POLITZER
Department of Chemistry, Louisiana State University in New Orleans
CHARLES H. WALKINSHAW
NASA-Manned Spacecraft Center, Lunar Receiving Laboratory, Houston, Texas 77058

References and Notes

1. I. R. Politzer, G. W. Griffin, J. L. Laseter, *Chem. Biol. Interactions* **3**, 73 (1971).
2. H. R. Rawls and P. J. Van Santen, *Ann. N.Y. Acad. Sci.* **171**, 135 (1970); *J. Amer. Oil Chem. Soc.* **47**, 121 (1970); *Tetrahedron Lett.* (1968), p. 1675.
3. R. T. Holman and S. I. Greenberg, *J. Amer. Oil Chem. Soc.* **35**, 707 (1958).
4. R. Cortesi and O. S. Privett, *Lipids* **7**, 715 (1972).
5. J. L. Laseter, J. Weete, D. J. Weber, *Phytochemistry* **7**, 1177 (1968); J. Oró, J. L. Laseter, D. Weber, *Science* **154**, 399 (1966).
6. A. A. Lamola, T. Yamane, A. M. Trozzolo, *ibid.* **179**, 1131 (1973).
7. J. J. Bufalini, *Environ. Sci. Technol.* **6**, 837 (1972); T. C. Frankiewicz and R. S. Berry, *ibid.*, p. 837; J. J. Bufalini and A. P. Alshuller, *Curr. Res.* **1**, 133 (1967).
8. T. Frankiewicz and R. S. Berry, *Environ. Sci. Technol.* **6**, 365 (1972).
9. I. T. N. Jones and K. D. Bayes, *Chem. Phys. Lett.* **11**, 163 (1971).
10. Concentrations of fungal spores and pollen may vary from a few to several hundred per cubic foot, and occurrences have been reported to altitudes of 40,000 feet (1 foot ≈ 0.3 m).
11. W. M. Hess, D. J. Weber, J. V. Allen, J. L. Laseter, in preparation; J. L. Laseter, G. C. Lawler, C. H. Walkinshaw, J. D. Weete, *Phytochemistry*, in press; J. L. Laseter and J. D. Weete, *Science* **172**, 864 (1971).
12. W. R. Morrison and L. M. Smith, *J. Lipid Res.* **5**, 600 (1964).
13. J. L. Laseter and B. Dowty, unpublished results.
14. J. W. Coomber and J. N. Pitts, Jr., *Environ. Sci. Technol.* **4**, 506 (1970); R. H. Kummler and M. H. Bortner, *ibid.* **3**, 944 (1969).
15. R. H. Hites and K. Bieman, *Science* **178**, 158 (1972).
16. P. B. Merkel and D. R. Kearns, *J. Amer. Chem. Soc.* **94**, 7244 (1972).
17. The authors wish to thank C. S. Foote for helpful discussions. We are grateful to R. J. Dinus for providing pollen samples used in this study. G.W.G. was aided by grant DRG-1196 from the Damon Runyon Memorial Fund for Cancer Research for partial support of this work. This work was also supported in part by the Cancer Association of Greater New Orleans (J.L.L.) and by National Aeronautics and Space Administration grant NAS 9-12941 (J.L.L.).

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Collagen-Induced Platelet Aggregation: Involvement of an Active Glycopeptide Fragment (α1-CB5)

Abstract. *It is widely held that the tertiary structure of collagen is essential for induction of platelet aggregation. However, we have found that the purified α1 chain prepared from denatured chick skin collagen aggregates platelets. This activity appears to be confined to a distinct region of the molecule representing less than 4 percent of the length of the α1 chain. Of all of the cyanogen bromide peptides of the α1 chain tested, only one (α1-CB5) was active. This glycopeptide, devoid of any ordered tertiary structure, contains only 36 amino acids and one residue of* O-α-D-*glucopyranosyl-(1 → 2)*-O-β-D-*galactopyranosyloxy-(1 → 5)-lysine (Glc-Gal-Hyl). Blocking experiments strongly suggest that the Glc-Gal-Hyl is one of the structural determinants involved in collagen-induced platelet aggregation.*

Collagen-induced platelet aggregation is a phenomenon that is presently attracting considerable attention (*1–4*). Jamieson *et al.* (*2*) and Bosmann (*4*) have suggested that collagen-induced platelet aggregation is mediated by an interaction between a glycosyltransferase on the surface of the platelet membrane and a carbohydrate moiety of collagen. Therefore, it was of interest to examine in a systematic manner those structural features of collagen that might be involved in this process. In this report we describe the results we obtained from studies of collagens from various phyla and tissues. We also report, contrary to previously published papers (*1*, *3*), that denatured collagen from chick skin has platelet aggregating activity. Furthermore, one of the cyanogen bromide peptides (α1-CB5) (*5*) from chick skin collagen is active; none of the other of these peptides from chick skin are active, including those from the α2 chain. Results from blocking experiments with pure Glc-Gal-Hyl (*6*) and other sugars strongly suggest that this group is one of the structural determinants of the α1-CB5 peptide.

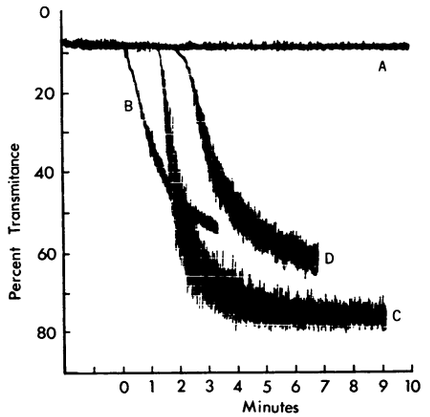

Fig. 1. Superimposed recordings of four typical results. (Curve A) *Metridium dianthus* insoluble collagen. This tracing was indistinguishable from all of the other negatively acting collagens listed in Table 1. (Curve B) Aggregation induced by adenosine diphosphate; (curve C) α1-CB5 chick skin peptide; and (curve D) α1 chain of chick skin collagen. Details, including amounts added, are given in (*7*) and in Table 1.

Platelet aggregation was measured in a Payton aggregometer, model 300A, with the use of a Goerz (Gelman) recorder, according to standard techniques (*7*). Typical recordings are shown in Fig. 1, and our results are summarized in Table 1. Under the test conditions employed, none of the