Evidence For The Photochemical Production of Superoxide Mediated By Saponified Chlorophyll*

Leland S. Jahnke** and Albert W. Frenkel

Department of Botany, University of Minnesota
St. Paul, Minnesota 55108

Received July 8,1975

Summary: The aerobic reduction of nitro blue tetrazolium by saponified chlorophyll, irradiated with red light, was inhibited by the addition of superoxide dismutase. Removal of molecular oxygen also inhibited the reduction of NBT, but the addition of superoxide dismutase did not further depress this anaerobic NBT reduction. These observations point to a chlorophyll mediated photoproduction of superoxide in the presence of molecular oxygen. EDTA is not required in this reaction, as is the case when free flavins mediate the photoproduction of superoxide ions.

Introduction

The superoxide anion can be generated in a number of diverse photochemical reactions. Ballou *et al.* (1) and Massey *et al.* (2) demonstrated the production of O_2^- upon reoxidation of photochemically reduced flavins; this ion also can be formed by illuminated chloroplasts (3,4). Gaffron (5), already in 1927, had observed a light dependent uptake of O_2, mediated by ethylchlorophyllide dissolved in iso-amylamine. Gaffron identified hydrogen peroxide and tentatively an amine peroxide, but he excluded the formation of chlorophyll peroxide.

---

*Supported in part by a grant from the National Institutes of Health, United States Public Health Service (AI-02218), and a grant from the Graduate School, University of Minnesota (to AWF).

**Present address: Department of Biological Sciences, Central Washington State College, Ellensburg, WA 98926.

/ Abbreviations: NBT, nitro blue tetrazolium; O_2^-, superoxide anion radical.

On the basis of these observations, it was considered worthwhile to test water soluble chlorophyll preparations for their ability to photochemically generate superoxide. Recent reviews by Fridovich (6, 7) provide extensive information on superoxide and superoxide disumtase and on the photochemical formation of superoxide (7).

Materials and Methods

Preparation of saponified chlorophyll: Eight healthy leaves of young spinach (*Spinacea oleracea L.*) or bean (*Phaseolus vulgaris L.*) plants were washed with cold distilled water and then macerated at high speed for 45 seconds in a pre-cooled blender containing 150 ml of 100% acetone and 1 g solid potassium bicarbonate. The resulting slurry was filtered through four layers of cheesecloth into a separatory funnel containing 50 ml petroleum ether (60-70^{o}) and 100 ml distilled water. Upon mixing, the water-acetone fraction was discarded and the petroleum ether layer was washed 8-10 times with distilled water thereby removing most of the acetone from the petroleum ether layer. The petroleum ether fraction was filtered through No. 1 Whatman filter paper to remove colorless precipitates. The chlorophyll fraction then was saponified by the addition of 5 ml - 7% potassium hydroxide in ethanol. The saponification was terminated after 2 minutes by the addition of 200 ml distilled water. The aqueous layer, containing chlorophyllide, was drawn off into another separatory funnel which contained 30 ml diethyl ether; thereupon it was titrated with 10% aq. acetic acid under constant agitation, care being taken not to go beyond pH 7.0 to prevent formation of phaeophorbides. The hydrolyzed chlorophyll (primarily chlorophyllide a and b) then was permitted to distribute itself between the ether and water

layers. The ethyl ether phase, containing most of the saponified chlorophyll was transferred to an evaporating dish to which 10 ml - 0.05 M potassium phosphate buffer (pH 7.8) had been added. The dish was placed into a vacuum desiccator and the ethyl ether drawn off by evacuation with a water aspirator. The aqueous stock solution of saponified chlorophyll was refrigerated under nitrogen in the dark, and was diluted in the reaction mixture to give a final absorbance at 645 nm of between 0.2 to 0.35.

Illumination system and absorbance measurements: The photochemical reduction of NBT was measured with a Cary model 14 dual beam spectrophotometer at 560 nm recorded on an extended scale (0 to 0.1 absorbance full scale). Actinic illumination was provided by a 400 watt Tungsten Halogen lamp focussed through 5 cm of water and a broad band red, far-red filter (cut-off at 600 nm). (Intensity: 1.5×10^5 to 6×10^5 ergs/cm^2). A green filter, transmitting between 485 and 595 nm, with maximum transmission at 535 nm, was placed in front of the photomultiplier tube to prevent fluorescent light from the reaction mixture to reach the detector.

Detection system for superoxide: The presence of the superoxide anion in aqueous media may be demonstrated indirectly by its ability to reduce cytochrome c (8), epinephrine (3,9), or NBT (10), and through the inhibition of these reductions by superoxide dismutase (4,6-10). The reduction of NBT and the inhibition of this reduction by superoxide dismutase was used here to assay for the formation of the superoxide anion. NBT was obtained from Sigma Chemical Co. and Erythrocuperin from Sigma Chemical Co.

Results and Discussion

The reduction of NBT in the light in the presence of saponi-

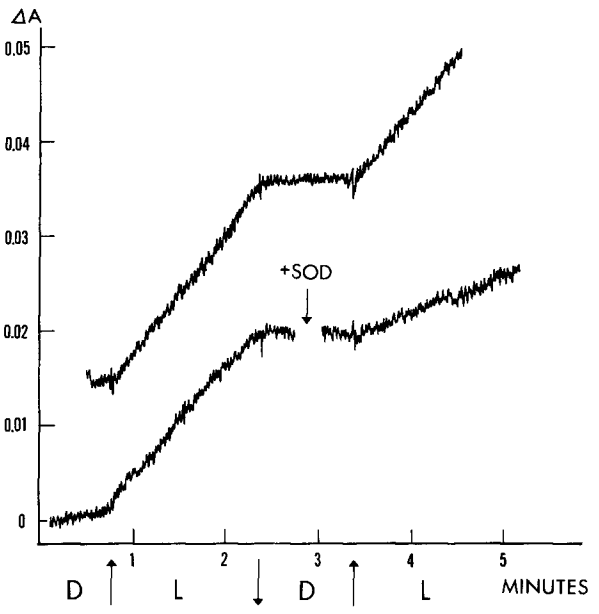

Fig. 1. Photoreduction of NBT by red light mediated by saponified chlorophyll under aerobic conditions. - Inhibitory effect of superoxide dismutase (SOD). Lower recorder tracing: SOD added in the dark (D) after the initial light (L) period. Upper recorder tracing: no SOD added. Absorbance changes (Δ A) measured at 560 nm. Reaction mixture described in legend for table I.

↑ Lights on. ↓ Lights off.

fied chlorophyll is easily observable (fig. 1, table I, II). No reduction of NBT was observed in the light or dark in the absence of saponified chlorophyll; furthermore, the reduction of NBT in the dark in the presence of saponified chlorophyll was negligible (fig. 1, table I). To test for the possible participation of superoxide in this light dependent reduction was examined. Added superoxide dismutase consistently produced an inhibition of the photoreduction of NBT in the presence of O_2 (fig.1, table I). After evacuation in anaerobic Thunberg cuvettes, the photoreduction of NBT was greatly inhibited and the addition of

Table I

Photoreduction of NBT by red light mediated by saponified chlorophyll under aerobic and anaerobic conditions.

		Superoxide dismutase mg	Rate ΔA_{560}/min	Inhibition Per cent
Aerobic	dark	0	0.0	
	light	0	0.011	
	light	0.13	0.004	64
Anaerobic	dark	0	0.0	
	light	0	0.004	
	light	0.13	0.004	0

The reaction mixture contained saponified chlorophyll (A-645 = 0.20) in 2.9 ml - 0.05 M potassium phosphate buffer (pH 7.8), to which NBT was added to give a final concentration of 0.14 mM. Total volume: 3.0 ml.

superoxide dismutase had no further effect on this low rate of light dependent NBT reduction (table I), consistent with the observations of Beauchamp and Fridovich (10).

The addition of EDTA is not required to bring about this light dependent reduction of NBT (table II). To check the validity of our experimental procedures, experiments with riboflavin as the light mediating pigment confirmed the requirement for EDTA in the latter reaction system (table II) (2,10).

It has not yet been established whether one of the chlorophyll derivatives or an impurity in this system acts as an electron donor in this light dependent reduction of oxygen to superoxide. The mechanism of this reaction will require further

Table II

The effect of EDTA on the light dependent reduction of NBT with saponified chlorophyll or with riboflavin as light absorbing pigment.

EDTA concentration	Saponified[1] chlorophyll	Riboflavin[2]
	ΔA_{560}/min	
5×10^{-4} M	0.046	0.072
1.25×10^{-4} M	0.040	0.025
0	0.048	0.001

Reaction misture: 2.9 ml - 0.05 M potassium phosphate buffer (pH 7.8) containing NBT to give a final concentration of 40 μM, plus additions as indicated. Final volume: 3.0 ml.

[1]Saponified chlorophyll, final absorbance (A_{645}) = 0.33.

[2]Riboflavin, final concentration: 7 μM.

elucidation. Many of the photooxidation and photoreduction reactions involving chlorophyll have been reviewed in some detail by Seely (11).

References

1. Ballou, D., Palmer, G., and Massey, V. (1969) Biochem. Biophys. Research Comm., 36, 898-904.
2. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., Sullivan, P. A., (1969) Biochem. Biophys. Research Comm., 36, 891-897.
3. Asada, K., and Kiso, K. (1973) Agr. Biol. Chem., 37, 453-454.
4. Elstner, E. F., and Kramer, R. (1973) Biochem. Biophys. Acta, 314, 340-353.
5. Gaffron, H. (1927) Ber. Dtsch. Chem. Ges. B., 60 2229-2238.

6. Fridovich, I. (1974) *in* Advances in Enzymology (Meister, A. ed.), Vol. 41, pp. 35-97, Intersience-Wiley & Sons, New York.
7. Fridovich, I. (1974) *in* Molecular Mechanisms of Oxygen Activation (Hayashi, O., ed.), pp. 453-477, Academic Press, New York.
8. McCord, J. M., and Fridovich, I. (1968) J. Biol. Chem., 243, 5753-5760.
9. Misra, H. P., and Fridovich, I. (1972) J. Biol. Chem., 247, 3170-3175.
10. Beauchamp, C., and Fridovich, I. (1971) Anal. Biochem., 44, 276-287.
11. Seely, G. R. (1966) *in* The Chlorophylls (Vernon, L. P. and Seely, G. R., eds.) pp. 523-568, Academic Press, New York.