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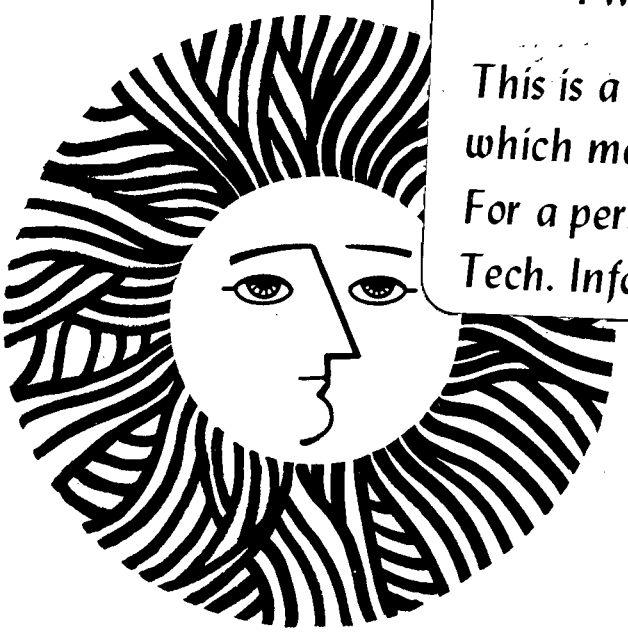

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PHOTOOXIDATIVE DAMAGE TO MAMMALIAN CELLS AND PROTEINS BY VISIBLE LIGHT

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During the last few decades mechanisms for the initiation and propagation of cellular damage by ultraviolet and ionizing radiation have received special attention. Photooxidative damage by visible irradiation ($\geq$ 400 nm) has received less attention in biological systems. Most investigations have focused on dye photosensitized reactions, rather than on chromophores found *in situ*, such as flavins and hemes, which normally act as cofactors in biological oxidation-reduction reactions. The visible light system can serve as an amplified model portraying oxidative stress in aerobic cells in that pro-oxidant substances (O_2^-, $^1O_2$, $\cdot OH$, H_2O_2, etc.) produced during normal metabolism, are easily generated under photooxidative stress. However, in itself the effect of visible light on biological systems has marked relevance in that it is a factor to which almost all organisms are exposed and must contend. In the present article we will review studies carried out in our laboratory on the effects of visible irradiation and O_2 in a variety of target systems ranging from cultured mammalian cells to purified catalase. We will relate these studies of photooxidative damage to a scheme for the propagation of intracellular damage (Fig. 1) which traces a number of the possible pro-oxidant and anti-oxidant pathways found in the cell.

Prooxidative reaction pathways. For visible light to affect cellular components it must first be absorbed. Hemes with a $\lambda_{max} \simeq 450$, $\varepsilon = 28{,}750\ M^{-1}cm^{-1}$ (for catalase heme) (1) and flavins with a $\lambda_{max} \simeq 445$, $\varepsilon = 12{,}500\ M^{-1}cm^{-1}$ (for FMN) (2) are the most probable sites of visible light absorption and oxygen activation. The excited sensitizer can chemically react directly with other compounds by a Type I process (3); Eqn 1.

$$\text{Sens} \xrightarrow{h\nu} {}^1\text{Sens} \longrightarrow {}^3\text{Sens} \xrightarrow{\text{substrate}} \text{H or } e^- \text{ transfer} \qquad (1)$$

Inasmuch as these photosensitizers serve as enzymatic cofactors, their excitation and reaction at the active site could lead to enzyme inactivation.

However, in the presence of oxygen, Type II reactions are likely to occur producing $^1O_2$ and O_2^- (Eqns 2 and 3):

$$^3\text{Sens} \xrightarrow{O_2} {}^1O_2 + \text{Sens}. \quad (2)$$

$$^3\text{Sens} \xrightarrow{O_2} O_2^- + \text{Sens}^+ \quad (3)$$

Both flavins and hemes have been shown to participate in Type I and II reactions.

As illustrated in Fig. 1, reactions of nonprotein and protein-bound coenzymes and metals with O_2 or with H_2O_2 can serve as routes of production of damaging oxygen radical species. H_2O_2 can be formed from the dismutation of O_2^- (Eqn 4):

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (4)$$

which can then form $\cdot OH$ by a one electron reduction process. For example, Equations 5 and 6

$$O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 \quad (5)$$

$$Fe^{+2} + H_2O_2 \longrightarrow \cdot OH + OH^- + Fe^{+3} \quad (6)$$

give reactions that have considerable experimental support (4), although the interaction of O_2^- with H_2O_2 may be Fe^{+3} mediated ($Fe^{+3} + O_2^- \rightleftharpoons O_2 + Fe^{+2}$). Other evidence supports the idea that O_2^- and H_2O_2 can give rise to $^1O_2$ as well as $\cdot OH$ by a reaction similar to equation 5 (5-7); however the actual mechanism of $^1O_2$ generation is unknown.

Hydroxyl radical ($\cdot OH$), with a reaction rate of $k \approx 10^9 M^{-1}sec^{-1}$ for most organic compounds, is probably the most reactive and damaging species found in biological systems. Singlet oxygen, although more selectively reactive than $\cdot OH$, reacts rapidly with compounds with amine groups or double bonds such as unsaturated fatty acids, amino acids, and nucleic acids. Some of the possible pro-oxidant pathways beginning with O_2, O_2^- and $\cdot OH$ are depicted in Fig. 1; these represent the most probable pathways of oxidative damage in mitochondria.

Antioxidative reaction pathways. The prevention of damage in cellular systems can be considered a two-level process. First, the cell would minimize the production and availability of pro-oxidant factors and substances. The compartmentalization of pro-oxidant enzymes in organelles such as mitochondria, and the sequestering of transition metals by specific proteins are examples of this level of defense. The second level of defense involves the scavenging and neutralization of pro-oxidants. These anti-oxidant pathways in mitochondria are depicted by darker lines in

Fig. 1, and show the quenching of $^1O_2$ by vitamin E, the dismutation of O_2^- by superoxide dismutase (SOD), the conversion of H_2O_2 to $H_2O_2 + O_2$ by glutathione peroxidase, and the scavenging of free radicals by antioxidants such as ascorbic acid in the aqueous phase and α-tocopherol in the lipid phase. These two antioxidants can work synergistically in preventing membrane damage (8). We will attempt to relate these proposed pathways of cellular damage to the patterns of damage actually found in our visible light studies.

Results

Cultured mammalian cells and isolated hepatocyte studies. There have been several recent reports of the damaging effects of visible light exposure (>400 nm) on various microorganisms (9,10) and cultured mammalian cells (11-13). We have reported that human diploid cells on exposure to visible light (14) and oxygen (>10%) (15) lost the ability to proliferate, while ultrastructural studies showed the presence of numerous damaged mitochondria in the illuminated cells (16). WI-38 human fibroblasts show a gradual decline in growth rate with exposure to visible light with younger cells (14) being more susceptible to photokilling, with partial protection observed on the addition of dl-α-tocopherol (vitamin E).

Studies with isolated hepatocytes (17) have led us to a characterization of the pattern of intracellular damage. Exposure of rat hepatocytes to visible light (400-720 nm) of intensity 300 mW/cm^2 over a 12 hr period results in a selective pattern of subcellular damage (Fig. 2). Virtually no release of lactate dehydrogenase or uptake of trypan blue was observed. The plasma membrane enzymes 5'-nucleotidase and β-leucyl naphthylamidase were only slightly inactivated. The plasma membrane thus appears highly resistant to damage.

Under the same conditions, however, other intracellular enzymes were markedly inactivated. Mitochondrial damage was indicated by a decrease in latency of chtochrome c oxidase and destruction of various enzyme activities in the following order: succinic dehydrogenase > succinate oxidase > glutathione peroxidase > NADH-cytochrome c oxidase > cytochrome c oxidase (Fig. 2). This pattern of inactivation is similar to the one found upon light exposure of isolated mitochondria (18), suggesting that continued studies with the in vitro system are indeed warranted. Lysosomal damage was also extensive, as indicated by the loss of latency and activity in the enzymes cathepsin c, acid phosphatase and N-acetyl-β-glucosaminidase. Some evidence of damage to microsomal membranes was indicated by a decline in glucose-6-phosphatase activity. The most light-sensitive enzyme was found to be catalase, an enzyme associated with the peroxisomal fraction. Another peroxisomal enzyme, urate oxidase, was relatively less suspectible to light damage. It is interesting to note that two

of the most light sensitive enzymes, catalase and succinate dehydrogenase, contain a heme and a flavin moiety, respectively, at their active sites. Although further studies demonstrated the oxygen dependence of their inactivation, scavengers of $^1O_2$, O_2^-, and $\cdot OH$ failed to protect, indicating that damage occurs at the active site itself. Significantly, complete protection was afforded by substrates in both cases (18,19). Inactivation of other enzymes lacking photosensitive cofactors presumably occurs by more indirect reaction pathways. In addition to inactivation of enzymes, destruction of membrane lipids was indicated by lipid peroxidation measurements.

Attempts to prevent visible light damage of succinate dehydrogenase showed that the addition of succinate + KCN was maximally effective. EDTA was effective in preventing succinate dehydrogenase inactivation and lipid peroxidation as measured by the TBA test. It is well known that succinate + KCN and EDTA both can act as reductants. Reducing conditions may protect against visible light damage by reducing flavins, which cannot act as efficient photosensitizers since they absorb very little visible light (ε = 870 $M^{-1}cm^{-1}$ for FMN at 445 nm (2)). dl-α-Tocopherol and butylated hydroxytoluene appeared effective against lipid damage but only the latter antioxidant appreciably affected the pattern of enzyme (succinate dehydrogenase) inactivation. Hepatocytes isolated from rats fed with a vitamin E-deficient diet showed a marked increase in susceptibility to lipid peroxidation compared to rats fed a vitamin E-supplemented diet.

Isolated mitochondria. Chance et al. (20) have shown that the absorption spectrum of whole cells is qualitatively similar to that of isolated mitochondria. Thus, visible light absorption by cells may involve mitochondrial flavins or hemes as endogenous photosensitizers. In isolated mitochondria we found that the inner energy transducing membrane can be extensively photooxidatively damaged. Such bioenergetic parameters as maintenance of proton and electrical potential gradients associated with coupling to ATP synthesis were progressively inhibited following exposure of isolated mitochondria to light, whereas samples kept under identical conditions for 12 hr in the dark showed no such changes (18). These bioenergetic parameters show an interesting pattern of change. Almost immediately after light exposure, uncoupling is detected as shown by a stimulation of respiration, loss of ATP synthesis, and increased ATP hydrolysis, all of which indicate uncoupling of electron transport from energetic gradients. Membrane potential changes seem largely dependent upon protein inactivation and occur at an earlier time period than the surface potential changes. Membrane potentials are unaffected by lipid-soluble antioxidants, whereas the surface potential changes occur at a later time exposure and are partially reversed by antioxidants, indicating that membrane lipids contribute to the surface electrical potential when measured with amphipathic spin labeled probes.

Electron transport components examined by spectrophotometric and EPR methods identified the major target of photooxidative attack as the flavin dehydrogenases (18). These complexes contain in addition to flavins, iron sulfur (FeS) clusters and active SH groups as important functional components and these also showed evidence of destruction. Quinones, some of which are bound to dehydrogenase complexes or which exist as a quinone pool, also showed photoinactivation. However, all of the heme-containing cytochromes of the bc complex and of the cytochrome oxidase complex, and cytochrome c showed no inactivation despite their visible light absorption. Hence, flavin-, FeS- and quinone-mediated photooxidative processes appear involved in initiation and propagation of damage.

Experiments with water-soluble spin labels added to mitochondrial inner membranes have demonstrated that photodestruction (not reduction) of spin signal occurs with an action spectrum coinciding with flavins. This suggests that some of the membrane protein-bound flavin coenzyme is released following light/O_2 exposure, and that released flavin radicals can be detected in solution. Released flavins could initiate photosensitized reactions that would accelerate the photoinactivation process. Indeed, our previous studies (14) indicate that maximum photokilling of WI-38 cells occurred by illumination in the wavelength region of maximum absorption by flavins. This evidence supports the idea that the cytochromes, despite their possession of heme groups, do not mediate visible light damage by acting as photosensitizers. Propagation of damage likely involves radicals in both the lipid and aqueous phases. Evidence of peroxidized lipids can readily be discerned, but this can largely be prevented by adding membrane-soluble antioxidants such as vitamin E or butylated hydroxytoluene which apparently prevent lipid peroxidation but leave the pattern of enzyme damage largely unaffected. Other studies with submitochondrial particles demonstrated an oxygen dependence for photoinactivation of all mitochondrial enzymes tested (18).

Catalase photoinactivation. Studies demonstrated that the inactivation of catalase is oxygen dependent and can be prevented by substrates (100 μM methanol or ethanol), while antioxygenic substances in general (sucrose for •OH, histidine for $^1O_2$, and 10 μg/ml superoxide dismutase for O_2^-) have little protective effect (see Table I) (19). Superoxide dismutase does, however, partially protect purified catalase added to the mitochondrial fraction, indicating that O_2^- produced during photooxidation of the mitochondrial fraction can inactivate catalase, probably by converting the active Compound I form to the inactive Compound II. Since catalase is a key enzyme in H_2O_2 metabolism, the importance of its inactivation both in in vivo and in vitro to the overall metabolic protective capacity of cells needs to be carefully characterized to identify its significance in the time sequence of damaging events. It is interesting to note that light with a wave-

length responsible for maximal catalase inactivation, has been implicated as having a major role in photooxidative damage of cultured cells (21).

Discussion

Photooxidative damage pathways. Based upon the results with cells and mitochondria, it is possible to construct a scheme to account for the various possible pathways of initiation and propagation of damage to lipids and proteins by photooxidative processes. Our results suggest a definite order of events in biological photooxidation processes. First, one sees a rapid inactivation of enzymes containing light sensitive cofactors at the active site, a process that is oxygen dependent and apparently not susceptible to inhibition by exogenously added scavengers of active oxygen species but which can be totally prevented by the addition of substrates. Examples of such enzymes are succinic dehydrogenase (FAD), NADH dehydrogenase (FMN) and catalase (heme). As their inactivation requires oxygen this implies that a type II process occurs at the photosensitive cofactor, which produces an active form of oxygen ($^1O_2$, O_2^-, etc.) which reacts with a susceptible group at the active site, causing damage and loss of activity. The production of such oxidative species by bound photosensitizer would obviously have much less effect on areas distal from the active site. However, photooxidative damage eventually can cause the release of the photosensitizer group, which could then cause a far more generalized pattern of damage in the cell.

The release of free photosensitizers, such as flavins, would be expected to act as $^1O_2$ generators (Eqn 2). Also, autooxidation of flavins and certainly quinones generate O_2^- in mitochondria (as in Eqn 3). The degree to which the "Haber-Weiss reaction" (Eqn 5) and Fenton reaction (Eqn 6) occur *in vivo* is still uncertain. Currently, experiments are underway in several laboratories to obtain quantitative information on •OH radical generation by Equations 5 and 6 using, in particular, spin trapping methods. Thus, the characteristic •OH radical adduct of the spin trap DMPO (5,5-dimethyl-1-pyrroline-N-oxide) can be shown to occur in mitochondria exposed to visible light (24) but it is still unclear from which stage in the propagation of damage that these •OH radicals arise.

In mitochondria our data indicate that a substantial release of free flavins does occur (18). It would be primarily from such free photosensitizers that damage to enzymes without photoactive groups, and lipid peroxidation, would occur. The propagation of damage through the initiation of oxidized lipid peroxides and alkoxy radicals is also an area in which quantitative information is required. Methods are now becoming available that use artificial lipid vesicles (22) and monolayer systems (23) to investigate the rate, extent and nature of free radical mediated oxidative reactions in lipids which can determine how damage spreads

in the vertical and lateral modes through membranes. Propagation of cellular damage could occur in both the cytosol and lipid phases of the cell, acting synergistically in causing the total damage profile. Figure 1 indicates the multifarious damage processes and interactions that may occur after the release of free photosensitizers. A pattern for the chemical defense against photooxidative damage can be recognized, and knowledge of prooxidant and antioxidant pathways may help in devising nutritional means which could afford increased protection against oxidative damage. At the present time, however, it is clear that many unanswered questions remain as to both the existence and importance of the many possible oxidative mechanisms of biological damage processes in vivo.

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Table I. Photoinactivation of Mitochondrial Fraction Catalase. Specificity of Protection[a]

Conditions	Activity (per cent)	
	Dark	Light
0 time	100.0 ± 0.4	100.0 ± 0.4
Complete system	89.3 ± 1.4	23.6 ± 2.5
+ Superoxide dismutase (10 μg/ml)	85.7 ± 1.3	22.4 ± 0.5
+ Histidine (1 mM)	98.7 ± 0.6	31.4 ± 2.1
+ Ethanol (100 μM)	104.2 ± 9.4	96.6 ± 2.7

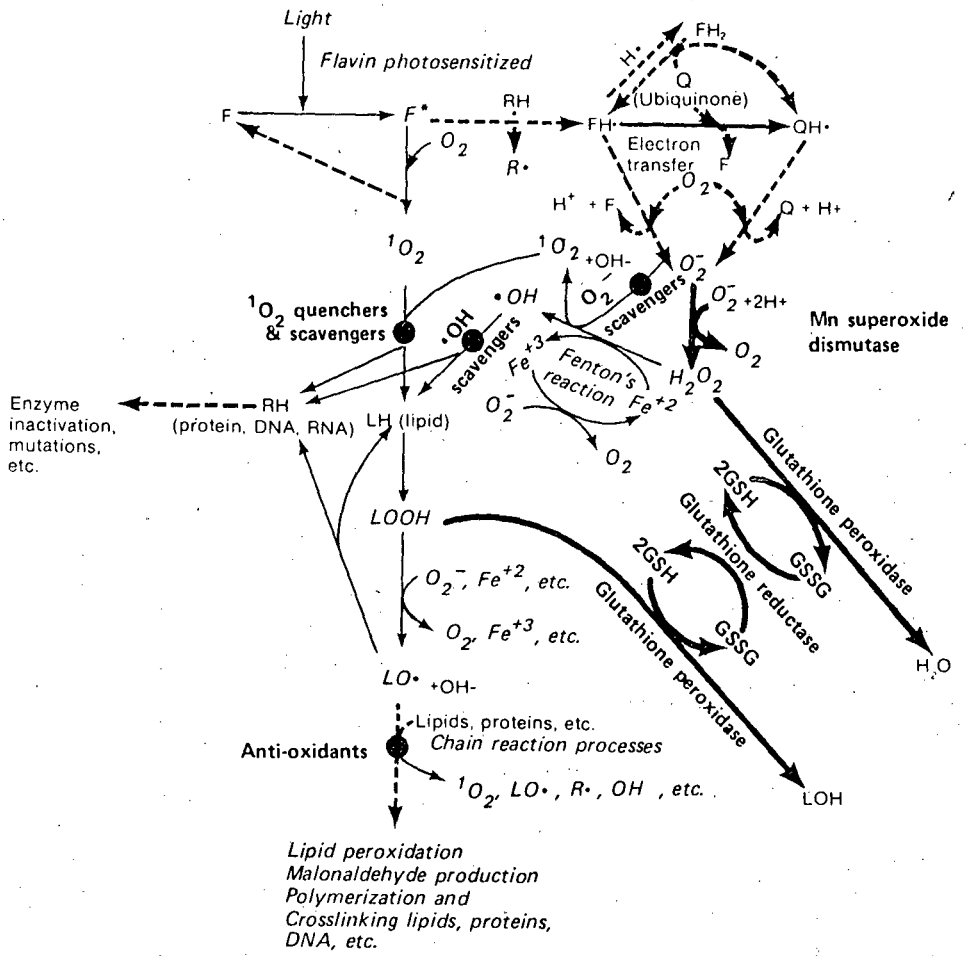
[a] The complete incubation system contained isolated mitochondria resuspended in 0.25 M sucrose to a final concentration of 0.5 mg/ml protein. Light samples were exposed to an incandescent light source (a bank of 50 watt G.E. reflector bulbs) with an intensity of about 15 mW/cm^2 for 2 hr. Six ml samples were incubated in a slowly shaking water bath at 34° C; small aliquots were removed for catalase assays. Samples run in duplicate.

Legends for figures

Fig. 1 - Possible pathways of oxidative damage in mitochondria.

Fig. 2 - Enzyme photoinactivation in isolated hepatocytes (17).

Hepatocytes in 0.25 M sucrose at 8-10° C were illuminated in a shaking water bath at 300 mW/cm^2 visible light (400-720 nm).



Pro-oxidant reactions, reactants

Pro-oxidants — Fe^{+2}, Cu^{+2}, free flavins and hemes, light

Anti-oxidant reactions, reactants

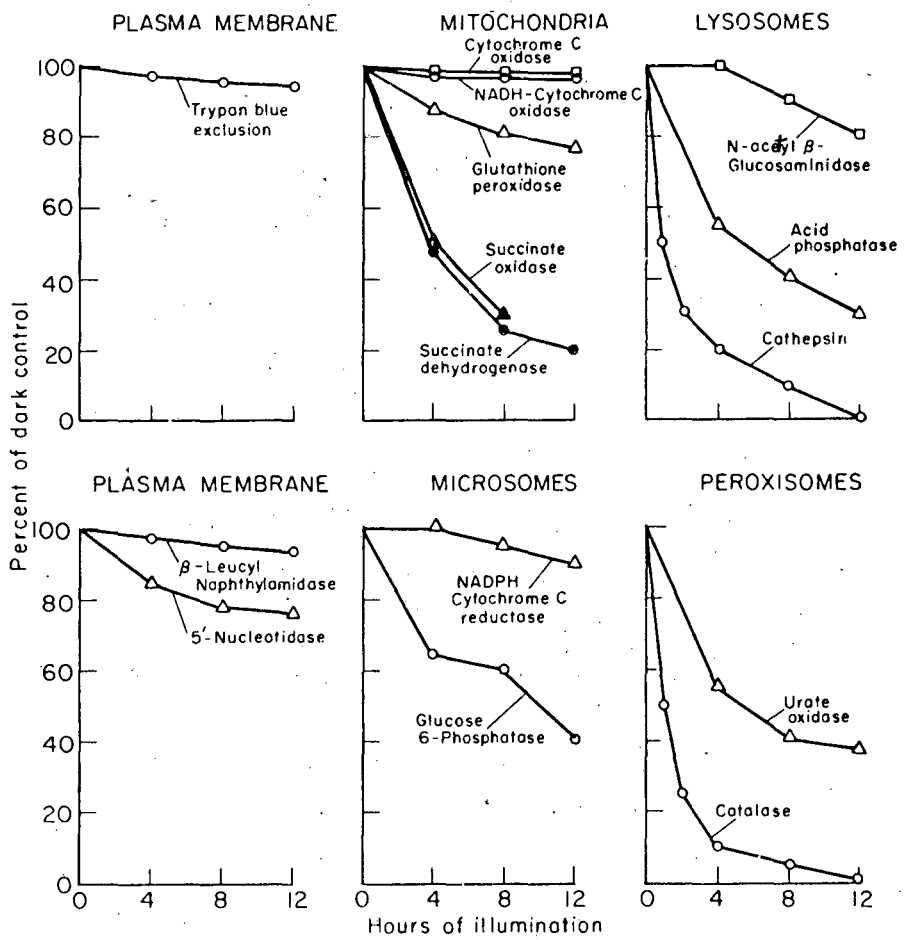
Anti-oxidants — α-tocopherol, glutathione, ascorbic acid

Superoxide Scavengers — ascorbic acid, glutathione, cytochromes, α-tocopherol

Singlet Oxygen Scavengers and/or Quenchers — β-carotene, α-tocopherol, histidine, tryptophan, guanine, uric acid

Hydroxyl Radical Scavengers — most organic compounds
$k \cong 10^{10}\ M^{-1}s^{-1}$

Other reactions


PLASMA MEMBRANE
MITOCHONDRIA
LYSOSOMES
Cytochrome C oxidase
NADH-Cytochrome C oxidase
Glutathione peroxidase
Succinate oxidase
Succinate dehydrogenase
Trypan blue exclusion
N-acetyl β-Glucosaminidase
Acid phosphatase
Cathepsin
PLASMA MEMBRANE
MICROSOMES
PEROXISOMES
β-Leucyl Naphthylamidase
5'-Nucleotidase
NADPH Cytochrome C reductase
Glucose 6-Phosphatase
Urate oxidase
Catalase
Percent of dark control
100
80
60
40
20
0
0
4
8
12
Hours of illumination

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