Singlet Oxygen–Induced Arrhythmias
Dose- and Light-Response Studies for Photoactivation of Rose Bengal in the Rat Heart

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In a study of aerobically perfused rat hearts, the in situ photoactivation (530–590 nm) of rose bengal (a process that leads to the production of singlet oxygen and superoxide) has been shown to lead to the rapid development of electrocardiographic abnormalities and arrhythmias. With rose bengal concentrations of 1,000, 500, 250, 100, and 50 nmol/l (n=6/group), photoactivation (3,600 lx) led to electrocardiographic changes (inversion of the T wave, Q-T prolongation, or both) after 3.8±0.9, 4.5±0.7, 11.8±2.1, 24.8±3.9, and 65.3±6.0 seconds, respectively; ventricular premature beats occurred in 100% of hearts after 0.5±0.2, 1.1±0.3, 2.2±0.7, 4.4±0.8, and 6.6±1.2 minutes, respectively. Ventricular tachycardia occurred in 83%, 83%, 83%, 67%, and 50% of hearts after 2.1±0.2, 2.1±0.4, 2.8±0.7, 5.7±2.0, and 11.2±1.9 minutes, respectively, and complete atrioventricular block in 100%, 100%, 100%, 100%, and 67% of hearts after 3.8±0.7, 6.5±1.0, 5.5±0.9, 13.8±1.0, and 14.1±0.9 minutes, respectively. With a fixed concentration (250 nmol/l) of rose bengal, similar light-response relations were observed. Photoactivation of rose bengal had no effect on heart rate but caused a transient (0–4 minutes) vasodilation followed by a progressive vasoconstriction. In further studies in which rose bengal was washed out for 10 minutes before photoactivation, several arrhythmias still developed, indicating that rose bengal binds strongly to tissue and acts as a cellular level rather than in the vascular compartment. To assess the reversibility of rose bengal–induced effects, hearts (n=6/group) were perfused with rose bengal (250 nmol/l) for 1, 2, 4, 6, and 20 minutes followed by perfusion in the dark for 19, 18, 16, 14, and 0 minutes, respectively. During dark perfusion, the incidence of arrhythmias declined and any decrease in coronary flow was reversed. However, analysis of contents of adenosine triphosphate, creatine phosphate, lactate, and creatine kinase leakage indicated the occurrence of severe injury that did not abate on termination of photoactivation. Finally, although many arrhythmias developed before the onset of vasoconstriction, the reduction in flow with consequent ischemia was shown to exacerbate vulnerability to arrhythmias. In conclusion, short-lived reactive oxygen intermediates such as singlet oxygen and superoxide, which are produced during the photoactivation of rose bengal, can cause rapid and major damage to the heart and its function. (Circulation 1989;80:1432–1448)

Photoactivation of rose bengal, by illumination with green light, results in the production of reactive oxygen intermediates (predominantly singlet oxygen with some superoxide radical). We have previously reported that illumination of isolated rat hearts, being perfused aerobically with rose bengal, results in the rapid development of electrocardiographic (ECG) changes (discernible after less than 15 seconds) that lead to the development of various arrhythmias (ventricular premature beats [VPBs] within 2.2±0.7 minutes, ventricular tachycardia [VT] within 2.8±0.7 minutes, and complete atrioventricular [AV] block within 5.5±0.9 minutes). These deleterious effects occur under aerobic conditions in the absence of ischemia or reperfusion.

Our original studies with rose bengal were stimulated by those of Borgers et al the first to exploit the photodynamic properties of rose bengal in the context of myocardial injury. Their work demonstrated that, when illuminated in a medium containing rose bengal (50 nmol/l), myocytes rounded up and developed severe ultrastructural injury. Further studies showed that the injury could be attenuated by cinnarizine, flunarizine, and lidoflazine but not by slow calcium channel blockers.

We and others have suggested that reactive oxygen intermediates (e.g., superoxide and hydroxyl
radicals) produced in “bursts” during ischemia and reperfusion may cause membrane injury that can lead to the genesis of severe arrhythmias. Our initial observations with rose bengal would lend further support to this concept. In consequence, we have now undertaken detailed dose-response and light-response studies with rose bengal in the isolated aerobically perfused rat heart to learn more about the novel effects of this fluorescein derivative. We also investigated whether the injury induced by rose bengal photoactivation is reversible and whether the electrophysiologic changes are accompanied by any metabolic changes.

**Methods**

**Animals and Reagents**

Male rats (220–280 g body wt) were obtained from Bantin and Kingman. Purified rose bengal (3′,4′,5′,6′-tetrachloro-2,4,5,7-tetraiodofluorescein; molecular weight, 1,018) was obtained from Aldrich (UK). The animal studies conformed to the guiding principles of the American Physiological Society.

**Perfused Heart Preparation and Exclusion Criteria**

Animals were anesthetized with diethyl ether and injected with sodium heparin (200 units i.v.). Thirty seconds later, hearts were excised and placed in cold (4°C) perfusion medium until contraction had ceased (approximately 15 seconds). Each heart was then cannulated via the aorta and perfused at constant perfusion pressure equivalent to 100 cm H₂O.

All hearts were subjected to an initial 10-minute equilibration period during which they were perfused in the absence of rose bengal. At the end of this period, heart rate and coronary flow were measured. Hearts were excluded from the study if they exhibited any disturbances of rhythm during the 10 minutes. Also excluded were hearts that, at the end of this period, had heart rate less than 280 beats/min or more than 420 beats/min and/or coronary flow less than 8.5 ml/min or more than 15 ml/min. The proportion of hearts excluded in this study was less than 5% (1–2% on account of disturbances of rhythm and 1–2% on functional grounds).

**Perfusion Fluids**

Bicarbonate buffer (pH 7.4, 37°C) containing (mmol/l) NaCl, 118.5; NaHCO₃, 25.0; KCl, 3.2; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 1.2; and glucose, 11.1, was the standard perfusion fluid. This was gassed with 95% O₂-5% CO₂. Before use, all perfusion fluids were filtered (pore size, 5 μm).

To define the dose-response characteristics of rose bengal, five concentrations (50, 100, 250, 500, and 1,000 nmol/l) were studied. The rose bengal was added to the perfusion medium before filtration. The reservoir containing these solutions, as well as all perfusion lines, were covered with aluminum foil to prevent interference by ambient light.

**Fiber Optic Illumination System**

As shown in Figure 1, a heart illumination system (available from Cardiovascular Research Equipment Facility, The Rayne Institute, St. Thomas’ Hospital) was constructed in 200 fiber optic cables (1-mm diameter each) arranged in a uniform array around a specially constructed, temperature-regulated heart perfusion chamber. Each cable (100 cm long) delivered green light (530–590 nm) from two air-cooled 90-W dichroic light sources equipped with appropriate heat and interference filters. The intensity of light delivered in the heart chamber was controlled by a rheostat and, in these experiments, could be varied between 350 and 6,600 lx. Preliminary studies revealed that, at full intensity, illumination had no significant effect on myocardial temperature.

**Experimental Time Course**

**Dose-response studies.** At the end of the equilibration period, the perfusion fluid was changed to one containing 50, 100, 250, 500, or 1,000 nmol/l rose bengal, and perfusion was maintained for a further 5 minutes. Perfusion with rose bengal was maintained for a further 20 minutes during which hearts (n=6/group) were continuously illuminated with light of constant intensity (3,600 lx).

**Light-response studies.** At the end of the equilibration period, the perfusion fluid was changed to one containing 250 nmol/l rose bengal, and perfusion was maintained for a further 5 minutes. Perfusion with rose bengal was maintained for a further 20 minutes during which the hearts (n=6/group) were continuously illuminated with varying intensities of light (350, 1,400, 3,600, or 6,600 lx).

**Washout studies.** At the end of the equilibration period, the perfusion fluid was changed to one containing 250 nmol/l rose bengal, and perfusion was maintained for a further 5 minutes. The perfusion fluid was then switched back to rose bengal-free buffer for a further 10 minutes. Perfusion with rose bengal-free buffer was then continued for another 20 minutes during which the hearts (n=6) were continuously illuminated with light (3,600 lx).

**Reversibility studies.** The protocol used for these studies is depicted in Figure 2A. At the end of the equilibration period, the perfusion fluid was changed to one containing 250 nmol/l rose bengal, and perfusion was maintained for 5 minutes. Perfusion with rose bengal was then maintained for a further 1, 2, 4, 6, or 20 minutes (n=6/group) during which the hearts were continuously illuminated (3,600 lx). After the light was switched off, perfusion with rose bengal solution was continued in the dark for the time remaining up to 20 minutes.

**Metabolic studies.** The protocol used for these studies is depicted in Figure 2. At the end of the equilibration period, the perfusion fluid was changed to one containing 250 nmol/l rose bengal, and perfusion was maintained for 5 minutes. Perfusion with
rose bengal was then maintained for a further 1, 2, 4, 6, or 20 minutes (n=6/group) during which hearts were continuously illuminated (3,600 lx). At the end of the period of illumination, hearts were either freeze-clamped immediately (Figure 2B) or after perfusion with rose bengal–containing buffer had continued in the dark for the time remaining up to 20 minutes (Figure 2A). Values were compared with those from control hearts (n=6/group) that were freeze-clamped after 5 minutes of perfusion with rose bengal without light or freeze-clamped after 20 minutes perfusion with or without rose bengal in the absence of light.

**Indexes Measured**

**Coronary flow and heart rate.** Coronary flow was measured throughout each experiment by a timed collection of coronary effluent. Heart rate was also recorded throughout the experimental period. When the protocol required the photoactivation of rose bengal with consequent development of arrhythmias, heart rate was assessed only during the period preceding the onset of the arrhythmia (i.e., generally within 1–6 minutes of illumination).

**Identification and quantification of arrhythmias.** Silver ECG electrodes were attached to the ventricular apex, the appendage of the right atrium, and the aortic cannula in all hearts. Both ventricular and atrial high resolution ECGs were recorded throughout the experimental period and analyzed for early changes in ECG morphology and the time to onset of these changes; the incidence and time to onset of arrhythmias developing before the onset of complete AV block, including VPBs, VT, and ventricular fibrillation [VF]; and the incidence and time to onset of complete AV block. Arrhythmias were defined and quantified in accordance with the Lambeth Conventions.10

**Biochemical studies.** The tissue contents of adenine triphosphate (ATP), creatine phosphate (CP), and lactate were determined by standard enzymatic assay in hearts freeze-clamped after various durations of illumination. Coronary effluent was collected throughout the experiment for the measurement of lactate and creatine kinase leakage with standard biochemical procedures.

**Statistics**

Statistical analysis was based on the guidelines described by Wallenstein et al.11 Gaussian-distributed variables were expressed as the mean±SEM. A one-way analysis of variance was
first carried out to test for any differences between the mean values of all groups. If a difference was established, the groups were compared by Tukey’s test. An analogous procedure was followed for binomially distributed variables (e.g., incidence of VPBs). An overall $\chi^2$ test for a $2 \times n$ table was

**Figure 2.** Protocols used to study the reversibility of the effect induced by rose bengal photoactivation. Before the events shown in the figures, hearts were subjected to 10 minutes of perfusion with rose bengal–free buffer followed by 5 minutes of perfusion with rose bengal (250 nmol/l) in the absence of light. The perfusion with rose bengal was then continued in the presence of light (3,600 lx) for various durations (1, 2, 4, 6, or 20 minutes), after which the light was switched off and (Panel A) the perfusion with rose bengal–containing buffer was continued in the dark for the remaining period up to 20 minutes, at which time the hearts were freeze-clamped, or (Panel B) the hearts were freeze-clamped immediately.
constructed, followed by a sequence of $2 \times 2 \chi^2$ tests with the Yates' correction to compare individual groups. A $p$ value of less than 5% was considered significant.

Results
Nature of Electrophysiologic Disturbances Induced by Rose Bengal Photoactivation

Figure 3A shows ECG recordings obtained at the end of the equilibration period, just before illumination, and in the seconds and minutes after the onset of illumination (3,600 lx) in rat hearts perfused aerobically with or without rose bengal (1,000 nmol/l) added to the perfusate. In the presence of rose bengal but in the absence of light, the morphology of the ECG remained essentially unchanged. In contrast, after the onset of illumination, changes in morphology could be discerned in as short a time as 3.8±0.9 seconds (i.e., less than 20 beats). These changes, which were characterized by the inversion of the terminal portion of the T wave and/or the progressive prolongation of the Q-T interval, then deteriorated to arrhythmias. Figure 3B shows the nature of the arrhythmias developing at various times after the onset of illumination. In this experiment, VPBs appeared after only 0.5±0.2 minutes, multiple bursts of VT (note the short duration) after only 2.1±0.2 minutes, and complete AV block after only 3.8±0.7 minutes. In control hearts exposed to rose bengal alone or to illumination alone, no ECG changes or arrhythmias were observed.

Dose-Response Studies

After 10 minutes of perfusion with a rose bengal–free buffer, hearts (6/group) were perfused for 5 minutes with rose bengal (50, 100, 250, 500, or 1,000 nmol/l) before being illuminated (3,600 lx) for a further 20 minutes. Coronary flow and ECG were recorded throughout the experimental period, and heart rate was measured until the onset of the first arrhythmia.

Heart rate and coronary flow. Figure 4A shows that at all doses studied (until the onset of an arrhythmia), rose bengal had no effect on spontaneous heart rate measured at the end of the period of equilibration, during the 5-minute period of perfusion with a rose bengal–containing buffer in the absence of light, or during the period of illumination.

**Figure 3.** Electrocardiographic recordings obtained from hearts perfused with buffer containing rose bengal (1,000 nmol/l) and illuminated at 3,600 lx. Panel A: Electrocardiographic recordings obtained just before and after illumination to show the rapid changes in morphology induced by light in hearts perfused with rose bengal. Panel B: Electrocardiographic recordings obtained after several minutes of illumination, showing various arrhythmias (VPBs, VT, and complete AV block).
However, on illumination, a dose-dependent and time-dependent decline in coronary flow was observed. Thus, with 1,000 nmol/l, coronary flow fell to 20%–30% of the baseline value within 10.8±0.6 seconds. In all hearts, except those in the lowest-dose group, an arrhythmogenic period developed after 0.5±0.2 seconds. At the other doses, a complete AV block occurred after 0.9±0.1 seconds, which increased to 67% at 100 nmol/l and to 83% at all other doses.

Changes in ECG morphology. Figure 5A shows the mean time to onset of the first detectable change in the ECG morphology (the inversion of the terminal portion of the T wave and/or the prolongation of the Q-T interval), the duration decreased with increasing doses of rose bengal. At the lowest concentration (50 nmol/l), no changes were detectable until 65.3±6.0 seconds after the onset of illumination; however, when the dose was doubled, this duration declined to 24.8±3.9 seconds, and at 1,000 nmol/l the changes could be detected within 3.8±0.9 seconds of the onset of illumination (i.e., less than 20 beats).

Arrhythmias. The changes in ECG morphology were rapidly followed by the development of arrhythmias, usually VPBs. Figure 5B shows the relation between the dose of rose bengal and the mean time to onset of VPBs, VT, and complete AV block. At the highest dose, VPBs developed after 0.5±0.2 minutes. Even at the lowest dose of rose bengal, VPBs were detected after only 6.6±1.2 minutes, VT after 11.2±1.9 minutes, and complete AV block after 14.1±0.9 minutes.

Table 1 shows the relation between the dose of rose bengal and the incidence of VPBs, VT, and complete AV block. VPBs developed in all hearts at all doses, and complete AV block occurred in all hearts except those in the lowest-dose group, in which the incidence was 67%. The incidence of VT at the lowest dose was 50%; this increased to 67% at 100 nmol/l and to 83% at all other doses. Also shown in Table 1 are the relations between the dose of rose bengal and the duration of the longest episode of VT and the number of episodes of VT before the development of complete AV block. In contrast to the other results, no dose-response relation was apparent, with similar short repetitive bursts of VT developing at all doses (mean duration of the longest episode ranging from 0.9±0.1 seconds with 250 nmol/l to 2.1±1.0 seconds with 500 nmol/l).

Light-Response Studies
To assess the relation between the arrhythmogeneity of rose bengal photoactivation and the inten-
sity of illumination, hearts (6/group) were perfused aerobically for 10 minutes in the absence of rose bengal. This was followed by a 5-minute perfusion with rose bengal (250 nmol/l) in the absence of light and then by 20 minutes of perfusion with rose bengal in the presence of light. The intensity of light in the heart chamber was set at 350, 1,400, 3,600, or 6,600 lx. Coronary flow and the ECG were recorded throughout the experiment, and heart rate was measured until the onset of an arrhythmia.

Heart rate and coronary flow. As shown in Figure 6A, heart rate (measured during the equilibration period, the 5 minutes of perfusion with rose bengal-containing buffer in the absence of light, and the illumination period up to the onset of an arrhythmia) was unaffected by illumination at any intensity studied.

As shown in Figure 6B, coronary flow was constant during the perfusion periods preceding illumination. On illumination, flow fell in an intensity-dependent and time-dependent manner. Thus, with maximal illumination (6,600 lx), flow fell by 96% (from 11.5±0.2 to 0.5±0.1 ml/min) over the 20-minute period of photoactivation. This decline was less severe in the 3,600-lx group (to 2.0±0.4 ml/min after 20 minutes) and the 1,400-lx

<table>
<thead>
<tr>
<th>Rose bengal dose (nmol/l)</th>
<th>Incidence of arrhythmias (%)</th>
<th>Mean duration of longest VT episode (sec)</th>
<th>Mean number of VT episodes (/min)</th>
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<tr>
<td></td>
<td>VPBs</td>
<td>VT</td>
<td>Complete AV block</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>50</td>
<td>67</td>
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Light intensity, 3,600 lx.
Values given are mean±SEM of hearts that exhibit arrhythmia or incidence out of the group of six.
A group with coronary flow illumination. 7A, ure in nation 6,600 ml/min and that illumination there were no than 37.6° myocardial on change of ment morphology were the between to fell the lowest time to onset in some cases, this was associated with increasing intensity of illumination. Table 2 shows the relation between light intensity and the incidence of arrhythmias; it is of interest that at the highest intensity, VF developed in one of the six hearts (after 3.8 minutes of illumination).

Also shown in Table 2 is the relation between light intensity and the duration of the longest episode of VT as well as the number of episodes of VT before the development of complete AV block. As was found for the dose-response relation (Table 1), increasing light intensity was associated with increasing incidences of arrhythmias, but there was no such relation with the duration of the longest episode of VT, which was uniformly short (range, 0.6–1.4±0.2 seconds) but with multiple bursts at all light intensities.

Maximum Light Intensity Plus Maximum Dose of Rose Bengal

Hearts (six) were subjected to the same protocol as in the preceding sections except they were
exposed to the highest light intensity (6,600 lx) while being perfused with the highest dose (1,000 nmol/l) of rose bengal. Although a profound arrhythmogenic effect was observed, the results did not differ fundamentally from those obtained with an identical dose of rose bengal but a lower intensity of illumination. Thus, changes in ECG morphology could be detected after 3.8±1.1 seconds (i.e., 20 beats) and the mean times to onset of VPBs, VT, and complete AV block were 0.4±0.2, 0.5±0.3, and 4.5±0.5 minutes, respectively. As would be expected under these severe conditions, coronary flow fell dramatically (from 13.1±1.0 to 0.4±0.1 ml/min after 20 minutes of illumination).

**Rose Bengal Washout Studies**

To assess whether rose bengal acts when bound to tissue or in the freely soluble state in the vascular compartment, a series of washout studies were undertaken. At the end of the equilibration period, hearts (six) were perfused with rose bengal (250 nmol/l) for 5 minutes, perfusion was then switched back to the rose bengal–free buffer solution for 10

<table>
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<tr>
<th>Light intensity (lx)</th>
<th>Incidence of arrhythmias (%)</th>
<th>Complete AV block</th>
<th>Mean duration of longest VT episode (sec)</th>
<th>Mean number of VT episodes / min</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>67</td>
<td>17</td>
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<td>6.7</td>
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<tr>
<td>1,400</td>
<td>83</td>
<td>67</td>
<td>83</td>
<td>1.2±0.2</td>
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<td>0.9±0.1</td>
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<td>6,600</td>
<td>67</td>
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<td>100</td>
<td>1.4±0.2</td>
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Values given are mean±SEM of hearts that exhibit arrhythmia or incidence out of the group of six.
minutes at which time the hearts were illuminated (3,600 lx) for 20 minutes.

Heart rate and coronary flow. Heart rate remained relatively constant throughout the experiment with a mean value of 319±12 beats/min at the end of the equilibration period, 310±13 beats/min at the end of the rose bengal–perfusion period, 308±14 beats/min at the end of the washout period, and 291±14 beats/min during the illumination period before the development of arrhythmias. Again, these results were similar to those obtained with rose bengal perfusion and simultaneous illumination.

Coronary flow decreased from its value of 10.2±0.4 ml/min at the end of the 5-minute period of perfusion with rose bengal (no light) to 8.6±0.3 ml/min at the end of the rose bengal–washout period. On illumination, there was an initial increase in coronary flow that reached 10.9±0.3 ml/min after 2 minutes of illumination. Flow then progressively decreased to 10.3±0.4, 10.0±0.6, 9.8±0.7, 9.5±0.7, 9.4±0.9, 8.9±1.0, 8.8±0.8, 8.5±0.9, and 8.0±0.8 after 4, 6, 8, 10, 12, 14, 16, 18, and 20 minutes of illumination, respectively. Thus, despite the absence of rose bengal in the perfusion fluid, the changes in coronary flow were similar to those seen when rose bengal was present during illumination (100 nmol/l in Figure 4B and 1,400 lx in Figure 6B).

Arrhythmias. Despite the 10-minute washout period and the maintenance of relatively high coronary flow, illumination of hearts that had previously been exposed to rose bengal still resulted in ECG changes (within 36.7±3.9 seconds) and the development of VPBs, VT, VF, and complete AV block (in 83%, 67%, 67%, and 67% of the hearts, respectively). However, compared with the results obtained with hearts that were simultaneously illuminated and perfused with 250 nmol/l rose bengal (Figure 5B), the time to onset of the arrhythmias was delayed (VPBs, VT, VF, and complete AV block developed after 5.0±1.0, 9.5±1.8, 12.4±2.3, and 15.3±1.4 minutes, respectively).

Reversibility Studies

To assess the reversibility of injury induced by rose bengal photoactivation, we conducted studies according to the protocol depicted in Figure 2A. At the end of the equilibration period, the perfusion fluid was changed to a solution containing rose bengal (250 nmol/l). After 5 minutes, hearts (6/group) were illuminated (3,600 lx) for 1, 2, 4, 6, or 20 minutes. After the light was switched off, perfusion with rose bengal solution was continued in the dark for the time remaining up to 20 minutes.

Heart rate and coronary flow. In common with the observations in the preceding sections, no significant changes in heart rate were recorded before the onset of an arrhythmia.

As shown in Figure 8, hearts subjected to a short period of illumination (1, 2, 4, or 6 minutes) did not subsequently suffer the decline in coronary flow that was seen with continuous illumination (Figures 4B and 6B and open boxes in Figure 8). In fact, in hearts subjected to 1 or 2 minutes of illumination, the initial vasodilation continued to increase even after the light was switched off (open circles and solid triangles in Figure 8). In the hearts subjected to 6 minutes of illumination, the expected initial vasodilation (from 9.0±0.2 before illumination to 10.9±0.3 ml/min after 2 minutes of illumination) was followed by an increasing vasoconstriction (with a mean coronary flow of 8.0±1.1 ml/min after 6 minutes of illumination). However, at the end of the period of illumination, the vasoconstriction ceased and coronary flow reverted to its pre–rose bengal control value. Thus, the vascular effects of rose bengal photoactivation appear to be fully reversible up to at least 6 minutes of illumination.

Arrhythmias. As can be seen from Figure 9, increasing the duration of illumination (indicated by the solid bar over each histogram) resulted in an increasing incidence of arrhythmias (calculated per minute) during and after the period of illumination. Comparison of the incidence of arrhythmias per minute in the 6-minute illumination group (Figure 9D) with those in the 20-minute illumination group (Figure 9E) reveals that the vulnerability of the heart to arrhythmias declined when the light was switched off. This suggests that some degree of electrophysiologic “recovery” may be occurring because at the end of 6-minute illumination, more than 80% of hearts were arrhythmic, but in the succeeding 14 minutes, this incidence fell to less than 20%.

It should also be noted that with very short durations of illumination (1 or 2 minutes), arrhythmias may suddenly, although infrequently, develop 10 or
15 minutes after the end of illumination, sometimes after a relatively long period of stable rhythm.

Metabolic Studies

The protocols used for these studies are depicted in Figure 2. At the end of the equilibration period, the perfusion fluid was changed to a solution containing rose bengal (250 nmol/l) for 5 minutes. In the first series of studies (Figure 2B), perfusion with rose bengal solution was then continued for an additional 1, 2, 4, 6, or 20 minutes during which the hearts (6/group) were continuously illuminated (3,600 lx). Hearts were freeze-clamped at the end of each period of illumination. In the second series (Figure 2A), perfusion with rose bengal was continued after the end of illumination for the remaining period up to 20 minutes. Coronary effluent was collected throughout the 20-minute perfusion period, and hearts (6/group) were freeze-clamped at the end of perfusion (Figure 2A). Tissue ATP, CP, and lactate contents were measured and compared with those from control hearts (6/group), which were freeze-clamped after 5 minutes of perfusion with rose bengal without light, or with those that were freeze-clamped after 20 minutes of perfusion with or without rose bengal in the absence of light. Lactate efflux and creatine kinase leakage were measured and compared with those from control hearts that were perfused for 20 minutes with rose bengal in the absence of light.

Tissue ATP, CP, and lactate contents. As shown in Figure 10A, myocardial ATP content declined progressively with increasing durations of rose bengal photoactivation (open circles). However, during the first few minutes, the decline was small and did not achieve a level of statistical significance until 4 minutes or more of illumination had elapsed. However, with 20 minutes of continuous illumination, the ATP content fell to 5.2±0.3 μmol/g dry wt. Of particular interest are the hearts in which rose
Figure 10. Metabolic changes induced by rose bengal photoactivation. Rose bengal (250 nmol/l) perfused hearts were subjected to various periods of illumination as indicated in Figure 2, after which they were either immediately freeze-clamped (○) or perfused in the absence of light for the time remaining up to 20 minutes (●). The tissue content (μmol/g dry wt) of ATP (Panel A), CP (Panel B), and lactate (Panel C) were then measured. *p<0.05 versus hearts freeze-clamped at the end of the 5-minute period of perfusion with rose bengal in the absence of light.

bengal perfusion was maintained after the illumination was terminated (solid circles); in these hearts, the decline in ATP content continued in a manner similar to that in hearts that were continuously illuminated. This would suggest that while metabolic injury is sustained during the period of photoactivation, it does not recover but continues to intensify during the subsequent period when photoactivation has ceased. Similar observations can be made in relation to the decline in tissue CP content (which fell more rapidly than ATP content) (Figure 10B) and the increase in tissue lactate content (Figure 10C).

ATP and CP contents were also measured in nonilluminated hearts freeze-clamped after 20 minutes of perfusion with or without rose bengal. ATP contents were comparable in both groups (19.4±0.4 μmol/g dry wt in the presence of rose bengal and 21.5±0.4 μmol/g dry wt in its absence). However, although CP content was well maintained in hearts perfused for 20 minutes in the absence of rose bengal and light (19.2±0.6 μmol/g dry wt), the value in hearts perfused for 20 minutes with rose bengal in the dark was somewhat reduced (13.1±1.4 μmol/g dry wt, p<0.05), suggesting an effect of rose bengal, independent of the photoactivation process. However, this effect took some time to develop because the CP content of hearts perfused for 5 minutes with rose bengal in the absence of light was 23.4±1.0 μmol/g dry wt.

Lactate production and creatine kinase leakage. Total lactate and creatine kinase release to the coronary effluent was measured during the period of illumination and the subsequent period of dark perfusion up to 20 minutes in the protocol indicated in Figure 2A. The results (Table 3) show that lactate can be detected in the coronary effluent after even 1 minute of illumination and that its production increased with increasing durations of illumination. Production also continued after the light had been switched off; thus, with 6 minutes of illumination and 14 minutes of dark perfusion, the total lactate produced was comparable to that observed with 20 minutes of continuous illumination. With period of illumination up to 6 minutes, the greater the duration of illumination, the greater was the total release of lactate during 20 minutes of perfusion. The lactate results would suggest that maximal metabolic damage may be inflicted after 6 minutes of photoactivation and that termination of photoactivation does not lead to metabolic recovery.

Creatine kinase leakage could be detected after 1 minute of photoactivation; even after this short period, subsequent leakage was extensive such that total leakage with 1 minute of photoactivation and 19 minutes of perfusion was similar to that seen with 20 minutes of continuous photoactivation. This, again, would suggest continuing damage beyond the period of photoactivation.

It should be noted that the total lactate and creatine kinase detected in the 20-minute illumination group probably underestimate the extent of damage in this group of hearts due to the coincident impairment of coronary flow that acts to limit the extent of washout.

Coronary Vasocostriction Induced by Rose Bengal Photoactivation as a Contributing Factor in Arrhythmogenesis

As described earlier, it was observed that in hearts perfused with rose bengal and illuminated for
6 minutes followed by 14 minutes of dark perfusion (Figure 9D), the incidence of ventricular arrhythmias progressively decreased to a low level during the 14-minute recovery period. In contrast, in hearts that were illuminated for 20 minutes (Figure 9E) a 100% incidence was recorded during the last 10 minutes of illumination. In the 6-minute group, coronary flow returned to control levels, whereas in the 20-minute illumination group, it fell to a very low level (Figure 8). To assess whether this decline in coronary flow contributed to the process of arrhythmogenesis, rose bengal–perfused hearts (six) were illuminated for 6 minutes and then, for the following 14 minutes, coronary flow was progressively reduced so as to mimic the reduction of flow that would have been observed had the hearts been illuminated throughout the 20-minute period.

Heart rate and coronary flow. No significant changes in heart rate were observed before the onset of an arrhythmia.

Coronary flow at the end of the equilibration period was 10.1±0.2 ml/min; at the end of 5-minute perfusion with rose bengal without light, the value was 9.4±0.2 ml/min. On illumination, as observed previously, coronary flow increased during the first 2 minutes to a value of 10.9±0.2 ml/min and then fell to 8.3±0.4 and 7.2±0.5 ml/min after 4 and 6 minutes of illumination, respectively. After the light was switched off, coronary flow was further decreased by manually adjusting the infusion pump such that mean coronary flow at 8, 10, 12, 14, 16, 18, and 20 minutes was 5.7±0.4, 4.7±0.1, 4.2±0.1, 3.2±0.1, 2.3±0.1, 1.7±0.1, and 1.3±0.1 ml/min, respectively.

Arrhythmias. At the end of the 6-minutes of illumination, 83% of hearts had developed arrhythmias (Figure 11A). During the subsequent 14 minutes of dark perfusion, the incidence declined progressively to 17% in the group of hearts in which coronary flow was allowed to recover spontaneously. Similarly, in hearts that were illuminated for the entire 20 minutes, the incidence of arrhythmias after 6 minutes was 83% and after 11 minutes, it was 100%, at which level it continued for the remainder of the experiment (Figure 11B). Hearts subjected to the progressive impairment of flow between the sixth and the 20th minute in the absence of photoactivation (Figure 11C) also showed a very high incidence of arrhythmias. This would support the view that “ischemia” due to the reduction in coronary flow induced by rose bengal photoactivation may exacerbate the vulnerability of the heart to arrhythmias detected in the later periods of photoactivation.

Metabolism. Tissue ATP content in the 6-minute illumination group with flow reduction, measured at the end of the 20-minute perfusion period, was similar to the value in the 20-minute illumination group (5.0±0.6 versus 5.2±0.3 µmol/g dry wt). As in the 20-minute illumination group (CP, 3.2±0.3 µmol/g dry wt; lactate, 1.2±0.1 µmol/g dry wt), the CP content in the flow reduction group was severely depressed (1.9±0.5 µmol/g dry wt), and lactate content was elevated (8.8±1.3 µmol/g dry wt).

Discussion

In this study with the aerobically perfused rat heart, we have shown that photoactivation of rose bengal with green light (530–590 nm), a process that generates singlet oxygen and superoxide,12–14 results in the rapid development of severe arrhythmias. These arrhythmias, which are dose and light dependent, develop in the absence of ischemia and reperfusion; however, with prolonged illumination, there is a decline in coronary flow that exacerbates the vulnerability to arrhythmias.

Rapidity of Injury

While it is generally accepted that free radicals and other reactive oxygen intermediates can be injurious to tissue and lead to potentially arrhythmogenic changes in cardiac electrophysiology,15,16 it has been suggested17 that these changes may be too slow to account for the occurrence of some arrhythmias. We have already reported1 that photoactivation of rose bengal can result in the rapid induction of arrhythmias; one of the objectives of the present study was to define the dose- and light-response characteristics of this phenomenon. Our present results show that discernible ECG
changes can occur after less than 4 seconds of photoactivation and that arrhythmias can develop after less than 0.5 minutes. The time to onset of the arrhythmias and the nature of the arrhythmias (usually VPBs, VT, and AV block) are highly reproducible. VPBs usually deteriorate to VT, which typically occurs in short (mean duration, approximately 1 second) repetitive bursts (Figure 3). Although the mean time to onset and the incidence of occurrence of VT in a group of hearts were both dose and light dependent, there was no such dependency for the number of bursts or the length of each burst within each heart. This last observation might suggest that although the reactive oxygen intermediates may play a role in the genesis of the arrhythmias, they do not contribute to the mechanisms that are responsible for the maintenance of the arrhythmias.

**Possible Mechanism of Electrophysiologic Injury**

The rose bengal–induced arrhythmias were always preceded by distinct changes in the morphology of the ECG, usually inversion of the terminal portion of the T wave, the prolongation of the Q-T interval, or both. Many studies have associated prolongation of the Q-T interval with disturbances of potassium conductance, leading to early afterdepolarizations and ventricular arrhythmias. However, in preliminary studies with isolated muscle preparations, we have reported that the photoactivation of rose bengal leads to the development of late afterdepolarizations, which would be more indicative of disturbances of calcium homeostasis.

Calcium is undoubtedly involved in the rose bengal–induced injury. As shown by the work of Borgers et al in myocytes and our own work in isolated rat hearts, ultrastructural changes (including severe contracture) occur, which are consistent with calcium redistribution and intracellular calcium overload. This has been confirmed by calcium cytochemistry, which demonstrates a striking rose bengal–induced redistribution of calcium from the sarcolemma to the intracellular
space, particularly the mitochondria. Although it is not clear whether this perturbation of calcium homeostasis occurs because of major nonspecific membrane injury or specific changes in membrane pumps, it is likely that, in either case, alterations in sodium and potassium fluxes would also occur and contribute to the observed electrophysiologic changes. Using giant nerve axons from the lobster, Pooler and Valenzeno have shown that the illumination of dyes such as eosin, methylene blue, and rose bengal can block sodium and, to a lesser extent, potassium channels.

**Vascular Injury**

In addition to inducing ECG changes and arrhythmias, the photoactivation of rose bengal results in dose- and light-dependent changes in coronary flow that, at high doses and intensities, may be large. The changes in coronary flow are characterized by an initial transient (up to 4 minutes) vasodilation followed by a progressive time-dependent vasoconstriction. These changes, as discussed previously, are consistent with progressive calcium overload of vascular smooth muscle and vascular compression as a consequence of contracture occurring within the myocytes surrounding coronary vessels. With high-dose or high-light intensity, rose bengal photoactivation can lead to large reductions in flow (from more than 10 to less than 2 ml/min), which are sufficient to generate tissue ischemia. The possibility, therefore, arises that this ischemia may itself be responsible for inducing the arrhythmias or increasing the vulnerability of the tissue to arrhythmias triggered by other factors. It is important to stress, however, that the decline in coronary flow cannot be responsible for initiating arrhythmias because, in many preparations, they arise before there is any significant reduction of flow. Furthermore, in the studies with low doses of rose bengal and low or intermediate levels of light, there were no changes in coronary flow; nonetheless, arrhythmias still occurred.

Although the rose bengal–induced coronary vasoconstriction is unlikely to account for the initiation of arrhythmias, there seems little doubt that, after extended periods of photoactivation, this factor can exacerbate the vulnerability of the heart to arrhythmias or support their maintenance. This was demonstrated (Figure 11) in studies in which we subjected hearts to 6 minutes of illumination followed by 14 minutes of perfusion in the dark. In comparison to hearts that were continuously illuminated (in all of which coronary flow fell and severe arrhythmias developed), coronary flow recovered to its control value, and vulnerability to arrhythmias declined. However, if the hearts were subjected to 6 minutes of illumination and 14 minutes of perfusion in the dark during which coronary flow was mechanically reduced to the level seen in the continuous illumination group, then the incidence of arrhythmias was maintained at a high level.

**Reversibility of Injury**

As speculated previously, the mechanism underlying the genesis of arrhythmias triggered by reactive oxygen intermediates might involve free radical–mediated membrane lipid peroxidation or changes in the redox state of thiol groups controlling the activity of membrane carrier proteins. In addition, these highly reactive intermediates might damage a variety of macromolecular structures. Some of these changes (e.g., those in redox state) might be expected to recover on removal of the oxidant stress, whereas others might be irreversible or take some time to revert to normal. Our studies (Figures 2, 8, 9, and 10) with short-term illumination might indicate that, for periods up to 6 minutes, the heart appears to be able to “recover” in terms of both coronary flow and electrical stability. However, in terms of tissue high-energy phosphate content, lactate production, and creatine kinase leakage, the termination of the photoactivation process was unaccompanied by improvement. While these results may appear to be in conflict, it may well be that the injury induced by the photoactivation process is heterogeneous and that irreversible injury is induced in an initially small but growing population of cells. Thus, on termination of photoactivation, the undamaged cells can return to normal electrical activity, whereas the irreversibly injured cells become electrically excitable but continue to deteriorate metabolically. Such a proposition gains some support from our ultrastructural observations of localized injury in the vicinity of major blood vessels.

**Site of Injury**

Ultrastructural studies indicate that the injury associated with rose bengal photoactivation appears to radiate out from major blood vessels. This might suggest that some toxic intermediate (e.g., hydrogen peroxide, which may migrate a limited distance into the tissue) is produced in the vascular space or, alternatively, that rose bengal progressively diffuses from the vascular compartment, generating a gradient for photoactivation-related injury. Our present studies with short-term perfusion with rose bengal followed by rose bengal washout and subsequent illumination provide strong evidence that substantial quantities of rose bengal are firmly bound to the tissue, creating injury at that site at the time of photoactivation. Whether the site at which rose bengal accumulates is in the endothelium, the smooth muscle, or the myocyte or whether it binds to cell membranes or is taken up into the intracellular compartment remains to be resolved. However, studies by Allison et al suggest that the molecule is most likely to be bound to the cell surface. Support for this possibility comes from the recent studies of Ver Donck et al, who demonstrated that rose bengal binds firmly to myocytes and cannot be washed away unless albumin is used. They concluded that the site of binding was the sarcolemma and that on photoac-
tivation, singlet oxygen was produced that acted in its immediate vicinity together with superoxide, which might migrate for some distance.

**Identity of Reactive Species**

In our previous studies in which rose bengal was illuminated just before its entry to the heart, we concluded that the damaging intermediate had a very short half-life. Possible candidates were the activated form of rose bengal or singlet oxygen and superoxide that, under the conditions of our experiment, must also be formed. Although, in photodynamic terms, it is inevitable that singlet oxygen is formed under these conditions, it is difficult to prove its existence in biological tissue. It has an exceptionally short half-life and unlike many free radicals cannot be selectively captured with spin traps or detected with electron spin resonance techniques. We have, however, obtained preliminary evidence (unpublished results), using the technique of laser time-resolved phosphorescence, for the generation of singlet oxygen in hearts perfused with rose bengal dissolved in deuterium oxide (a procedure that increases the half-life of singlet oxygen by approximately eightfold). Finally, our observations that antioxidants such as superoxide dismutase, glutathione, ascorbate, dimethylthiourea, and methionine do not limit rose bengal-induced arrhythmias but that histidine (an effective quencher of singlet oxygen) is protective lead us to conclude that singlet oxygen is the most likely culprit.

**Conclusions**

Our results provide further evidence that reactive oxygen intermediates such as oxygen-derived free radicals and singlet oxygen (all of which can be formed in the heart under various pathologic conditions) can induce severe myocardial injury. This injury can develop with great rapidity and may result in damage to contractile function, vascular control, and electrical stability. The mechanism by which these agents exert their damaging effects remains to be resolved, but membrane lipid peroxidation, changes in redox state, or damage to proteins, enzymes, and other macromolecules may be involved.

**Acknowledgments**

The advice and discussion of Dr. D.C. Neckers and Dr. MAJ Rodgers, Centre for Photochemical Sciences, Bowling Green State University, Ohio, is gratefully acknowledged, as is the collaboration with Dr. R.V. Bensasson, Laboratoire de Biophysique, Museum National d'Histoire Naturelle, Paris, France, over his studies with laser time-resolved phosphorescence.

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KEY WORDS: *free radicals* • *singlet oxygen* • *superoxide* • *membrane injury* • *arrhythmias* • *metabolism*
Singlet oxygen-induced arrhythmias. Dose- and light-response studies for photoactivation of rose bengal in the rat heart.

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_Circulation_. 1989;80:1432-1448
doi: 10.1161/01.CIR.80.5.1432

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/80/5/1432

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