possible for intrastrand DNA breaks within a certain critical distance of each other to be repaired, or a break near some other lesion, such as a protein-DNA cross-link, might be inaccessible to repair enzymes. As is the case with double-strand breaks, high-LET radiations should induce these hypothetically non-repairable double lesions more efficiently than low-LET radiations.

Cell killing by ionising radiation can be characterised by two independent mechanisms having lethal, single-hit and sublethal, multi-hit attributes. Our results offer a molecular explanation of the lethal, single-hit component, relating it to non-rejoining DNA strand breaks. The identity of other lesions responsible for the sublethal component remains undefined, although it has been postulated that these lesions are rejoinable DNA single-strand breaks[17,29].

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1 Munson, R. J., Neary, G. J., Bridges, B. A. & Preston, R. J. *Int. J. radiat. Biol.* **13**, 205–224 (1967).
2 Powers, E. L., Lyman, J. T. & Tobias, C. A. *Int. J. radiat. Biol.* **14**, 313–330 (1968).
3 Sayeg, J. A., Birge, A. C., Beam, C. A. & Tobias, C. A. *Radiat. Res.* **10**, 449–461 (1959).
4 Manney, T. R., Brustad, T. & Tobias, C. A. *Radiat. Res.* **18**, 374–388 (1963).
5 Barendsen, G. W. *Int. J. radiat. Biol.* **8**, 453–466 (1964).
6 Deering, R. A. & Rice, R., Jr *Radiat. Res.* **17**, 774–786 (1962).
7 Skarsgard, L. D., Kihlman, B. A., Parker, L., Pujara, C. M. & Richardson, S. *Radiat. Res. Suppl.* **7**, 208–221 (1967).
8 Todd, P. *Radiat. Res. Suppl.* **7**, 196–207 (1967).
9 Raju, M. R., Gnanapurani, M. & Richman, C. *Br. J. Radiol.* **45**, 178–181 (1972).
10 Barendsen, G. W. in *The Initial Effects of Ionizing Radiations on Cells* (ed. Harris, R. J. C.) 183–194 (Academic, New York, 1961).
11 Lawrence, J. & Tobias, C. in *Mod. Trends Radiother.* **1**, 260–276 (1967).
12 Tobias, C. A. & Todd, P. in *National Cancer Institute Monograph* 24, *Conference on Radiobiology and Radiotherapy*, 1–15 (US Department of Health, Education and Welfare, 1967).
13 Sinclair, W. K. *Cancer Res.* **27**, 297–308 (1967).
14 Mortimer, R. Brustad, T. & Cormack, D. V. *Radiat. Res.* **26**, 465–482 (1965).
15 Puck, T. *Prog. Biophys. biophys. Chem.* **10**, 237–258 (1960).
16 Barendsen, G. W., Walter, H. M. D., Fowler, J. F. & Bewley, D. K. *Radiat. Res.* **18**, 106–119 (1963).
17 Chadwick, K. H. & Leenhouts, H. P. *Phys. med. Biol.* **18**, 78–87 (1973).
18 Todd, P. thesis, Univ. California, Berkeley (1964).
19 Todd, P. W. *Radiat. Res.* **61**, 288–297 (1975).
20 Kapp, D. S. & Smith, K. C. *J. Bact.* **103**, 49–54 (1970).
21 Painter, R. B., Young, B. R. & Burki, H. J. *Proc. natn. Acad. Sci. USA* **71**, 4836–4838 (1974).
22 Cleaver, J. E., Thomas, G. H. & Burki, H. G. *Science* **177**, 996–998 (1972).
23 Burki, H. J., Roots, R., Feinendegen, L. E. & Bond, V. P. *Int. J. radiat. Biol.* **24**, 363–375 (1973).
24 Howard-Flanders, P. *Adv. Biol. med. Phys.* **6**, 553–603 (1958).
25 Christensen, R. C., Tobias, C. A. & Taylor, W. D. *Int. J. radiat. Biol.* **22**, 457–477 (1972).
26 Cole, A., Shonka, F., Corry, P. & Cooper, W. G. in *Molecular Mechanisms for Repair of DNA* (eds Hanawalt, P. C. & Setlow, R. B.) 665–676 (Plenum, New York, 1975).
27 Ehmann, U. K. & Lett, J. T. *Radiat. Res.* **54**, 152–162 (1973).
28 Cleaver, J. E. in *Meth. Cancer Res.* **11**, 123–165 (Academic, New York, 1975).
29 Dugle, D. L., Gillespie, C. J. & Chapman, J. D. *Proc. natn. Acad. Sci. USA* **73**, 809–812 (1976).

# Electrical demonstration of rapid light-induced conformational changes in bacteriorhodopsin

BACTERIORHODOPSIN (a purple pigment) is a light-driven proton pump[1,2], found in patches of the cell membrane of *Halobacterium halobium*[3,4], and is chemically similar to the visual pigment rhodopsin of animal eyes[4]. In both molecules the chromophore retinal is linked as a protonated Schiff base to a lysine residue of the apoprotein opsin[1,5,6]. Light induces bacteriorhodopsin to move protons from the inside to the outside of the cell. The resulting electrochemical proton gradient across the membrane is finally used to synthesise ATP. We report here the application of a new technique for the direct measurement of small capacitative charge displacements in oriented molecules. Our photoelectric measurements using asymmetrically aligned bacteriorhodopsin demonstrate that this protein undergoes reversible conformational changes after absorption of light.

Flash spectroscopy shows that bacteriorhodopsin undergoes a cycle through at least five different spectroscopically defined intermediates[7]. About 8 ms after bleaching the pigment returns to its original form. This cycling time is in good agreement with the maximal 'proton pumping' rate at saturating light intensities. Measurements show that a single protein molecule can transport up to 100–200 protons per second[8].

It is conceivable that the excited bacteriorhodopsin molecule, in addition, passes through a conformational transition sequence to accomplish the vectorial proton transport[9]. Such conformational changes are expected to generate charge displacements within the protein. To measure this effect electrically, two basic requirements have to be fulfilled. First, the molecules must be oriented in parallel but asymmetrically and second, they must be synchronisable, that is, they have to start with the change at a common zero time.

The most favourable system for fulfilling these conditions is found in photoreceptor cells of animals. Here, rhodopsin molecules are strongly oriented in lamellar cell structures, and synchronisation is achieved by means of short flashes. Fast capacitative photopotentials from a wide variety of photoreceptor cells have been measured, and have come to be known as 'early receptor potentials' (ERPs) (ref. 10). The signals are generally attributed to conformational changes within the rhodopsin molecule as it passes through its bleaching sequence[11].

We have succeeded in developing a model system in which rhodopsin from bovine retinal rod outer segments is reoriented on to one side of a thin Teflon film separating two aqueous phases[12]. Flashes to the layer evoke fast photovoltages and photocurrents (depending on the measuring device). Since the layer, having virtually infinite resistance when arranged between two aqueous phases, can be considered as a capacitor, the observed photoelectric response is due to pure capacitative charge displacements. Such model layers offer, for instance, the advantage of allowing the measurement of true transition time courses unaffected by cell membrane time constants. We have now applied this approach to bacteriorhodopsin layers.

Surface-active material from the aqueous suspension of purple membrane fragments was obtained by agitation until foam formed. About 100 μl of the foam was transferred to one side of a two-compartment Teflon cell containing a 4 M NaCl subphase. Thereafter it was overlaid with hexane. The surface film in the water–hexane interface was apposed to a vertically mounted Teflon septum (6 μm thick) by slowly increasing the water level. The chamber on the other side of the septum contained only electrolyte. Thus, the model system consists of the spatial sequence: electrolyte–bacteriorhodopsin–Teflon–electrolyte. The Teflon septum between the two half-cells acts as an electrical insulator and couples the compartments only capacitatively. The two identical electrolyte solutions were connected by silver–silver chloride electrodes to a high resistance input electrometer ($10^{14}\Omega$; 20 pF) (ref. 12).

The voltage responses of this layer when illuminated with bright white flashes are illustrated in Fig. 1. The individual oscilloscope pictures originate from sequential flashes to the same layer. They differ in the time axis to delineate the full time course of the signal.

Analysing the photopotential time course for exponentials leads to, at least, three clearly distinguishable transitions: the signal increases with a fast time constant $\tau_1 \sim 1$ ms and a slower one, $\tau_2 \sim 15$ ms. The main decay back to the initial potential occurs with $\tau_3 \sim 1$ s. Correlating these time constants to those of the spectral intermediates is not feasible at the present time and with the present technique. In spite of this, the close agreement of $\tau_2$ with the 412 nm→570 nm transition ($\tau \sim 12$ ms) is striking. Also, millisecond spectral transitions have been observed which could coincide with $\tau_1$ (ref. 9).

Since bacteriorhodopsin undergoes a cyclic photoreaction, the photovoltage would be expected to be reversible; and indeed it is. The bleaching cycle in the purple membrane is

completed in approximately 10 ms, whereas in the model layer about a second is required. This discrepancy of two orders of magnitude is tentatively attributed to the different arrangement of the protein in the two systems: *in situ*, a lipid bilayer matrix, two sides of the protein contact aqueous media[13]; whereas *in vitro*, the protein is in contact with water only on one side—the more hydrophilic one. If a water-soluble reactant (presumably protons) is required for regeneration at the side where the bacteriorhodopsin molecule touches the Teflon surface, then the hindered accessibility would account for the inhibition of regeneration.

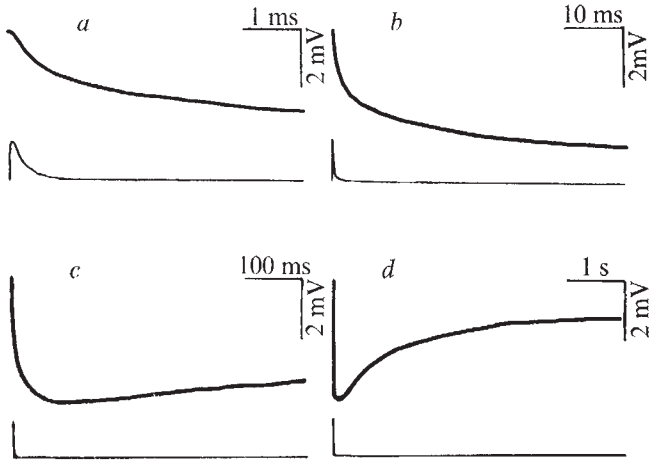


**Fig. 1** Examples of the photovoltage of a bacteriorhodopsin layer, 6 mm in diameter, in response to white light flashes. Purple membrane fragments, prepared according to Oesterhelt and Stoeckenius[14], were suspended in 3.0 ml basal salt sodium 4.35 M NaCl, 0.275 M KCl, 0.08 M $MgSO_4$, 9 mM Na citrate, *p*H 7.0) at a protein concentration of 0.5 mg $ml^{-1}$. The aqueous subphase contained 4 M NaCl and 10 mM imidazole adjusted to *p*H 7.1 with HCl. The experiments were carried out at room temperature (22 ± 1 °C). Each oscilloscope trace includes the time course of the flash at the bottom. Subsequent flashes were separated by more than 1 min. The following amplifier time constants were chosen: *a*, 1 Hz–10 kHz; *b*, 0.1 Hz–3 kHz; *c*, d.c.–1 kHz; *d*, d.c.–0.1 kHz.

The polarity of the voltage is negative on that side where the layer is formed. This is the expected polarity for protons moving from the aqueous phase to the bacteriorhodopsin layer. If this were so, then a strong increase in photovoltage amplitude should result from reducing the ionic strength. Furthermore, the time constants recorded above should depend on proton concentration. Changing the electrolyte (while the layer remains apposed to the Teflon), from 4 M NaCl to 4 mM NaCl and then back to 4 M NaCl, however, did not alter the photovoltage amplitude nor the time constants $\tau_1$, $\tau_2$, $\tau_3$, at any stage of the manipulations. Apart from stressing the stability of the bacteriorhodopsin layer this experiment rules out an origin of the photovoltage from a diffused ion double layer at the aqueous interface. More likely, the effect is based on charge or dipole reorientations in the bacteriorhodopsin molecule itself.

Changes of *p*H in the subphase at constant ionic strength did not significantly affect the time constants, $\tau_1$ or $\tau_2$ (range, *p*H 4–9). One effect of increased proton concentration is a faster decay of the photovoltage transient to zero. For instance, at *p*H 7, $\tau_3 = 2$ s and at *p*H 4, $\tau_3 = 0.5$ s. This gives further support to the above mentioned explanation for the slow regeneration rate: the increased proton concentration at lower *p*H compensates for the hydrophobic location of the hypothetical regeneration site.

At low *p*H the photovoltage amplitude increases slightly. A layer, which displays at *p*H 9 a maximal amplitude of 3 mV displays at *p*H 4 about 7 mV. At *p*H 3 the shape of the photovoltage response changes dramatically; the negative phase is

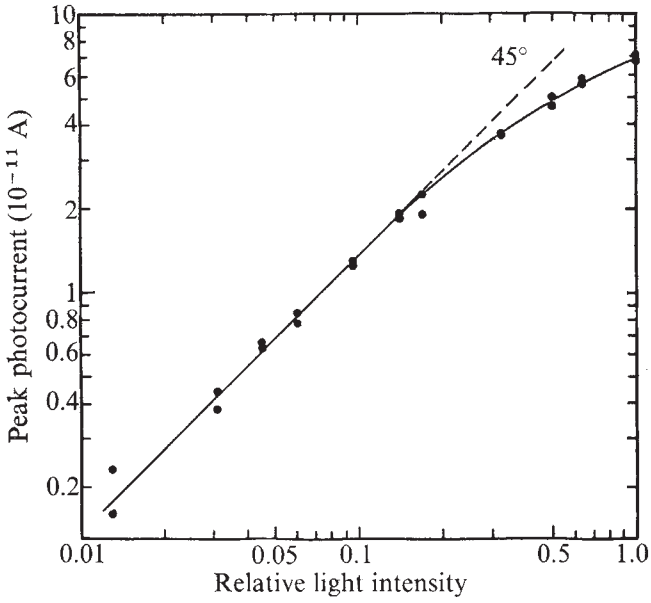


**Fig. 2** Peak photocurrent as a function of the flash intensity. The increase in amplifier current time constant was limited to 1 ms. Neutral density filters were used to reduce the flash intensity. Data were obtained from one layer.

reduced in its amplitude and a very fast positive transient of 3 mV appears. The whole signal has a duration of 200 ms. These experiments indicate a proton contribution to the generating mechanism; since this is already localised in the protein (independent of ionic strength), the photovoltage or a part of it may directly reflect a proton movement in the protein molecule.

Since the bleaching of bacteriorhodopsin is reversible, it is possible to apply many flashes to the same layer, retrieving reproducible responses: one layer can be exposed a hundred times to a saturating flash without significant alterations of the photovoltage.

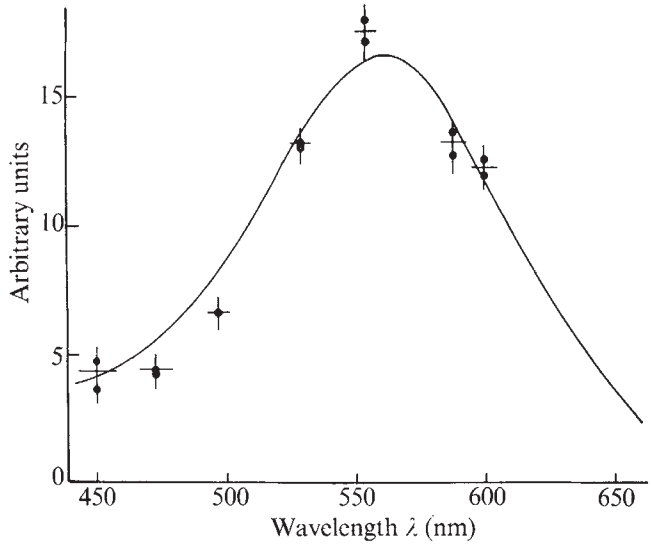


**Fig. 3** Action spectrum of a bacteriorhodopsin layer. Solid line represents absorption spectrum of aqueous suspensions of bacteriorhodopsin. Each data point derives from the peak photocurrent at the given wavelength, normalised to the number of photons within the flash. Horizontal bars indicate the interference filter bandwidth at 50% transmission, and vertical bars represent the estimated experimental error.

The photoelectric effect can also be detected as a photocurrent if the electrodes are connected to an ammeter. In this case a photocurrent transient, which is exactly the first time derivative of the photovoltage, is measured. Therefore, the peak in the current transient reflects the steepest slope of the voltage. Since reducing the flash light intensity decreases the photovoltage amplitude, without affecting the shape, a dependence on light intensity could also be measured as the peak photocurrent amplitude. This method provides a better signal-to-noise ratio and is therefore used in the experiment illustrated in Fig. 2. For dim flashes the peak photocurrent is directly proportional to the light intensity. Brighter flashes lead to sublinear responses, indicating a saturation behaviour.

To further establish that the photoresponses result from bacteriorhodopsin bleaching, an action spectrum was obtained (Fig. 3), using the 'current' mode described above. The individual peak photocurrents lie without exception within the linear portion of the curve shown in Fig. 2. The absorption spectrum of the bacteriorhodopsin suspension used in the experiments (solid line) is fitted with its amplitude to the date points. The good agreement of both identifies bacteriorhodopsin as the molecular species responsible for the photoelectric effects.

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1 Oesterhelt, D., Stoeckenius, W. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2853–2857 (1973).
2 Stoeckenius, W. *Sci. Am.* **234**, 38–46 (1976).
3 Oesterhelt, D. & Stoeckenius, W. *Nature new Biol.* **233**, 149–152 (1971).
4 Blaurock, A. E. & Stoeckenius, W. *Nature new Biol.* **233**, 152–155 (1971).
5 Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H. & Stoeckenius, W. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4462–4466 (1974).
6 Lewis, A. & Spoonhower, J. in *Neutron, X-Ray and Laser Spectroscopy in Biophysics and Chemistry* (eds Yips, S. & Chen, S.) (Academic, New York 1974).
7 Lozier, R. H., Bogomolni, R. A. & Stoeckenius, W. *Biophys. J.* **15**, 955–962 (1975).
8 Oesterhelt, D. *Angewandte Chemie* **15**, 17–24 (1976).
9 Slifkin, A. M. & Caplan, S. R. *Nature* **253**, 56–58 (1975).
10 Cone, R. A. & Pack, W. L. in *Handbook of Sensory Physiology* **1** (ed. Loewenstein, W. R.) 345–365 (Springer, Heidelberg, 1971).
11 Rüppel, H. & Hagins, W. A. in *Biochemistry and Physiology of Visual Pigments* (ed. Langer, H.) 257–261 (Springer, Heidelberg, 1973).
12 Trissl, H.-W., Darszon, A. & Montal, M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 207-210 (1977).
13 Henderson, R., & Unwin, P. N. T. *Nature* **251**, 28–32 (1975).
14 Oesterhelt, D. & Stoeckenius, W. *Methods Enzym.* **31**, 667–678 (1974).

# Catalytic effect of nitrosophenols on *N*-nitrosamine formation

THE effect of phenolic compounds on the rate of nitrosation of secondary amines is not a simple one. Tannins inhibit nitrosamine formation[1], but in suitable conditions gallic acid catalyses the nitrosation of diethylamine, the rate being dependent on *p*H and gallic acid concentration[2]. Some phenolic constituents of smoked foods[3] inhibit nitrosation of morpholine at *p*H 3.0. Phenolic compounds react with nitrite at a much faster rate than do secondary amines[4], but chlorogenic acid and 4-methylcatechol catalyse[5] the nitrosation of piperidine at gastric *p*H. We report here that nitrosophenols can catalyse *N*-nitrosamine formation.

Nitrosophenols are formed by the nitrosation of phenols and their presence in cured smoked meats has been demonstrated[6]. The reaction we studied was that of 0.15 M sodium nitrite with 0.15 M pyrrolidine in citrate buffer, *p*H 5.0, at 37 °C. Perchloric acid was used to bring the pyrrolidine solution to *p*H 5.0 before mixing. The effect of *p*-nitroso-*o*-cresol on this reaction was studied at 0.25, 0.50, 0.75 and 1.0 mM concentration using the method of initial rates. Aliquots were taken from the reaction mixtures at 5-min intervals for 20 min. Each aliquot was quenched with a solution of ammonium sulphamate in 2 N $H_2SO_4$ to remove unreacted nitrite, and the nitrosopyrrolidine (NPy) was extracted into dichloromethane and determined by gas–liquid chromatography. Each reaction was repeated in triplicate. For each reaction a plot of NPy concentration against time gave a straight line, the slope of which gave the rate of formation of NPy. Figure 1 shows a plot of the rate of NPy formation against nitrosocresol concentration. Figure 2

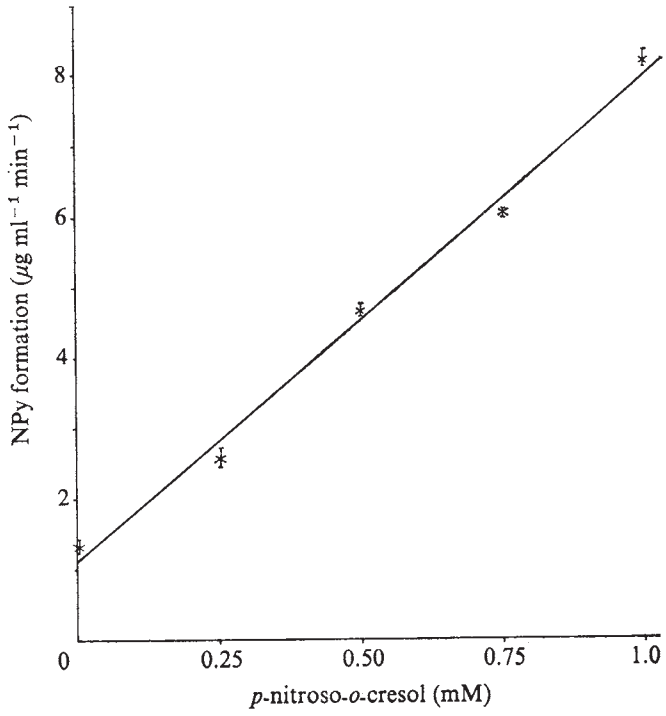


**Fig. 1** Dependence of the rate of formation of *N*-nitrosopyrrolidine on the concentration of *p*-nitroso-*o*-cresol.

shows a plot of the logs of these two properties. That the points in Figs 1 and 2 do not fall exactly on straight lines may be due partially to the poor solubility of *p*-nitroso-*o*-cresol in the citrate buffer solution used. The slope of the line in Fig. 2 calculated by the method of least mean squares is 0.81, and this represents the order of the reaction with respect to *p*-nitroso-*o*-cresol. The two other nitrosophenols we have tested, *p*-nitrosothymol and *l*-nitroso-2-naphthol, also catalysed NPy formation at *p*H 5.0. No reaction takes place between *p*-nitroso-*o*-cresol and pyrrolidine at *p*H 5.0 in the absence of nitrite. The formation of NPy therefore does not occur simply by a trans-nitrosation process from nitrosocresol to pyrrolidine. We found that *N*-nitrosation does not occur in the organic phase during work-up by reduced nitrite species. Such nitrosamine formation requires the presence in the organic phase of nitrosating species and pyrrolidine. The acidic quenching procedure employed in these experiments, besides removing unreacted nitrite, will protonate unreacted amine. The protonated amine should remain in the aqueous layer during dichloromethane extraction. To check that this was so we prepared a 0.15 M solution