PHOTODYNAMIC HEMOLYSIS* AT LOW TEMPERATURES

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A large number of organic dyes may act as photosensitizers for oxidations by molecular oxygen; the substrate may be any one of a wide variety of organic or inorganic substances. When such photosensitized oxidation takes place in living systems it is commonly referred to as photodynamic action; the results are generally destructive. The name photodynamic action is not altogether appropriate since it seems to assign too general significance to the phenomenon. The term photooxidation, on the other hand, may be confusing since these same dyes may forward other photooxidations that do not produce the destructive effects characteristic of photodynamic action (see Blum, 1941).

In photosensitized oxidation the dye is the light absorber, but how the energy of the absorbed quantum is employed in bringing about the oxidation of the substrate by molecular oxygen is not clearly understood. The over-all reaction may be written in the following way

$$^{h\nu}_{D} + O_2 + X = X_{ox} + D \qquad (1)$$

in which D is the dye molecule, X the substrate, and X_{ox} the oxidized substrate after reacting with O_2. The symbol $^{h\nu}_{D}$ is used to indicate that it is the dye molecule which captures the light quantum, but without commitment as to the way in which the energy of the quantum is used in forwarding the oxidation. D appears on the right hand side of the equation, indicating that the dye is unchanged at the end of the reaction, which may go in a cycle, the dye capturing successive quanta and repeatedly bringing about the oxidation of X molecules by O_2 molecules.

The evidence that a cycle occurs is very good. In simple inorganic solution the dye may photooxidize many times its equivalent of oxidizable substrate, say iodide ion. This is not a chain reaction; the quantum yield approaches but never exceeds unity. In the case of photodynamic hemolysis the same number of quanta must be absorbed per red cell by the dye ($\simeq 10^{10}$ quanta per cell) in order to produce hemolysis, regardless of the concentration of the dye or the

* Hemolysis by photosensitized oxidation.

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intensity of the light. At lower concentrations a single dye molecule absorbs more quanta than at high concentrations, indicating that the dye operates in a cycle, repeatedly oxidizing substrate molecules. At very low dye concentration thousands of quanta may be absorbed per dye molecule (Blum and Gilbert, 1940).

The present study is concerned with hemolysis at low temperatures of erythrocytes photosensitized with rose bengal, and with whether the same mechanism operates under these conditions. The question was suggested by recent experiments of Heinmetz, Vinegar, and Taylor on methylene blue–photosensitized bacteria in the frozen state (1952). These investigators suggest that a complex of dye, O_2, and cellular substrate is involved in photodynamic action. Our experiments do not support this idea, as will be pointed out in the discussion.

EXPERIMENTAL

For studying photosensitized hemolysis at low temperatures rabbit erythrocytes were suspended in solutions of 70 per cent glycerol and 30 per cent water containing 0.9 per cent NaCl. The suspensions were frozen rapidly with solid CO_2 or liquid nitrogen. The 70 per cent glycerol solution solidifies without gross evidence of crystal formation; crystals form if the concentration of glycerol is less than about 65 per cent. The cells do not hemolyze on freezing in solutions of lower glycerol concentration,[1] but the crystallization gives poor optical conditions and results in redistribution of dye between phases.

To avoid hemolysis by sudden osmotic changes the glycerol was added slowly. The usual procedure was to add glycerol containing 0.9 per cent NaCl to a suspension of washed red cells in 0.9 per cent NaCl, taking about 75 minutes to reach a glycerol concentration of 70 per cent. To this mixture was added the desired amount of dye dissolved in 0.9 per cent NaCl solution. The concentration of the erythrocytes was adjusted so as to give approximately 3.5×10^4 cells per c. mm. in the final suspension. Cells so prepared may be frozen and thawed and still remain for 24 hours or longer without hemolyzing.

Experiments at −79°C.

Most of the experiments were carried out at the temperature of melting CO_2 (−78.5°C.).

The cell suspensions were placed in test tubes or Petri dishes and frozen quickly by plunging the vessels into crushed CO_2. After 20 minutes' freezing the vessels were transferred for illumination to a smooth bed of finely divided solid CO_2 about 3 inches thick, which was renewed as necessary. The source of light was a bank of two 15 watt "daylight" fluorescent lamps placed 3 inches from the vessels.

[1] According to Sloviter (1952) 15 per cent glycerol is sufficient to prevent hemolysis of the greater part of the cells on cooling.

After the exposure period the vessels were removed from the light and placed in water at room temperature to thaw the suspensions. The cells did not hemolyze immediately upon thawing; but, following adequate doses of light, hemolysis occurred at the end of 24 hours. Usually a series of samples was exposed to light for lengths of time increasing in increments of 1.5 times. The "threshold" was then determined as the lowest dose (measured in minutes, since intensity is constant) at which complete hemolysis was observed at the end of 24 hours. In some cases it was found best to make comparisons on the basis of equal degree of partial hemolysis. For comparison at room temperature similar series were illuminated with an aluminum reflector behind the vessels. Under these conditions the hemolytic effectiveness should be roughly comparable with that in the experiments described by Blum

TABLE I

Effect of Previous Freezing and Thawing on Photosensitized Hemolysis at Room Temperature

Freezing temperature −79°C. Photosensitizer 10^{-5} M rose bengal.

Experiment	Conditions	Temperature during illumination	Duration of illumination required to produce comparable hemolysis
		°C.	*min.*
22	Unfrozen	27	4 ½
	Frozen and thawed	27	2
23	Unfrozen	26	4 ½
	Frozen and thawed	26	7
26	Unfrozen	26	4 ½
	Frozen and thawed	26	4 ½

and Gilbert (1940); that is, for purposes of comparison we may assume that 10^{10} quanta absorbed per cell would be required for hemolysis at room temperature.

Controls identically treated but not exposed to light do not hemolyze at the end of 24 hours or more. To determine whether freezing of the photosensitized cells alters their sensitivity to light, samples were frozen in the usual manner, then thawed and illuminated at room temperature. Table I shows that the doses required to produce hemolysis do not differ greatly for frozen and thawed suspensions and unfrozen suspensions. The variability shown in the table suggests the accuracy with which thresholds for frozen and unfrozen states can be compared.

O_2 Requirements.—To assure that we deal with photooxidation by O_2 some of the suspensions were evacuated before they were frozen and illuminated. For this purpose the suspensions were placed in Thunberg tubes and evacuated until the color of the cells changed, indicating that the partial pressure of O_2 had been markedly reduced. This treatment raised the threshold to illumination

both at room temperature and at −79°C., as shown in Table II, showing that O_2 is essential for photodynamic hemolysis under both conditions.

Comparison of Hemolysis at Room Temperature and −79°C.—Table III describes three experiments in which hemolysis was compared at room temperature and at −79°C. in terms of the length of exposure to light. Much longer

TABLE II

Effect of O_2 Lack on Hemolysis

Photosensitizer 10^{-5} M rose bengal.

Experiment	Conditions	Temperature during illumination	Duration of illumination	Hemolysis*
		°C.	min.	
17	Air	25.5	2½	+
	Evacuated	25.5	2½	−
	Air	−79	86	+
	Evacuated	−79	86	−

* +, complete hemolysis; −, no hemolysis; 20 hours after exposure.

TABLE III

Comparison of Hemolysis at Room Temperature and −79°C.

Photosensitizer 10^{-5} M rose bengal.

Experiment	Temperature during illumination	Duration of illumination required to produce comparable hemolysis
	°C.	min.
16	27.5	3
	−79	86
20	23.3	1½
	−79	68½
17	25.5	2½
	−79	86

exposures are required at −79°C. than at room temperature. The interpretation of this difference will be considered in the Discussion.

Persistence of Hemolytic effect at Low Temperature after Exposure.—It is conceivable that the dye might enter some long lived intermediate state which would persist for a long time at low temperatures, and on return to room temperature would react with the cell to cause hemolysis. To examine this possibility sensitized cells were exposed to light at −79°C., and this low temperature was maintained for varying times after the end of exposure. As seen in Table IV, the cells hemolyzed when kept at −79°C. for as long as 2 hours after the exposure. It is unlikely that an "activated state" of the dye would have such a long

lifetime even at this temperature. If there is any intermediate formed it must be quite stable. It seems more probable that the hemolysis results from photochemical change that is completed during the illumination of the cells, although the resulting damage to the cell does not manifest itself in the gross until many hours later.

TABLE IV

Persistence of Hemolytic Effect after Exposure at −79°C.

Photosensitizer 10^{-5} M rose bengal.

Experiment	Conditions	Duration of illumination	Duration at −79°C. after illumination	Hemolysis*
		min.	*min.*	
27	Illuminated	60	0	(+)
		60	30	(+)
		60	60	+
		60	120	(+)
	Control	0	0	(+)
		0	30	−
		0	60	−
		0	120	−
	Illuminated	90	0	(+)
		90	30	(+)
		90	60	+
		90	120	(+)
	Control	0	0	−
		0	30	−
		0	60	−
		0	120	−

* +, complete hemolysis; (+), partial hemolysis; −, no hemolysis; 24 hours after exposure.

Effect of Dye Concentrations.—Under appropriate conditions at room temperature, dye concentration and dose of illumination are approximately reciprocal (Blum and Gilbert, 1940). To give adequate doses to produce complete hemolysis at −79°C. using rose bengal concentrations lower than 10^{-5} molar was not feasible, but partial hemolysis was obtained with 10^{-6} molar rose bengal at −79°C. At lower concentrations the results were uncertain.

Experiments at −210°C.

A few experiments were carried out at the temperature of boiling N_2 (−209.86°C.). In these, test tubes containing the erythrocyte suspension, pho-

tosensitized with 10^{-5} molar rose bengal, were immersed in liquid N_2 and illuminated through the walls of an unsilvered Dewar flask. Illumination for 6 hours did not produce any trace of hemolysis; 24 hours after the end of the exposure the illuminated suspension was indistinguishable from controls kept in the dark at room temperature, and from a control in the dark kept at −210°C. for 6 hours and then at room temperature. At room temperature traces of hemolysis were observed with illuminations as short as 1 or 2 minutes. We conclude that lowering the temperature to −210°C. decreases the hemolytic effectiveness at least 1,000-fold.

Viscosity at Low Temperatures

As will be pointed out in the Discussion, viscosity at low temperature is an important factor in the interpretation of these experiments. In freezing to −210°C. the suspensions containing 70 per cent glycerol contract and fracture; their viscosity at this temperature is too high to measure. On the other hand, at −79°C. such suspensions display a certain amount of flow in the course of an hour. Determination of the viscosity of mixtures of 70 per cent glycerol, 30 per cent H_2O, and 0.9 per cent NaCl at the latter temperature was carried out for us by Professor Walter Kauzmann by measuring the flow in a tube of radius 0.15 cm. under a pressure difference of 1 atmosphere. He obtained the value 5×10^6 poises (estimated error 20 to 30 per cent), which falls in line with viscosities given by Miner and Dalton (1953) for other glycerol combinations and temperatures.

Photobleaching

When rose bengal and other fluorescein dyes are exposed to light in the absence of an oxidizable substrate the dye undergoes irreversible bleaching through oxidation; the dye takes the place of the substrate X in Equation 1. Bleaching of dyes dissolved in glycerol was easily demonstrable at room temperature, but not at −79°C. For example, under conditions of illumination comparable to those used in our experiments on hemolysis, 10^{-5} molar rose bengal in glycerol bleached 47 per cent (measured spectrophotometrically) in 4 hours at room temperature, whereas there was no detectable bleaching at −79°C.

DISCUSSION

The reaction scheme presented in the introduction (Equation 1) requires the simultaneous meeting of three molecules or two consecutive meetings. Thus the interval between molecular encounters, which is greatly lengthened at low temperatures, should be an important factor in determining the rate of such reactions. But another factor involved is the time the molecules remain able to react after being activated by a quantum of light. In the solid state the dye

molecules may, after capture of a quantum, enter into a very long lived phosphorescent state which Lewis *et al.* (1944, 1945) identify as a triplet or bi-radical. There may be other long lived intermediate states, and at low temperatures in the solid or semisolid state the low frequency of molecular encounters may be compensated to a certain extent by increase in the length of time the molecules remain able to react. Thus for a full interpretation of the effect of temperature on rate and photochemical efficiency under conditions such as those studied in this paper many factors would have to be taken into account.

Let us consider these conditions. Bleaching of the dye was studied in a 70 per cent solution of glycerol and water. The concentration of dye was 10^{-5} moles per liter, that of O_2 was 8.9×10^{-5} moles per liter.[2] The average distance between rose bengal and O_2 molecules should be about 2×10^{-5} cm. The diffusion constant of O_2 may be estimated from the Einstein equation (1926)

$$D = \frac{RT}{N} \times \frac{1}{6\pi\eta\rho} \tag{2}$$

in which D is the diffusion constant, R the gas constant, N Avogadro's number, T the absolute temperature, η the viscosity, and ρ the radius of the molecule. The viscosity of the 70 per cent glycerol mixture at 25°C. is 1.8×10^{-1} poises; at −79°C. it is 5×10^{5} poises.[3] Taking the radius of the oxygen molecule as 0.5 Angstrom units, D would be about 2.4×10^{-6} sq. cm. per second at room temperature, 5.7×10^{-13} sq. cm. per second at −79°C. The average distance moved by the molecule in time t can be estimated from the equation (Einstein, 1926)

$$u = \sqrt{2Dt} \tag{3}$$

in which u is the velocity and t the time. The average distance moved by an oxygen molecule in 1 second should be 2.2×10^{-3} cm. at 25°C. and 1.1×10^{-6} cm. at −79°C. The movement of rose bengal should be slower because the molecule is larger. The movement of either molecule at 25°C. should be about 2,000 times as great in unit time as that at −79°C. Considering the distance between the molecules, the reduction of the diffusion rate at the lower temperature may well be the limiting factor as regards bleaching, which requires encounters between dye and O_2 molecules.

In the case of the red blood cells the situation is somewhat different, the O_2 and dye molecules being much closer together in the cell than in solution. The rabbit red cell has a volume of about 8×10^{-11} cc. and contains when in equilibrium with air about 6×10^{8} molecules of O_2.[4] Assuming uniform concentration,

[2] Based on solubility of oxygen in glycerol solutions at 25°C., given by Seidell (1940); it is assumed that with rapid freezing this concentration remains unchanged.

[3] Value for 25°C. from Miner and Dalton (1953); value for −79°C. from the present paper.

[4] Based on values given by Prosser (1950).

the distance between O_2 molecules would be 5×10^{-7} cm. Assuming that about 5×10^{-16} moles of rose bengal is taken up per red cell[5] the molecules should be on the average 3×10^{-6} cm. apart; but the dye is probably concentrated near the surface of the cell, in which region it may have a much higher concentration. The O_2, on the other hand, is for the most part loosely combined with the hemoglobin and is distributed more uniformly. There are reasons to believe that only a very small fraction of the O_2 is used up in photodynamic hemolysis, and this is presumably the part closest to the surface of the cell. Under these circumstances the diffusion of the oxygen molecule should be much more important than that of the rose bengal molecule; but if this alone were the limiting factor we might expect a greater reduction in the efficiency of photodynamic hemolysis in going from room temperature to −79°C. than we have observed (30- to 50-fold in our experiments, Table III). Longer lifetimes of "intermediate" states at lower temperatures should tend to increase the photochemical efficiency, however. The viscosity within the cell could be considerably lower than that in the 70 per cent glycerol medium outside; particularly if the site of photochemical reaction is the cell membrane, which is composed largely of protein and lipids. When the temperature is lowered to −210°C. the glycerol mixture has a very high viscosity, say 10^{12} poises, permitting virtually no movement of molecules. At this temperature there would probably be little or no movement of molecules in any part of the cell, and it is not surprising that there is no photodynamic hemolysis under these conditions.

The importance of molecular encounters would be more or less ruled out if we could accept the idea of Heinmetz, Vinegar, and Taylor (1952) that the dye, O_2, and substrate are in some way combined at the time the dye captures the quantum. But in that case the efficiency of the reaction might be expected to be unaffected, or even to increase at low temperatures, because of increased lifetimes of intermediate states; whereas in our experiments there is a great reduction in efficiency and this also seems to be true in those of Heinmetz *et al.* Moreover, some photosensitized hemolysis might be expected at −210°C., but this we did not find. There seems to be a still more serious objection to the conclusions of Heinmetz *et al.* as applied to the case of photodynamic hemolysis with rose bengal. As pointed out in the introduction, the dye molecule acts cyclically. At the highest concentration used in our experiments (10^{-5} M rose bengal) the molecule goes through about 50 cycles at room temperature; at lower concentrations the number increases proportionately (based on the data of Blum and Gilbert, 1940). The binding of the dye, substrate, and O_2 does not help to explain how this cycle could go on at −79°C. if there were no movement of molecules.

Fiala (1949) postulated a loose binding between dye and oxygen. Our experi-

[5] Estimated from Gilbert and Blum (1942), for cell suspensions in phosphate buffer.

ments which showed no photobleaching at −79°C. seem to make this unlikely, particularly since photodynamic hemolysis goes on at this temperature.

When we compare our experiments on photodynamic hemolysis with the photosensitized killing of bacteria at −15°C. described by Heinmetz *et al.*, it seems probable that the basic mechanism is the same, but that there are minor points of difference. In the first place, although ice at −15°C. has a very high viscosity (about 10^{13} poises) which would virtually prevent all movement of molecules, it does not seem necessary to assume that the movement of molecules within all the phases of the cell is so restricted at this temperature. Heinmetz *et al.* offer considerable evidence for some form of binding between methylene blue and the bacterial substance. This is also true for rose bengal and the red blood cell,[6] but does not constitute evidence in either case for combination with O_2. An important difference between photodynamic hemolysis and killing of bacteria is the number of molecules involved; the dose-survivor curve given by Heinmetz *et al.* indicates that at most only a few molecules have to be put out of commission to prevent the bacterial cell from reproducing.[7] In the case of photodynamic hemolysis with rose bengal the hemolytic process involves many molecules.[8] When all these matters are taken into account there may be little discrepancy between our findings and those of Heinmetz *et al.* as regards the basic mechanism of photodynamic action.

The photochemist is reluctant to assign an intimate reaction mechanism even for photochemical reactions taking place in very much less complicated systems than those we study here. A large number of alternative schemes can usually be written for a particular photochemical reaction. The variety of possible reactions of dyes after activation by light is sketched by Franck and Livingston (1941); see also Uri (1952). Most of the mechanisms suggested for photosensitized oxidation meet with difficulties. There are physical objections to the idea that the activated dye molecule transfers its energy of activation to either the O_2 molecule, or to the substrate molecule. The idea that an intermediate peroxide is formed, either a peroxide of the dye or hydrogen peroxide, is ruled out on various grounds (Fiala, 1949; and with regard to photodynamic action see: Blum, 1941). Under appropriate conditions dye-leucobase equilibria are shifted by light so that both photooxidation and photoreduction may be demonstrated; but such reactions appear to have little to do with photosensitized oxidations by molecular oxygen, in which the oxidation-reduction poten-

[6] The general importance of uptake of dye by the cell for photodynamic action has been emphasized elsewhere (Blum (1941); Gilbert and Blum (1942)).

[7] In the case of ultraviolet radiation without photosensitizer only one molecule has to be affected in some cases.

[8] Preliminary measurements made by Mr. John S. Cook in this laboratory indicate that a large number of molecules have to be changed before hemolysis occurs. This fits with other aspects of photodynamic hemolysis (see Blum, 1941).

tial of the dyes seems of no direct importance as regards the efficiency of the reaction. For example, rose bengal is very difficultly reducible, whereas the oxidation-reduction reactions of methylene blue are highly reversible, yet both are efficient photosensitizers.

Uri (1950) has recently suggested that a free radical, O_2^-, formed by reaction of O_2 with the triplet state of the dye, may be an intermediate in these reactions. This has attractive aspects. The scheme presented by Uri includes the formation of H_2O_2, based on the studies of Blum and Spealman (1933), who showed this substance to be formed and suggested it as an intermediate. Subsequent studies indicate that H_2O_2 is probably a by-product (see Blum, 1941; Fiala, 1949), but the formation of this substance as an intermediate does not seem essential to Uri's scheme. The kind of oxidation that occurs in photodynamic action seems to be one in which the normal metabolism of the organism is not involved (see Blum, 1941); the over-all effect is a destructive one, probably involving materials which are not concerned in normal oxygen metabolism. Again Uri's idea of the formation of the free radical O_2^- is suggestive in this regard, since such an intermediate is presumably not produced in the normal metabolism of the organism.

SUMMARY

It is shown that photodynamic hemolysis may occur at −79°C. if the erythrocytes are suspended in a solution containing 70 per cent glycerol which prevents hemolysis by freezing; but that there is no hemolysis under the same conditions at −210°C. At the higher temperature the viscosity of the solution is still low enough to permit appreciable movement of molecules, whereas at the lower temperature the molecules must be virtually immobile. The findings are compatible with the idea that the dye molecule acts in a cycle, bringing about successive oxidations by O_2 molecules, as has been shown for photodynamic hemolysis at room temperature. The assumption of a combination between dye, O_2, and substrate does not explain photosensitized hemolysis in the semi-solid state. The mechanism of photosensitized oxidation by O_2 is discussed.

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