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PEROXIDASE ACTIVITY IN HUMAN RED CELL: A BIOLOGICAL MODEL FOR EXCITED STATE MOLECULES GENERATION

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Summary. The presence of enzymically generated triplet acetone in red cells and energy transfer to eosin, rose bengal and 9,10-dibromoanthracene-2-sulfonate was indicate by: (1) product distribution; (2) $K_{ET}\tau^{0}$, similar to the 2-methylpropanal/peroxidase/O_2 system; (3) correlation between hemolysis, oxygen uptake and photon emission; (4) membrane protection by energy acceptors, and (5) by comparison of the 2-methylpropanal/peroxidase/O_2 system with 2-methylpropanal/red cells/membranes/O_2 and 2-methylpropanal/acid extractable protein from red cells membrane/O_2 systems, which have a high peroxidase activity.
This is the first report of a biological system producing a photohemolysis effect in the dark.

The generation of excited states in biochemical systems is under investigation in our laboratories (1-23). The enzymic formation of triplet acetone from 2-methylpropanal, in high yield by a peroxidase system has been achieved probably via a dioxetane intermediate (5,17). It was shown that the electronic energy from excited acetone can be transferred to added acceptors, such as eosin, rose bengal, riboflavin and DBAS[1] (19) and used to perform chemical work, such as DNA single strand breaks (18,20), chlorpromazine oxidation (21) and phytochrome transformations (22).

This paper reports the formation of excited state molecules in a red cell by interaction with 2-methylpropanal and its involvement in the hemolysis.

MATERIALS AND METHODS

HRP (Type VI) and Triton X-100 were from Sigma. Eosin and rose bengal were from Merck. DBAS and DPAS were prepared by the published method (5). Sodium azide and DABCO and 2-methylpropanal were from Aldrich.

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[1] Abbreviations: HRP, horseradish peroxidase; DABCO, 1,4-diazobicyclo (2.2.2) octane; DBAS, 9,10-dibromoanthracene-2-sulfonate; DPAS, 9,10-diphenylanthracene-2-sulfonate.

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Human blood was collected from hematologically normal adult donors, utilizing sodium citrate as anticoagulant. The blood was then centrifuged for 15 min at 1500 x g. The plasma was carefully removed, including the coat of leukocytes, and washed 3 times with isotonic sodium chloride, pH 7.4. All sodium chloride solutions used were prepared by dilution of the buffered stock solution prepared as described by Parpart et al., (24). Red cells were resuspended in the wash solution to make finally 40% hematocrit value.

Red cell membranes were prepared by hemolysing washed red cells with 30 volumes of 10 mM Tris, pH 7.4, at 4°C. Subsequently, the membranes (0.1 ml) were dissolved in 0.05 ml of 0.2% Triton-X-100, diluted to 1 ml with 10 mM Tris, pH 7.4, and left 12 hours at 0°C. The acid extractable protein from red cell membranes was obtained by the method of Braganca et al., (25).

The standard reaction mixture was prepared as follows: To 50 µl of red cell suspension in 1.5 ml of 1.0 M phosphate buffer pH 7.4 and 1.0 ml of 0.1 M pyrophosphate buffer pH 7.4, was added 84 mM 2-methylpropanal and convenient amounts of energy acceptors at 37°C.

Absorption spectra were taken on a Zeiss DMR-21 recording spectrophotometer using 1 cm cells. The chemiluminescence was measured in a Beckman LS-250 liquid Scintillation Counter. Oxygen consumption was determined with a Yellow Spring Instrument Model 53 Oxygen monitor. The fluorescence spectra were taken on a Hitachi-Perkin Elmer MPF-4 Fluorescence Spectrophotometer.

RESULTS AND DISCUSSION

When 84 mM of 2-methylpropanal was added to red cells a 20% of membrane cleavage was observed, following the absorbance at 656 nm, where the hemoglobin absorption was negligible and the apparent absorption is due to high scattering by unhemolyzed cells (26). Similar results were obtained by following the appearence of hemoglobin in the medium (575 nm band) (Table 1). The hemolysis correlates within experimental error with oxygen uptake and photon emission occurring during the interaction of the 2-methylpropanal with the red cells (Fig. 1).

The product distribution showed that the 2-methylpropanal/peroxidase/O_2 system, and 2-methylpropanal/red cells/O_2 systems were similar, they produced acetone and formic acid as main products (5).

The DBAS/2-methylpropanal/red cell/O_2 system and DBAS/2-methylpropanal/peroxidase/O_2 system showed similar emission rates (Fig. 2). That the excited molecules generated during this interaction play an important role in the hemolysis was shown by using different energy acceptors. None of them produced any effect on the red cells in the absence of the aldehyde. Table 1, shows the percentage of membrane protection. Clearly, DBAS (20%), eosin (53%) and rose

TABLE 1. INFLUENCE OF VARIOUS SUBSTANCES ON THE INTEGRATED PHOTON EMISSION AND HEMOLYSIS DURING THE INTERACTION OF 2-METHYL PROPANAL AND HUMAN RED CELLS.

	INTEGRATED EMISSION AT 6 min COUNTS x 10^{-6}	ABSORPTION OF MEMBRANES FOLLOWED BY 656 nm BAND AT 6 min.	PERCENTAGE OF HEMOLYSIS FOLLOWED BY 575 nm BAND AT 6 min.	PERCENTAGE OF PROTECTION AT 6 min.
Red cells[a]	0.0	1.500	0.0	-
Red Cells/2-methyl propanal (84 mM)	12.3	1.395	20.0	-
+ DPAS (24 uM)	15.2	1.370	23.0	-
+ DBAS (10 uM)	40.0	1.415	16.0	20.0
+ Eosin (100 uM)	44.0	1.450	9.3	53.0
+ Rose bengal (25 uM)	42.0	1.400	16.6	17.0
+ Azide (10 mM)	8.0	1.422	15.0	25.0
+ DABCO (10 mM)	19.4	1.395	20.0	-
+ DABCO (20 mM)	21.7	1.395	20.0	-
+ HCO_3^- (20 mM)	14.0	1.395	20.0	-
+ Benzoate (10 mM)	10.0	1.395	20.0	-
+ Air	250.0[b]	-	-	-
+ Oxygen (5 min)	329.2[b]	-	-	-
+ Nitrogen (5 min)	248.9[b]	-	-	-

The reaction medium contained 50 ul of human red cells (40% in buffered isotonic sodium chloride) in 1 M phosphate buffer pH 7.4 and 0.1 M pyrophosphate buffer pH 7.4 at 37°C.

a) In the absence of acceptors. Bicarbonate, azide or gas alone did not altered the red cells at the reaction condition.

b) The time of counting was 30 min.

bengal (17%), partially protect the red cell from disruption. In the case of DBAS and eosin the double reciprocal plot (Fig. 3) of the enhancement of photon emission were measured and were linear with $K_{ET}\,\tau^{o}$ values of $3 \times 10^{4}\ M^{-1}$ and $2 \times 10^{4}\ M^{-1}$ respectively. These values are much larger than the value of $1.2 \times 10^{3}\ M^{-1}$ obtained for sorbic acid in the 2-methylpropanal/peroxidase/O_2 system, for which the act of quenching must surely require intimate donor acceptor con-

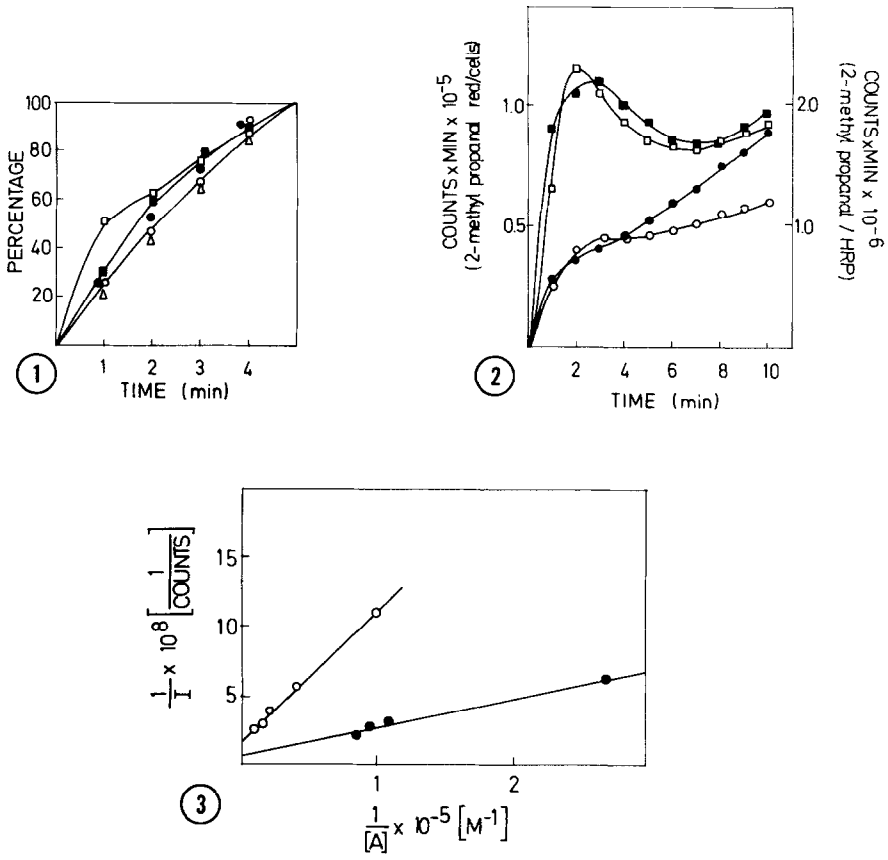

Fig. 1. Correlation between O_2 uptake (-○-), photon emission (-△-), hemolysis (followed at 575 nm absorption band of hemoglobin) (-■-), membrane modification (at absorption band of 656 nm) (-●-), extracellular potasium ion (-□-), when 50 μl of red cells was hemolyzed at standard conditions in the presence of 84 mM of 2-methylpropanal.

Fig. 2. DBAS effect on the photon emission rate by 2-methylpropanal/HRP/O_2 system and 2-methylpropanal/red cells/O_2 systems: (a) 2 μM DBAS, 42 mM 2-methylpropanal, 2 μM HRP (Type VI) (-□-), and in the absence of DBAS (-○-); (b) 8.5 μM DBAS, 84 μM 2-methylpropanal and 50 μl red cells (40%) (-■-), and in the absence of DBAS (-●-), at standard condition.

Fig. 3. Double reciprocal plot of the effect of DBAS (-●-) and eosin (-○-) upon the photon emission of 2-methylpropanal/red cells/O_2 system at standard conditions.

tact (19). They are, however, similar to the values of 7.5×10^4 M^{-1} and 3×10^4 M^{-1} obtained for the energy transfer to DBAS and eosin respectively in the 2-methylpropanal/peroxidase/O_2 system (17), an acceptor for which quenching mechanisms such as a "long range" energy transfer, which does not require intimate contact are operative.

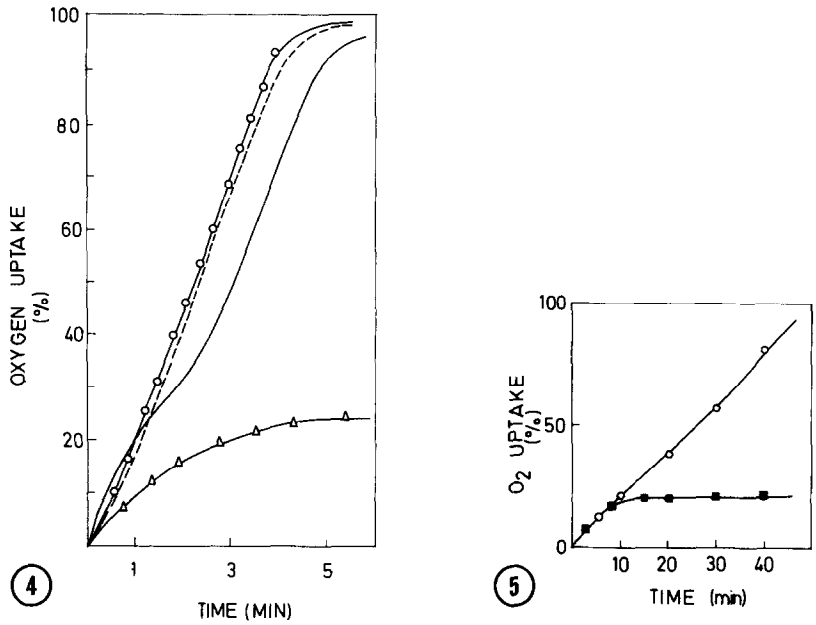

Fig. 4. Oxygen uptake of 84 mM of 2-methylpropanal in the presence of: (a) HRP, 2 nM (type VI) (-○-); (b) 0.1 ml dissolved membrane (- - -) and in the absence of enzyme (-△-). Oxygen uptake of incubated 0.1 ml dissolved membrane plus 84 mM 2-methylpropanal (—) at standard conditions.

Fig. 5. Oxygen uptake of 84 mM 2-methylpropanal and acid extractable protein from red cells hemolyzates: (a) 0.2 ml of protein solution (0.06 mg/ml), 30 mM Mn^{++} (-○-); (b) 2-methylpropanal and 30 mM Mn^{++} (-●-) at standard conditions.

The fact that DBAS, eosin and rose bengal partially protect the red cell membrane indicates that some of the hemolytic effect is produced by energy transfer from the excited acetone to some component of the membrane. A 10 mM sodium azide or cyanide protect in 25% the red cell membrane from hemolysis. This probably indicates the implication of a hemoprotein in the 2-methylpropanal activation. This was observed by us almost a year ago (27). Very recently Snyder et al., (28) reported that a small but significant amount of catalase (1.6%) was retained on human red cell membranes prepared by hemolyzing washed red cells. Membrane-bound catalase has a relative higher peroxidase activity compared with the soluble enzyme fraction. When 0.1 ml solubilized red cell membrane was used, as described in the Materials and Methods section an equivalent horseradish peroxidase activity of 2×10^{-9} M/0.1 ml of original red cell

membrane was found. This enzymatic reaction shows sensitivity to the sequence of addition of the component (Fig. 4). An acid extractable protein from the red cell membrane was found to be also active on 2-methylpropanal (Fig. 5).

A comparative study of activation of 2-methylpropanal with different hemoproteins was carried out. The results demonstrated similarities between the behaviour of peroxidase and the protein in the red cell membrane. No reaction was observed with hemoglobin, methemoglobin, and catalase in standard conditions.

The DABCO experiment on photon emission at 6 min reaction (Table 1) is indicative of some singlet oxygen generation in the red cell system (16). The absence of any effect of bicarbonate and benzoate eliminates the $OH^{\cdot}$ species as generator of membrane damage.

Thus, the observed hemolytic effect due to 2-methylpropanal activated by membrane-bound catalase (possible in a monomeric form) must be partially a "dark photobiochemical effect". This is supported by the occurrence of hemolysis, damage which can be induced photochemically (26,29). Photoprotection by eosin, rose bengal and DBAS which are efficient triplet acceptors, and correlation of hemolysis with oxygen uptake and photon emission support this point.

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