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PHOTOBIOCHEMISTRY IN THE DARK: PHOTOHEMOLYSIS OF RED CELLS SENSITIZED BY CHLORPROMAZINE-BIOENERGIZED TRIPLET ACETONE SYSTEM

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SUMMARY. Chlorpromazine is decomposed when it is treated with bioenergized triplet acetone from the 2-methylpropanal/red cells/O_2 system, forming chlorpromazine-5-oxide, with a concomitant strong hemolytic effect observed by a spectrophotometric method. Experiments with external superoxide dismutase, catalase, benzoate and bicarbonate indicate the absence of $O_2^{\cdot}$, H_2O_2 and OH· species as the precursor of the hemolytic effect.

Comparison between the 2-methylpropanal/peroxidase/O_2 system and the 2-methylpropanal/red cells/O_2 system in the presence of chlorpromazine, indicate that essentially the same type of mechanism occurs in both cases.

These results could explain the in vivo hemolytic and toxic effect of chlorpromazine in the dark.

The enzymic formation of triplet acetone in high yield, by a 2-methylpropanal/peroxidase/O_2 system (1-3) and from human red cells (4) has been achieved. It was shown that the electronic energy from excited acetone can be transferred to eosin, rose bengal, riboflavin and to DBAS[1] (5) and used to perform chemical work such as CPZ oxidation, phytochrome photoalteration, lysozyme-riboflavin adduct formation (6), single strand breaks in DNA (7) and red cells hemolysis (4).

CPZ produces several pharmacological effects (8) and it is phototoxic (9-11). The formation of CPZO and $CPZ^{+\cdot}$, which are known photoproducts of CPZ (12), together with the supression of acetone chemiphosphorescence generated by 2-methylpropanal/HRP/O_2 system (1-3), strongly indicated that energy was trans-

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[1] ABREVIATIONS. DBAS, 9,10-dibromoanthracene-2-sulfonate; CPZ, chlorpromazine; CPZO, chlorpromazine-5-oxide; $CPZ^{+\cdot}$, chlorpromazine radical cation; HRP, horseradish peroxidase; DPAS, 9,10-diphenylanthracene-2-sulfonate.

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ferred from triplet acetone to CPZ (13). This was the first report of photobiochemical effect without light.

Johnson (14), using washed normal human red cells in the presence of CPZ at pH 7.4, confirmed that photohemolysis can be observed with the drug. Blum (15) ascribes photohemolysis to damage to the red cell membrane caused by energy transfer from radiation induced excited drug molecules. In this regard, it is noted that photohemolysis of the red cells sensitized by CPZ proceeds by a non-singlet oxygen pathway (16). Recently it was postulated that CPZ photohemolysis may be due to the action of cytotoxic photoproduct which acts specifically on the red cell membrane (17).

Recently, a membrane-bound catalase (4) was found to generate triplet acetone in human red cells by interacting with 2-methylpropanal. It was interesting to couple this bioenergized process under these biological conditions.

This paper reports a dark photohemolysis due to the formation of $CPZ^{\ddagger}$ by energy transfer from bioenergized triplet acetone to CPZ at concentration where the hemolysis by the component is minimal.

MATERIALS AND METHODS

HRP (Type VI), superoxide dismutase and catalase were from Sigma Chem. Co. Eosin and rose bengal were from Merck. 2-Methylpropanal was from Aldrich and CPZ. HCl was from Rhodia Co. (Brazil).

Human blood was collected from hematological normal adult donor, utilizing sodium citrate as anticoagulant. Red cells were obtained by a published method (2).

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 Springs Instrument Model 53 Oxygen Monitor. The fluorescence spectra were taken on a Hitashi Perkin Elmer MPF-4 Fluorescence Spectrophotometer.

RESULTS AND DISCUSSION

When 84 mM of 2-methylpropanal was added to red cells which previously were incubated (30 sec) with 0.27 mM CPZ, a strong hemolytic effect was observed by following the absorbance at 656 nm (Fig. 1). The hemolytic effect

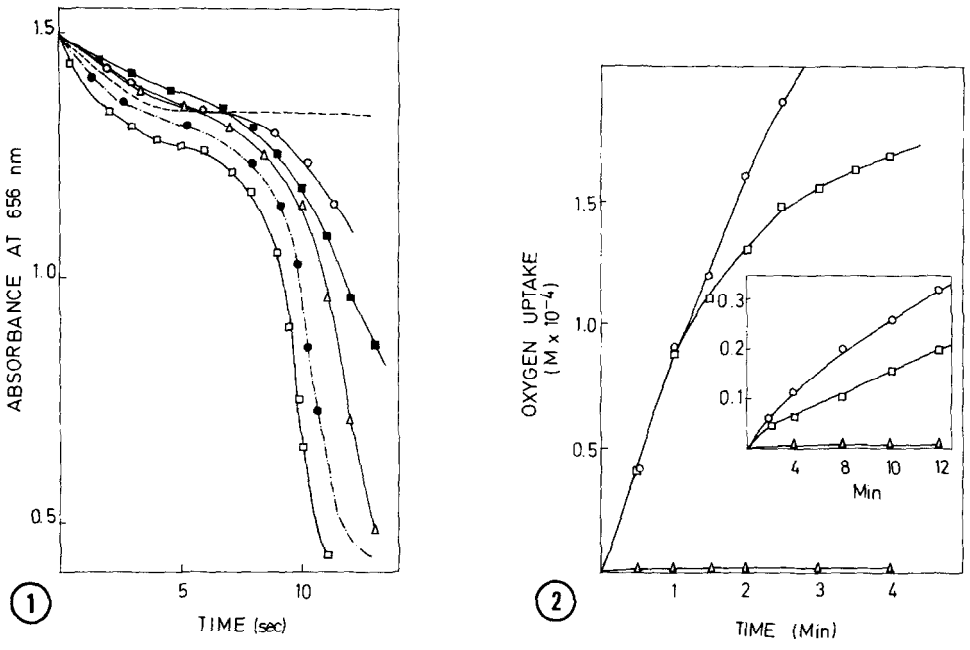

Fig. 1. Lysis of red cells by 0.27 mM CPZ and 84 mM 2-methylpropanal at 37°C. (a) red cells suspension (---); and 2-methylpropanal (---); and CPZ (---); (b) red cells suspension, 2-methylpropanal and CPZ; in the presence of: air (-●-); oxygen (-□-); nitrogen (-Δ-); 2 μM of HRP (-O-); 25 μM eosin (-■-). The standard conditions are asin Table 1.

Fig. 2. Oxygen uptake of 84 mM of 2-methylpropanal in the presence of: (a) 2 μM HRP (-O-); (b) 2 μM HRP and 1 mM CPZ (-□-); (c) The symbol in set (-□-) and (-O-), represent the oxygen uptake with and without CPZ respectively, in the presence of human red cells at standard conditions. CPZ and 2-methylpropanal in the absence of HRP (-Δ-).

is, to some extent, oxygen dependent and when external HRP was added the initial hemolytic rate was decrease. This result indicates that the hemolytic effect of CPZ is related to a process catalyzed by an internal enzymatic system.

Fig. 2 shows that oxygen consumption by the enzymatic and red cell systems are similar, both in the absence and presence of CPZ.

Fig. 3 shows the fluorescence spectra of CPZ and the formation of CPZO, which is the oxidated product of CPZ, in the presence of 2-methylpropanal and red cells, compared with the peroxidase system.

That the excited species is generated in the main reaction in the red cells, and not by adventitious biological chemiluminescence (19) coming from

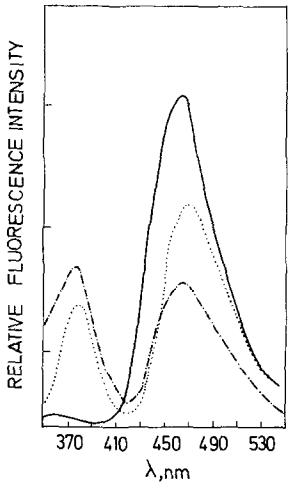

Fig. 3. Fluorescence spectra after 30 sec of interaction of 0.27 mM CPZ and 84 mM 2-methylpropanal (——); and after 2 μM HRP (-.-) and 50 μl (40% hematocrit value) of red cells (...) addition, at standard conditions.

the reagents, was further indicated by the failure of OH· radical (benzoate 10 mM), $O_2^{\cdot-}$ (superoxide dismutase, 435 units] and H_2O_2 (catalase, 300 units) scavengers to affect the emission. That these species did not play any role in the hemolytic effect was also shown by non-observation of membrane modification (increasing or decreasing the hemolytic effect following the 656 nm band) in the presence of benzoate, bicarbonate, catalase, and superoxide dismutase. Only external riboflavin (50 μM) slightly accelerates the initial rate of hemolysis.

The riboflavin effect presumably is due to generation of excited singlet riboflavin by triplet acetone energy transfer, as was observed before by Haun et al (20], to produce triplet riboflavin by intersystem crossing, and subsequent singlet oxygen or OH· radical generation as was observed by Michelson and Durosay (21).

We have shown previously that DBAS, eosin and rose bengal partially protect the red cell membrane from hemolysis (20%, 53% and 17% respectively) from triplet acetone, indicating that some of the hemolytic effect of 2-methylpropanal was produced by energy transfer from the excited acetone to some component of the membrane. In the presence of CPZ this energy presumably is

TABLE 1. INFLUENCE OF CPZ AND XANTHENE DYES ON THE INTEGRATED PHOTON EMISSION DURING THE INTERACTION OF 2-METHYL-PROPANAL AND HUMAN RED CELLS.

	Integrated emission at 10 min. Counts x 10^{-6}		Integrated emission at 10 min. Counts x 10^{-6}
Red cells	0.0	+ 2-methylpropanal eosin (25 μM), and CPZ (0.27 mM)	28.7
+ 2-methylpropanal (84 mM)	25.1		
+ 2-methylpropanal and CPZ (0.27 mM)	18.0	+ 2-methylpropanal and rose bengal (25 μM)	79.4
+ CPZ (0.27 mM)	0.0		
+ 2-methylpropanal and eosin (25 μM)	62.5	+ 2-methylpropanal rose bengal (25 μM) and CPZ (0.27 mM)	18.7

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

transferred efficiently to CPZ, as indicated by the quenching of the photon emission in the direct and enhanced emission (Table 1). Xanthene dyes give a mild protection from hemolysis, as is observed in Fig. 1. DBAS and DPAS produces a complex interaction when CPZ in the red cell was present and it was not possible to use them for protective purpose.

In order to attempt to assign the active species in the photohemolysis in the dark, in the presence of CPZ after energy transfer, we carried out experiments with the 2-methylpropanal/HRP/CPZ/O_2 system.

In red cells, three major enzymes are present to diminish the toxic effect which can be caused by uncontrolled levels of $O_2^{\cdot-}$ or H_2O_2 (derived from dismutation of $O_2^{\cdot-}$ o via other processes). These are superoxide dismutase, catalase and glutathione peroxidase. The superoxide dismutase (erythrocuprein) is entirely cytoplasmatic and in the mature red cells catalase is entirely soluble, whereas the glutathione peroxidase is apparently membrane bound (21). The catalase and superoxide dismutase activities in human red cells are quite high, and protect them efficiently from $O_2^{\cdot-}$ and H_2O_2 toxicity (22).

Experiments on the model of CPZ activation in Fig. 4, shows almost no effect with catalase and increasing effect with superoxide dismutase on the

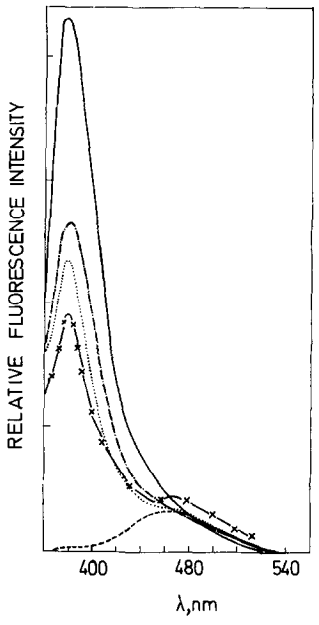

Fig. 4. Fluorescence spectra of 0,27 mM CPZ (---) and CPZO (-.-.) after addition of: superoxide dismutase (350 units) (——); catalase (150 units) (...); and riboflavin (25 μM) (-x-).

oxidation of CPZ, probably due to a protection of peroxidase from $O_2^{\cdot-}$ inactivation (Eq. 1)

$$^3(\overset{\diagup}{\underset{O}{C}}) + {}^3(CPZ...O_2) \rightarrow {}^1(\overset{\diagup}{\underset{O}{C}}) + {}^3(CPZ^{+\cdot}..,O_2^{\cdot-})$$

$$^3(CPZ^{+\cdot} ...O_2^{\cdot-}) \rightarrow CPZ^{+}\text{-}O\text{-}O^{-}$$

$$^3(CPZ^{+\cdot} ...O_2^{\cdot-}) \rightarrow CPZ^{+\cdot} + O_2^{\cdot-}$$

$$CPZ + CPZ^{+}\text{-}O\text{-}O^{-} \rightarrow 2\ CPZO$$

$$O_2^{\cdot-} + HRP \rightarrow HRP \text{ partial inactivated}$$

The decreasing CPZ oxidation in the presence of riboflavin is due to a competitive effect in energy transfer from triplet acetone.

Whether $CPZ^{+\cdot}$ or $O_2^{\cdot-}$, after energy transfer from triplet acetone to CPZ in the membrane, are the active species is still not fully understood but we have work in progress in EPR-labeling experiments with red cell membranes which should help clarify this problem.

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