Role of oxygen radicals scavenging enzymes in the protoporphyrin induced photohemolysis

A. Finazzi-Agrò, G. Floris, M. B. Fadda and C. Crifò

Institute of Medicine, Molecular Biology, Collemaggio, L'Aquila (Italy), Institute of Biological Chemistry, University of Cagliari, Cagliary (Italy), and C.N.R. Centre for Molecular Biology, Rome (Italy), 9 February 1979

Summary. Inhibition of superoxide dismutase by diethyldithiocarbamate or cyanide increases the rate of red blood cells lysis after irradiation in the presence of protoporphyrin IX. Catalase activity, which is decreased during the photohemolytic process, appears to be not essential for the lytic event. No relationship between catalase activity and hemolysis rate was found. Superoxide dismutase appears to prevent only in part catalase inactivation by singlet oxygen.

The photosensitizing effect of protoporphyrin on biological material is well known[1]. There is also a congenital pathological state, the erythropoietic porphyria[2] which is characterized by an abnormal increase of circulating protoporphyrin with concomitant photosensitization of the skin. The red blood cells in this disease undergo photohaemolysis when irradiated in vitro with visible or near UV light[3]. Similarly it is possible to induce the photohaemolysis by externally added protoporphyrin. The mechanism of this porphyrin-induced lysis is not at all clear, though the involvement of active oxygen species is most probable[4-7]. Most of this experimental work has been directed to the study of membrane damage. However, in a previous paper[8] we found that the lysis of human erythrocytes induced by protoporphyrin occurs after a significant loss of cellular catalase activity. This inactivation appears to be a fairly constant phenomenon. Since catalase is an intraerythrocytic enzyme, it was difficult to understand the relationship between its inactivation and the structural damage of the membrane required for the lysis. Furthermore it is known that catalase activity varies very much from species to species[9], and even among individuals of the same species. Thus it seemed worthwhile to extend the study to other enzymes concerned with oxygen radicals, in particular to superoxide dismutase. The present paper deals with the relationship between the level of these enzymes and the rate of photohaemolysis.

Materials and methods. Fresh human blood samples were obtained from the local blood bank. Animal blood samples were obtained by venipuncture and drawn in a test-tube containing oxalate as anticoagulant. Protoporphyrin IX was obtained from Calbiochem. S. Diego (Ca., USA), Catalase was from Sigma, St. Louis (Mo., USA). All the other chemicals were of the best available quality.

Red blood cells were counted in a Thoma-Zeiss chamber. Reticulocytes were stained with brilliant cresylblue.

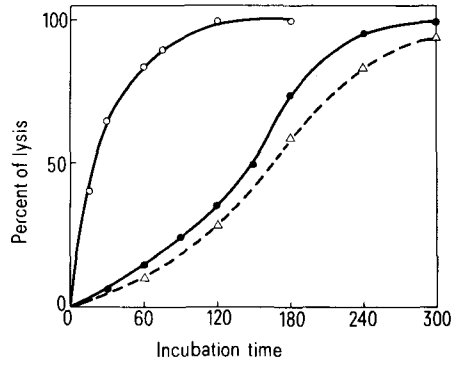
Protein concentration was measured by a biuret procedure[10]. Catalase and superoxide dismutase activities were determined according to Lück[11] and to Concetti et al.[12] respectively. Hemoglobin concentration was checked at 550 nm after reducing the samples to deoxyhemoglobin by addition of few crumbs of solid sodium dithionite.

The photohemolysis experiments were conducted as follows. The red cells were freed from plasma by centrifugation, washed 3 times with isotonic saline and incubated 1 h at 37 °C. The cells were then diluted 1:100 with isotonic sodium chloride to which 10^{-6} M protoporphyrin was added (stock solution 10^{-3} M in dimethylformamide). When required, other substances were also added. The samples were irradiated 15-30 min in a thermostated bath using 2 Osram 150-W white projector lamps placed at 20 cm distance from the samples. Tests and controls without protoporphyrin IX or incubated in the dark were always run in triplicate. After irradiation, the samples were stored in the dark. Aliquots were taken every 30 min and centrifuged. The optical density at 550 nm was recorded. When the lysis was complete, the activity of catalase and superoxide dismutase was determined. The supernatant obtained from osmotically haemolyzed erythrocytes was used as a control.

Results. Red blood cells from various species (see table 1) undergo photohaemolysis in much the same way as previously found for man[8]. Under our conditions, the irradiation of erythrocytes for 13-15 min in the presence of 10^{-6} M protoporphyrin induces the lysis of the cells after 2-4 h of incubation in the dark. As reported in table 1, the loss of catalase activity was variable in the various species under study. In fact it ranges from 0 to 40% of the control samples, indicating that inactivation of catalase is not essential for the lysis. Interestingly, the level of catalase activity is also variable in the various species, being low in dog and sheep and high in man and rabbit in keeping with the report of Maral et al.[9].

No difference in the extent and rate of photohemolysis was observed using blood of rabbits which have been made anemic by phenylhydrazine. In that case, as many as 90% reticulocytes were present. This indicates that younger cells do not withstand photohemolysis any better than older ones. Thus it appears that membrane composition and difference in steady state concentration of metabolites do not modify the photohemolytic process.

Catalase activity is not crucial in the photohemolytic process. In fact, as reported in the figure, azide-treated human erythrocytes appear to be slightly more resistant to photohemolysis. Here the catalase activity determined after photooxidative or osmotic lysis is zero. The small protective effect of N_3^- might be ascribed to the well-known quenching effect of azide on singlet oxygen which is formed by photosensitization in the presence of protoporphyrin IX. Azide is also a good ligand of other metal proteins, like superoxide dismutase, but with a lower affinity constant[14]. In the present experimental conditions, no effect of azide on superoxide dismutase activity was observed. This enzyme can also be inhibited by cyanide[14] or diethyldithiocar-



Effect of azide and cyanide on the rate photohemolysis. After 15 min of irradiation with white light in the absence (●——●) and presence of 1 mM cyanide (○——○) or 1 mM azide (△---△) the erythrocytes were allowed to stand in the dark. Samples were withdrawn at 15-30 min time intervals and the extent of hemolysis determined. For other experimental details, see text.

Table 1. Effect of photooxidative lysis on the level of catalase activity in the red cells of various animals

Species	Catalase activity* (H_2O_2 consumed: mmoles/mg/min)		Residual activity after photohemolysis (%)
	Osmotic lysis	Photooxidative lysis	
Cow	32.5	32.5	100
Rabbit	80.8	80.8	100
Horse	44.2	39.2	88.7
Rat	36.7	32.5	88.5
Mouse	24.2	18.3	82
Dog	4.2	3.3	78
Man	87	61	70
Sheep	15.8	10.8	68
Pig	42.5	26.7	63

* The above reported data are mean values of 3 different determinations.

Table 2. Effect of various inhibitors on the time of hemolysis and on the activity of catalase and superoxide dismutase

Addition	mM	Time required for 100% hemolysis (min)	Residual activity (%) Superoxide dismutase	Catalase
None	-	240	100	70
Diethyldithiocarbamate	2.4	180	0 (60)*	55
Disulfiram	2.4	210	65 (85)*	60
Penicillamine	2.4	300	140	60
KCN	1	90	45	48**
KCN	2	60	32	40**
KCN	3	30	0	30**

The activity of the enzymes was determined after the photooxidative lysis. * The value in parenthesis was determined at the end of irradiation time. ** No difference with controls obtained by osmotic lysis.

bamate even in vivo[15]. It was found that superoxide dismutase activity is strongly reduced by treating red cells with cyanide or diethyldithiocarbamate. In this case, the lysis occurred much earlier (see figure and table 2). The lag phase in the dark before lysis is reduced in a parallel fashion to the inactivation of superoxide dismutase. Photohemolysis is also accelerated by disulfiram (tetraethylthiuram disulfide) which gives a partial superoxide dismutase inhibition (table 2). When superoxide dismutase activity is lowered by inhibitors, the inactivation of catalase is more pronounced (i.e. 50% instead of 30%). Penicillamine, a therapeutically used sulfur-bearing copper chelating agent, does not accelerate the hemolysis nor inhibit superoxide dismutase activity which appears rather to be increased (table 2). A similar stimulation of superoxide dismutase activity by penicillamine was also observed with the purified enzyme (A. Rigo, unpublished results). A superoxide dismutase-like activity of a penicillamine copper complex has been described by Younes and Weser[16].

Discussion. The protoporphyrin sensitized photolysis of erythrocytes occurs most probably through the formation of singlet oxygen inside the membrane which causes oxidation of its lipid and/or protein components. However, it appears that 2 cytoplasmic enzymes like catalase and superoxide dismutase are involved in the process. In fact catalase is partly inactivated in the course of irradiation of human red cells in the presence of protoporphyrin[8]. It was proposed that catalase might play an active role in the cell defense against singlet oxygen formed, which only after a substantial decrease of catalase activity would attack the membrane structure. This hypothesis is strongly challenged by the above-reported data which show red cells of different species undergoing photohemolysis in the same way as the human ones but without any catalase inactivation. Furthermore, the level of catalase activity varies very much in the species examined (table 1). Finally the presence of N_3^-, which completely abolishes the activity of catalase, does not increase the rate of lysis. Thus catalase inactivation appears to be but one aspect of the overall cellular damage which eventually results in the lysis. In contrast, superoxide dismutase inhibition resulted in a pronounced increase of the hemolysis rate i â the dark phase after irradiation. Both the inhibitors used, diethyldithiocarbamate and CN^-, may well interact with other components of the erythrocytes, being not at all specific for superoxide dismutase. However, some considerations seem to indicate superoxide dismutase as the site of action of these substances. First, CN^- and diethyldithiocarbamate are effective on photohemolysis only in the range of concentration needed for superoxide dismutase inhibition. In fact, there is a clear relationship between their effectiveness toward superoxide dismutase and toward hemolysis. Second, related substances like N_3^- and penicillamine, which do not inhibit superoxide dismutase in the concentration used, do not influence hemolysis either. Other chemicals, which like CN^- and diethyldithiocarbamate may react with membrane thiols, have rather a protective effect[7].

Complementary results were reported by Stern and coworkers[17,18]. These authors found that drug-induced hemolysis follows superoxide dismutase inactivation counteracted by catalase. Both enzymes play a specific role against oxidant species, being in the meantime also attacked by some of them. Hydrogen peroxide is substrate of catalase and inactivator of superoxide dismutase[19]. Singlet oxygen produced in the course of photoirradiation is particularly reactive toward catalase[20]. It remains to ascertain whether superoxide dismutase is active directly on singlet oxygen, as previously suggested[21], or active on other radicals generated from it.

1 H. Gaffron, Biochem. Z. *179*, 157 (1926).
2 E.S. Peterka, R. Runge and R. Fusaro, Arch. Dermat. *94*, 282 (1966).
3 B.D. Goldstein and L.C. Harber, J. clin. Invest. *51*, 892 (1972).
4 A.A. Lamola, T. Yamane and A.M. Trozzolo, Science *179*, 1131 (1973).

5 A.F.P.M. De Goeij, T.H.J. Ververgaert and J. Van Steveninck Clin. chim. Acta *62,* 287 (1975).
6 A.W. Girotti, Biochem. biophys. Res. Commun. *72,* 1367 (1976).
7 R. Strom, C. Crifò, S. Mari, G. Federici, I. Mavelli and A. Finazzi-Agrò, Physiol. Chem. Phys. *9,* 63 (1977).
8 A. Finazzi-Agrò, M.B. Fadda, G. Floris, M.R. Dessì and C. Crifò, Experientia *34,* 181 (1978).
9 J. Maral, K. Puget and A.M. Michelson, Biochem. biophys. Res. Commun. *77,* 1525 (1977).
10 J. Goa, J. clin. Lab. Invest. *5,* 218 (1953).
11 H. Lück, in: Methods of Enzymatic Analysis, 2nd edn, p. 886. Ed. M.V. Bergmeyer. Verlag Chemie/Academic Press, London-New York 1974.
12 A. Concetti, P. Massei, G. Rotilio, M. Brunori and E.A. Rachmilewitz, J. Lab. clin. Med. *87,* 1057 (1976).
13 N. Hasty, P.B. Merkel, P. Radlick and D.R. Kearns, Tetrahedron Lett. *1972,* 49.
14 A. Rigo, P. Viglino, and G. Rotilio. Analyt. Biochem. *68* (1975).
15 R.E. Heikkila, F.S. Cabbat and G. Cohen, J. biol. Chem. *251,* 2182 (1976).
16 M. Younes and V. Weser, Biochem. biophys. Res. Commun. *78,* 1247 (1977).
17 B. Goldberg and A. Stern, J. biol. Chem. *251,* 6468 (1976).
18 S. Mc Mahon, S.G. Sullivan and A. Stern, Biochim. biophys. Acta, submitted.
19 R.C. Bray, S.A. Cockle, E.M. Fielden, P.B. Roberts, G. Rotilio and L. Calabrese, Biochem. J. *139,* 43 (1974).
20 A. Finazzi-Agrò and I. Mavelli, XV Congr. Soc. It. Biofis. Biol. Mol., Abstr., p. 18, 1978.
21 A. Finazzi-Agrò, C. Giovagnoli, P. De Sole, L. Calabrese, G. Rotilio and B. Mondovì, FEBS Lett. *21,* 183 (1972).

Pyrrolizidine alkaloid storage in African and Australian danaid butterflies

J.A. Edgar[1], M. Boppré[2] and D. Schneider[2]

CSIRO, Division of Animal Health, Private Bag No. 1, Parkville, 3052 (Australia), and Max-Planck-Institut für Verhaltensphysiologie, D-8131 Seewiesen über Starnberg (Federal Republic of Germany), 20 February 1979

Summary. 8 species of African and Australian danaid butterflies, captured in the field, were analyzed and found to contain pyrrolizidine alkaloids. It is suggested that these alkaloids, which are gathered by the adults from plants, contribute significantly to the chemical defences of the danaids.

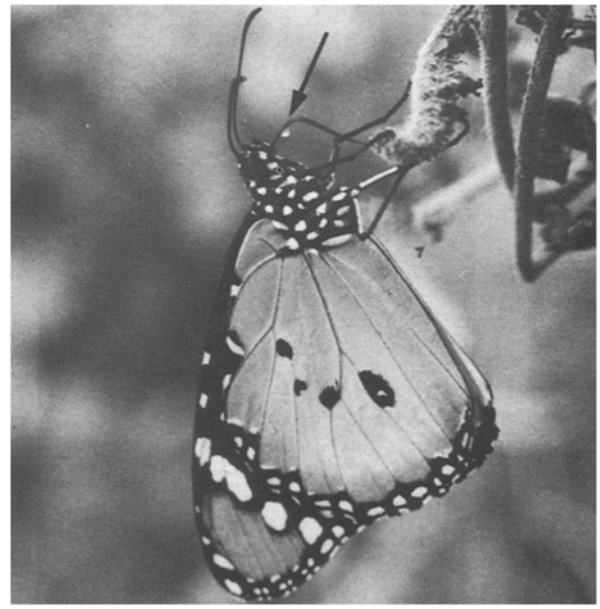
Danaid butterflies feed on exudates and on withered parts of plants containing pyrrolizidine alkaloids[3,4] (figure). These phytochemicals are partially metabolized by the males of many species of this family and are converted into dihydropyrrolizines[5-7] which are used as pheromones during courtship behaviour[8,9]. Recently it was shown that adult male and female *Danaus plexippus* (L), which do not produce such pheromones but still feed on plants containing pyrrolizidine alkaloids, are able to retain the alkaloids unmodified in their bodies for extended periods[10]. It was therefore suggested that pyrrolizidine alkaloids, as well as acting as pheromone precursors, may also be 'stored' by danaid butterflies as a protection against predators[10].

We report here on a chemical examination of 57 danaid butterflies, representing 5 African and 3 Australian species of 3 genera *(Amauris, Danaus, Euploea).* Our results (table) indicate that 'storage' of pyrrolizidine alkaloids occurs in both sexes. The butterflies were captured in the field (in Kenya/East Africa and Queensland/Australia) and kept alive without access to alkaloids for periods ranging from 2 to 21 days in order to ensure that alkaloids detected in their bodies were in fact 'stored' and not recently ingested gut contents.

The diversity of alkaloids found confirms that danaids use various plant scources of pyrrolizidine alkaloids. They have been reported to visit and feed on plants in the families Boraginaceae, Asteraceae, Leguminosae and Apocynaceae[3,4]. and pyrrolizidine alkaloid representatives of all these plant families were found in the bodies of the butterflies. However, it remains to be investigated whether or not the various danaid species prefer certain pyrrolizidine alkaloids and if the different types of pyrrolizidine alkaloids are equally appropriate as pheromone precursors and for storage.

Previous reports have emphasized the predominance of males among butterflies seen feeding on pyrrolizidine alkaloid plants[3,4]. It was therefore interesting to find that the samples of females we analyzed all contained alkaloids (albeit generally less than the male samples) indicating that, while they are apparently less frequent feeders, some of them at least acquire and store pyrrolizidine alkaloids in nature.

D. formosa (sample of 3 males) was the only species without detectable amounts of pyrrolizidine alkaloids in the male sex. It (like *D. plexippus*) has been previously reported not to possess a pyrrolizidine alkaloid-derived pheromone on its hairpencils[12]. However, W. Schäfer[13] recently detected a pyrrolizidine metabolite in some (not all) hairpencil extracts of *D. formosa.* Since the bodies of females of this species contained alkaloids it seems likely that the males



Danaus chrysippus (male) applying a droplet (arrow) to withered *Heliotropium steudneri* (Borginaceae) in order to extract pyrrolizidine alkaloids for ingestion. This is a common strategem of danaids in their quest for pyrrolizidine alkaloids.