These results are in agreement with those of several workers who have found an increased incorporation of ^{35}S-sulfate into glycosaminoglycans in aorta tissue incubated under atherosclerotic conditions[15–18]. Mammalian cells are known to incorporate sulfate only into proteoglycan, and the amount of sulfate incorporation is considered to be a good estimate of actual amounts of proteoglycans synthesized by the cells[19]. Therefore, it seems likely that elevated serum lipoprotein levels cause heightened production of proteoglycans in aorta cells. Phase microscopy studies of morphology indicate that the cell populations consist mainly of smooth muscle cells. The facts that the cells synthesize predominantly dermatan sulfate proteoglycan(s) (see below) supports this conclusion.

Radiolabeled proteoglycans from rabbit aorta cultures incubated with 3 times the normal human serum LDL were chromatographed on P-10 columns before and after hyaluronidase or nitrous acid treatment. After incubation with hyaluronidase, a retarded peak (mol. wt $< 20{,}000$) was found in all medium samples (figure 1), but not in any cell samples (figure 2, a). After nitrous acid degradation, the cell fraction also showed a retarded peak (figure 2, b). The untreated samples from cell and medium fractions showed only a single radioactively labeled peak migrating with the column void volume. Similar results were obtained from analyses of the monkey aorta proteoglycan fractions. With normal and reduced content of LDL in the serum, no change in the P-10 profile was found (data not shown). The secreted proteoglycan material also shows no significant differences in composition with different amounts of serum LDL in the cell growth medium.

We conclude from these results that the composition of cell-associated and extracellular proteoglycans does not vary significantly with the lipoprotein content of the medium. The cell-associated proteoglycans are not degraded by hyaluronidase (figure 2, a), although nitrous acid treatment degrades $\sim 45\%$ of this material (figure 2, b). The cellular proteoglycan material, therefore, consists of 55% dermatan sulfate proteoglycan (hyaluronidase, nitrous acid-resistant) and 45% heparan sulfate (nitrous acid-sensitive). The extracellular proteoglycans contain a portion that is degradable by hyaluronidase ($\sim 55\%$) and a portion resistant to enzyme degradation. We have found (results not shown) that this hyaluronidase-resistant material is not degraded by nitrous acid but is extensively degraded by chondroitinase ABC. These results indicate that the peak in the P-10 chromatograph eluting in the column void volume probably is composed of dermatan sulfate proteoglycan.

Compared to other glycosaminoglycans, dermatan sulfate, identified by its resistance to hyaluronidase and nitrous acid and susceptibility to chondroitinase ABC, has the greatest affinity for LDL under physiological conditions and has been found in high concentrations in arterial lesions[10]. Our results suggest a mechanism to explain the increased incidence of fatty lesions in aortas of animals whose serum lipid content is elevated. The increased serum LDL content in the aorta stimulates an increase in growth of those aorta smooth muscle cells exposed to blood flow due to prior injury of the arterial wall[3, 11]. The growth increase is accompanied by an increased synthesis and secretion of dermatan sulfate proteoglycan which complexes with the serum LDL to form insoluble products. The buildup of insoluble lipids causes loss of cell viability. Eventually, calcification may occur to render the proteoglycan-LDL complex even more resistant to removal from the lesions by enzymatic degradation. This hypothesis is consistent with the increase in dermatan sulfate found in arteries that develop lesions after extensive lipid infiltration[10].

Selective inactivation of catalase during protoporphyrin induced photohemolysis of human red blood cells

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

Institute of Biological Chemistry, University of Cagliari, via della Pineta 77, I–09100 Cagliari (Italy), and C. N. R. Center for Molecular Biology, Rome (Italy), 23 May 1977

Summary. The level of some enzymatic activities in red blood cells before and after photohemolysis induced by protoporphyrin IX was studied. A 30% decrease in catalase activity was found both in normal erythrocytes and those from patients affected by favism. Other proteins though present in larger amounts inside the erythrocytes are not affected by the photohemolytic process.

The photodynamic effect of protoporphyrin on red blood cells has recently been studied, in view of its significance as a model system for porphyrias[1]. A further interesting aspect is to identify the early damaged component of the membrane[2–6]. While the previous papers were concerned with the membrane damage in general, in the present communication we report about the inactivation of intraerythrocytic catalase during photohemolysis.

Materials and methods. Human red blood cells were obtained by venipuncture from healthy people and from patients affected by favism, using oxalate as anticoagulant. The red cells were collected by centrifugation and washed 3 times with isotonic saline. Then the packed cells were suspended in 100 vol. isotonic saline and incubated in the presence of 1 μM protoporphyrin IX (Sigma, St. Louis, Mo, USA). Samples were irradiated within a thermostated bath with 2 Osram projector 150 W lamps situated at 30 cm distance.

After 5–30 min incubation under light, the samples were stored in the dark for 2 h, then centrifuged and the extent of hemolysis was evaluated by measuring the absorbance of supernatant at 550 nm. Control samples were obtained either by incubation in the dark or by hypotonic hemolysis.

1 E. S. Peterka, W. J. Runge and R. M. Fusaro, Archs Derm. *94*, 282 (1966).
2 B. D. Goldstein and L. C. Harber, J. clin. Invest. *51*, 892 (1972).
3 A. A. Lamola, T. Yamane and A. M. Trozzolo, Science *179*, 1131 (1973).
4 A. F. P. M. De Goeij, J. Th. Ververgaert and J. Van Steveninck, Clin. chim. Acta *62*, 287 (1975).
5 A. W. Girotti, Biochem. biophys. Res. Commun. *72*, 1367 (1976).
6 R. Strom, C. Crifò, S. Mari, G. Federici, I. Mavelli and A. Finazzi-Agrò, Physiol. chem. Phys., *9*, 63 (1977).

Protein loss after photohemolysis of red blood cells

	Activity in the supernatant[e]		Recovery (%)	Activity in the membranes[f]		Recovery (%)
	H_2O hemolysis	Photohemolysis		H_2O hemolysis	Photohemolysis	
Catalase[a]	87,000 ± 3500	61,000 ± 2000	70	30,000 ± 2000	70,000 ± 2000	256
Glutathione peroxidase[a]	62 ± 10	60 ± 10	97	4 ± 1	4 ± 1	100
Acethylcholine esterase[b]	790 ± 150	840 ± 150	106	24,400 ± 100	8,500 ± 100	35
Carbonic anhydrase[a]	10.5 ± 1	11.4 ± 1	108	24 ± 1	24 ± 1	100
Superoxide dismutase[c]	0.1 ± 0.01	0.1 ± 0.01	100	0.15 ± 0.01	0.15 ± 0.01	100
Hemoglobin[d]	0.23	0.23	100	not determined		

[a] The activity is expressed in μmoles of substrate converted/mg protein min. [b] The activity is expressed as described by Ellman[11]. [c] The superoxide dismutase activity is expressed as the amount of protein which gives a 70% inhibition of epinephrine oxidation as described by Misra[8]. [d]) Oxyhemoglobin content was determined by its absorption at 540 nm. [e] Supernatant volume was 100 ml containing 2 mg protein/ml. [f] The ghosts were resuspended in 50 ml volume which contained 0.12 mg protein/ml.

The enzymic activities were determined in the supernatant and in the precipitate pellet after solubilization by 1% Triton X-100 with the following methods: catalase[7], superoxide dismutase[8], carbonic anhydrase[9], glutathione peroxidase[10] and acetylcholinesterase[11].

Results and discussion. Under the present experimental conditions, 13 min irradiation produces complete lysis of human normal red blood cells, a result similar to that reported by Girotti[5]. No difference was observed using blood samples from patients affected by the hemolytic disease favism, although in this case 15% of the cells appear to withstand the photohemolytic process. These patients show an inherited low level of glucose 6-P dehydrogenase in their erythrocytes[12], which apparently makes these cells more fragile. Many authors have studied the modifications induced by photohemolysis into the erythrocyte membrane[2–6], but while Lamola et al.[3] pointed to the formation of cholesterol hydroperoxide as responsible of the lytic effect, De Goeij et al.[4] and more recently Girotti[5] suggested the membrane proteins as the primary target of the photooxidative attack. In a previous paper[6], we have shown the presence of cholesterol hydroperoxide after photohemolysis and the possible repairing effect exerted by glutathione in bovine red cells.

In an attempt to gain further information concerning the mechanism of photohemolysis and the defense or repair systems of erythrocytes, we determined the activities of some red cell enzymes before and after the photohemolysis. The results are reported in the table. As previously described[2] the membrane-bound acetylcholinesterase shows a significant inactivation while the intra-erythrocytic activity of the enzyme is unchanged after photohemolysis. A 30% decrease of catalase activity was also observed with a concomitant increase of catalase activity into the membrane pellets which however accounts only for a small fraction of the lost catalase in terms of total activity (table). Other proteins, like carbonic anhydrase, hemoglobin, glutathione peroxidase and superoxide dismutase, do not show any decreased content or activity in the course of the experiment. Furthermore any significant formation of methemoglobin was found during the photohemolysis as determined by comparing the absorption spectra of samples obtained by osmotic lysis and photohemolysis. The irradiation of the red cell lysate in the presence of protoporphyrin IX under the experimental conditions used for the intact cells, does not produce any loss of catalase activity. This indicates that the enzyme inactivation is a specific process associated with the cell integrity. Control experiments made on purified samples showed that all the above-mentioned proteins, with the exception of superoxide dismutase[13], are inactivated by photo-oxygenation in the presence of protoporphyrin. The question may arise whether catalase inactivation occurs as a direct consequence of irradiation or as a secondary damage during incubation in the dark. To check this point, the red cells were disrupted by osmotic shock just after the irradiation at various times and the catalase activity assayed. It was in fact found that the remaining catalase activity is equal to that determined after 2 h dark incubation and spontaneous hemolysis. After reaching a maximum value of 30% inactivation, no further decrease of catalase activity was obtained even after 45 min irradiation (i.e. when the lysis occurs under irradiation).

It is worth noting that red blood cells from subjects affected by favism undergo photohemolysis in the same way as those from healthy people, indicating that this pathological state does not imply a different resistance of cell membrane to oxidative attack. In this case a 30–40% catalase inactivation was observed after photohemolysis. One third catalase loss seems to be a threshold value after which hemolysis occurs.

While it is impossible at the moment to understand whether this inactivation is a crucial step in the photohemolytic process or only a concomitant phenomenon, a few points can be made. Catalase is not associated with erythrocyte membrane as determined by controlled hemolysis[14]. Nevertheless during irradiation, the catalase activity is lost. Since the site of the photodynamic attack should be the membrane it seems that catalase could migrate toward it and there become selectively photoinactivated, perhaps by cross-linking to other membrane proteins[5].

7 H. Lück, in: Methods of Enzymatic analysis, 2nd ed., p. 886. Ed. M. V. Bergmeyer. Verlag Chemie/Academic Press, New York 1974.
8 H. P. Misra and I. Fridovich, J. biol. Chem. *247*, 3170 (1972).
9 V. Pocker and J. T. Stone, Biochemistry, *6*, 668 (1967).
10 E. D. Paglia and W. N. Valentine, J. Lab. Med. *70*, 158 (1967).
11 G. L. Ellman and E. Callaway, Nature *192*, 1216 (1961).
12 J. W. Harris and R. W. Kellermayer, in: The red Cell, p. 559. Harward Univ. Press, Harward 1970.
13 A. Finazzi-Agrò, D. Cocco, L. Calabrese, W. H. Bannister and F. Bossa, Int. J. Biochem., in press (1977).
14 A. Bozzi, I. Mavelli, A. Finazzi-Agrò, R. Strom, A. M. Wolf, B. Mondovì and G. Rotilio, Molec. Cell. Biol. *10*, 11 (1976).