tracts (*18*), these two clones were considered to be lacking both GPI and the F-19 chromosome. By these criteria, nine clones were positive for both GPI and F-19, 28 clones were negative for both, and no clones were positive for one and negative for the other (Table 1). The presence of human GPI did not correlate with the presence of any other human chromosome (Table 2).

Glucosephosphate isomerase is loosely linked to the $\beta$ chain of hemoglobin in the mouse (*6*). Since variants of GPI and of the $\beta$ chain are present in humans, this linkage could be investigated in man by classical family studies. The $\beta$ and $\delta$ chains of hemoglobin are very closely linked in man (*6*). If GPI and the $\beta$ chain are linked in man, this would place the genes for GPI and the $\beta$ and $\delta$ chains on the human F-19 chromosome.

Concordant segregation of human MPI and chromosome C-7 was also observed in most cases (Tables 1 and 2). When hybrid clones containing one mouse chromosome set plus one reduced human set (1s + 1s) were analyzed, MPI and C-7 were always present together or absent together (Table 1). Presence of human MPI did not correlate with the presence of any human chromosome other than C-7 (Table 2). When subclones were derived from one clone that expressed GPI and MPI, one subclone obtained (WAIaD6) had lost two human chromosomes, C-7 and F-19, and had also lost both enzyme phenotypes. These data support the linkage of the human gene for MPI to the C-7 chromosome.

Data for hybrid clones containing two mouse chromosome sets and one reduced human chromosome set (2s + 1s) failed to support the linkage of MPI to C-7. Five clones of this makeup contained C-7 chromosomes but did not express human MPI. Four of these five clones were derived from the RK hybrid series. Data for these same clones failed to support two other human gene-chromosome linkage relationships; in each case, the chromosome was present but the enzyme was not expressed. A subclone of one of these clones reexpressed a human enzyme that was anomalously unexpressed in the parental hybrid clone. These considerations suggest that some phenomenon in hybrids with the karyotype 2s (mouse) + 1s (human) may interfere with the expression of enzymes generally considered to be constitutively expressed (*19*). The data from 1s + 1s hybrids (Tables 1 and 2) consistently support the linkage of human MPI to chromosome C-7 and make the linkage of MPI to any other chromosome unlikely. Linkage of MPI to C-7 is therefore indicated, but is not as firmly established as the linkage of GPI to F-19.

We have reported the linkage of lactate dehydrogenase subunits A and B, which are very closely related in structure and function, to the morphologically similar human chromosomes C-11 and C-12, respectively (*20*). It has been suggested that chromosome duplication at some point in evolution could have provided the genetic raw material for producing new, but similar, chromosomes and polypeptide sequences (*21*). In the present case, two hexosephosphate isomerases have been linked to chromosomes that are at least superficially dissimilar (Fig. 1). Structural dissimilarity is also suggested for the enzymes; GPI is a dimer (*11*), whereas the electrophoretic pattern of MPI from hybrid mice and from interspecific somatic cell hybrids suggests that MPI is a monomer (*12*). Further research in somatic cell genetics may thus contribute to our understanding of molecular evolution as well as of human genetics.

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**References and Notes**

1. R. J. Klebe, T. R. Chen, F. Ruddle, *J. Cell Biol.* **45**, 74 (1970).
2. J. W. Littlefield, *Science* **145**, 709 (1964).
3. T. Kusano, C. Long, H. Green, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 82 (1971).
4. M. C. Weiss and H. Green, *ibid.* **58**, 1104 (1967).
5. F. Ruddle, *Advan. Hum. Genet.* **3**, 173 (1972); in *Long-Term Cultivation of Leucocytes* (National Foundation–March of Dimes, White Plains, N.Y., in press).
6. V. A. McKusick, *Mendelian Inheritance in Man* (Johns Hopkins Press, Baltimore, 1971), pp. xli and 232.
7. J. L. Hamerton, G. R. Douglas, P. Gee, B. J. Richardson, *Cytogenetics*, in press.
8. F. A. McMorris and F. Ruddle, in preparation.
9. F. Ricciuti and F. Ruddle, *Nature*, in press.
10. J. Tischfield and F. Ruddle, in preparation.
11. R. J. DeLorenzo and F. Ruddle, *Biochem. Genet.* **3**, 151 (1969).
12. E. A. Nichols, A. M. Chapman, F. Ruddle, *ibid.*, in press.
13. F. Ruddle and E. A. Nichols, *In Vitro* **7**, 120 (1971).
14. M. L. Pardue and J. G. Gall, *Science* **168**, 1356 (1970); T. R. Chen and F. Ruddle, *Chromosoma* **34**, 51 (1971).
15. A. T. Sumner, H. J. Evans, R. A. Buckland, *Nature New Biol.* **232**, 31 (1971).
16. T. Caspersson, G. Lomakka, L. Zech, *Hereditas* **67**, 89 (1971).
17. Chromosomes were identified in accord with the recommendations of the Fourth International Conference on Standardization in Human Cytogenetics, Paris, 1971.
18. R. J. Klebe, thesis, Yale University (1970).
19. F. Ricciuti, J. Tischfield, F. Ruddle, in preparation.
20. C. M. Boone, T. R. Chen, F. Ruddle, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 510 (1972); T. R. Chen *et al.*, *Amer. J. Hum. Genet.*, in press.
21. D. E. Comings, *Nature* **238**, 455 (1972).
22. We acknowledge the technical assistance of M. Geissler, E. Nichols, M. Hirschi, P. Eager, and S. Chen, and manuscript preparation by E. Creagan and M. Reger. Supported by NIH grant 5-R01-GM-09966.
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## Cholesterol Hydroperoxide Formation in Red Cell Membranes and Photohemolysis in Erythropoietic Protoporphyria

Abstract. *3β-Hydroxy-5α-hydroperoxy-Δ⁶-cholestene is produced in protoporphyrin-containing red blood cell ghosts irradiated with approximately 400-nanometer light in the presence of oxygen. Incorporation of this cholesterol photooxidation product into normal red blood cells leads to increased osmotic fragility and eventual hemolysis. These results may be relevant to photohemolysis associated with erythropoietic protoporphyria.*

The red blood cells (RBC) of patients possessing the genetic disorder erythropoietic protoporphyria (EPP) (*1*) contain a much larger amount of free porphyrin than is found in normal RBC (*2*). These porphyrinemic RBC undergo facile hemolysis upon irradiation in vitro with visible light, whereas normal cells do not (*3*). Although there is no direct evidence that photohemolysis is the basis for the severe cutaneous photosensitivity of EPP patients, the phenomenon has received much attention (*4–7*), largely because (i) in vitro photohemolysis serves as a useful diagnostic criterion for EPP (*8*); (ii) photohemolysis may be a useful model for the skin sensitivity; and (iii) photohemolysis is associated with damage to the RBC membrane, the structure of which is being widely studied.

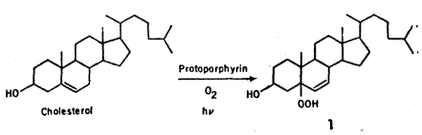
Wavelengths of light near 400 nm are the most effective for both the skin effects and photohemolysis (*1–4*). This

indicates, as expected, that both phenomena involve excitation of the protoporphyrin. Further evidence for this correspondence is provided by the observations that normal RBC can be rendered light-sensitive after incubation with protoporphyrin (*4*). Photohemolysis requires molecular oxygen (*4*) and is accompanied by the formation of "lipid peroxides" (*7*). That damage to the RBC membrane occurs is also indicated by the fact that cell $K^+$ is lost before hemolysis (*6*).

We were intrigued by the effectiveness of β-carotene as an inhibitor of the photoerythema in EPP patients (*9*) and the reports of significant inhibition of in vitro photohemolysis by β-carotene (*5, 10*). The demonstration by Foote *et al.* (*11*) that β-carotene is an effective quencher of singlet oxygen ($^1O_2$*) (specifically, the $^1\Delta_g$ state) in solution, taken together with the observations that protoporphyrin can sensitize $^1O_2$* production, suggested to us that $^1O_2$* may be involved in the photohemolysis. Thus we thought it worthwhile to consider the possible reactions of $^1O_2$* with the lipids of RBC membranes.

The structures of the lipids found in human RBC membranes (*12*) suggested that the so-called "ene-reaction" of $^1O_2$*, in which allylic hydroperoxides are produced (*13*), be given primary consideration. Furthermore, both reactivity considerations and the large content of cholesterol compared to that of unsaturated fatty acid side chains in the membrane (*12*) suggested to us that, in the absence of localization effects, the cholesterol should be the primary substrate for $^1O_2$*.

Indeed, Schenck *et al.* (*14*) and others have reported that cholesterol undergoes facile oxidation under conditions known to involve $^1O_2$* as the active oxygen species. It appears that a single hydroperoxide, 3β-hydroxy-5α-hydroperoxy-Δ⁶-cholestene (**1**), is obtained under conditions that do not allow isomerization. Both hematoporphyrin and protoporphyrin are effective sensitizers. The reactivity of cholesterol toward $^1O_2$* compared to that of various alkenes (*13, 14*) supports our contention that cholesterol should be more reactive toward $^1O_2$* than are the unsaturated fatty acid side chains.




1

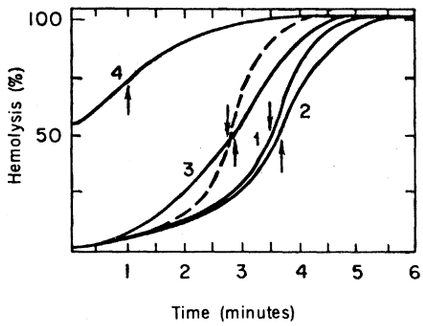



Fig. 1. Fragility curves. The solid curves show hemolysis of **1**-containing RBC that had been incubated at 37°C for 10 minutes (curve 1), 20 minutes (curve 2), 50 minutes (curve 3), or 80 minutes (curve 4). The dashed curve is the control. The arrows indicate the time for hemolysis of 50 percent of the RBC still intact at the beginning of the fragility measurement.

To assess the importance of this reaction in a heterogeneous system such as the cell membrane, we performed experiments with liposomes prepared by sonicating aqueous suspensions (10 ml) of egg lecithin (20 mg), cholesterol (12 mg), and protoporphyrin (0.5 mg). Irradiation of a suspension of these liposomes in 10 ml of oxygen-saturated saline solution (0.15*M* NaCl) for ½ hour at 25° to 30°C with filtered light (380 to 450 nm) from a 450-watt medium pressure mercury lamp placed 10 cm from the sample led to the conversion of 30 percent of the cholesterol to **1**. The amount of **1** was determined by using thin-layer chromatography (*15*) and [$^{14}$C]cholesterol. Alternatively, quantitative analysis of unlabeled samples was accomplished by gas chromatography after reduction of **1** to Δ⁶-cholestene-3β,5α-diol with triphenylphosphine.

Next we looked for **1** in irradiated RBC ghosts that contained protoporphyrin. To a stirred suspension of ghosts (*16*) from 100 ml of whole human blood in 20 ml of 0.5*M* NaCl (0.05*M* phosphate buffer, *p*H 7) was added 10 mg of protoporphyrin in 2 ml of methanol. After being stirred for 1 hour the mixture was centrifuged at 25,000*g* for 15 minutes, and the pellet of porphyrin-dyed ghosts was separated from the dark pellet of undissolved protoporphyrin at the bottom of the tube. Between 1 and 2 mg of the protoporphyrin was taken up by the various preparations of ghosts. A suspension of porphyrin-dyed ghosts in 20 ml of 0.5*M* NaCl (0.05*M* phosphate buffer, *p*H 7) was irradiated at 10°C as described above while oxygen was slowly bubbled through the sample. The ghosts became less colored as the irradiation proceeded and were only faintly colored after 6 hours. After irradiation the sample was centrifuged and the pellet of ghosts was lyophilized. The dried ghosts were extracted by stirring with 50 ml of fresh diethylether for 24 hours. The extracted material was analyzed for cholesterol and for **1** by thin-layer chromatography and gas chromatography. For various samples irradiated for 5 hours, 20 to 30 percent of the cholesterol was converted to **1**. No cholesterol hydroperoxide was detected in samples bubbled with oxygen for 5 hours in the dark, and only small amounts were found in irradiated samples that did not contain protoporphyrin.

To determine whether or not **1** should be considered relevant to the photohemolysis of protoporphyrinemic RBC, we investigated the hemolytic effect of **1** on RBC. Part of the cholesterol of normal RBC was exchanged with **1** by the procedure of Bruckdorfer *et al.* (*17*), as follows. Egg lecithin liposomes containing cholesterol and **1** were prepared by sonicating a mixture of lecithin (20 mg), cholesterol (6 mg), and **1** (10 mg) in 10 ml of buffered saline followed by centrifugation at 48,000*g* for 1 hour. When these liposomes were incubated (37°C) with washed RBC in buffered saline, **1** became incorporated into the RBC membranes. The total steroid content of the membranes could be kept unchanged by using the proper ratio of RBC and liposomes. We were able to follow the uptake of **1** by the RBC by using $^{14}$C-labeled **1**. Osmotic fragilities of RBC containing **1** were measured with a Fragiligraph model D-2 (Elron Electronic Industries, Israel) (*18*). Controls were RBC incubated in buffered saline or with saline suspensions of liposomes containing cholesterol but not **1**.

The RBC of various donors were rendered more fragile than the controls after incubation with liposomes containing **1** for 3 hours at 37°C. For example, exchange of 5 to 10 percent of the RBC cholesterol for **1** over a 3-hour period led to a decrease in the lysis time of up to a factor of 2. Experiments in which the RBC were exposed to a pulse of **1** were more informative. In these experiments, the RBC were incubated with a high concentration of **1**-containing liposomes for a short time (5 to 15 minutes), and then the liposomes were removed from the sample by centrifugation and washing.

The RBC containing **1** were then incubated in buffered saline at 37°C, and fragility measurements were made at intervals. Typical results are shown in Fig. 1, which includes several fragility curves. In these measurements, the hemolysis is monitored as a function of decreasing salt concentration, which occurs linearly with time. The dashed curve is the control, a sample incubated with liposomes containing cholesterol but not **1**. The control showed no change in osmotic fragility after 80 minutes in saline at 37°C. Curve 1 was obtained for RBC exposed to a pulse of **1** (about 5 percent of the RBC cholesterol was exchanged for **1**) and then kept at 37°C in saline for 10 minutes; corresponding times in saline were 20 minutes for curve 2, 50 minutes for curve 3, 80 minutes for curve 4.

These observations show that incorporation of **1** into normal RBC membranes leads at first to a decrease in osmotic fragility (*19*); but after incubation for a time at 37°C an increase in fragility and hemolysis is achieved. That an "induction period" is observed before increased fragility may be rationalized in two ways. It may be that **1** is the precursor of the species that causes the hemolysis. That is, a rearrangement or decomposition product of **1** may be responsible for the increased fragility of the RBC, or the decomposition of **1** may initiate nonselective free-radical chain oxidation of unsaturated membrane lipids. Alternatively, the action of **1** may at first cause $K^+$ ions to leak out of the RBC faster than $Na^+$ ions leak in. This would cause the RBC to shrink initially and appear less fragile until most of the $K^+$ is gone (*20*).

In summary, we have found efficient formation of **1**, the product of attack of singlet oxygen upon cholesterol, after irradiation of RBC ghosts containing protoporphyrin. Incorporation of **1** into normal RBC membranes leads to increased osmotic fragility and eventual hemolysis of the RBC. Photohemolysis of RBC from EPP patients can be rationalized on the basis of these observations. We are performing several kinds of experiments to determine whether the photohemolysis mechanism that does operate involves singlet oxygen and **1** as important intermediates.

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### References and Notes

1. D. P. Tschurdy, I. A. Magnus, J. Kalivas, in *Dermatology in General Medicine*, T. B. Fitzpatrick, K. A. Arndt, W. H. Clark, A. Z. Eisen, E. J. Van Scott, J. H. Vaughan, Eds. (McGraw-Hill, New York, 1971), pp. 1143–1166.
2. H. Langhof, H. Müller, L. Rietschel, *Arch. Klin. Exp. Dermatol.* **212**, 506 (1961); I. A. Magnus, A. Jarrett, T. A. Prankerd, C. Rimington, *Lancet* **1961-II**, 448 (1961).
3. L. C. Harber, A. S. Fleischer, R. L. Baer, *J. Amer. Med. Ass.* **189**, 191 (1964).
4. J. Hsu, B. D. Goldstein, L. C. Harber, *Photochem. Photobiol.* **13**, 67 (1971).
5. A. A. Schothorst, J. Van Steveninck, L. N. Went, D. Suurmond, *Clin. Chim. Acta* **28**, 41 (1970).
6. A. S. Fleischer, L. C. Harber, J. S. Cook, R. L. Baer, *J. Invest. Dermatol.* **46**, 505 (1966).
7. B. D. Goldstein and L. C. Harber, *J. Clin. Invest.* **51**, 892 (1972); A. A. Schothorst, J. Van Steveninck, L. N. Went, D. Suurmond, *Clin. Chim. Acta* **39**, 161 (1972).
8. E. S. Peterka, W. Runge, R. Fusaro, *Arch. Dermatol.* **94**, 282 (1966).
9. M. M. Mathews-Roth, M. A. Pathak, T. B. Fitzpatrick, L. C. Harber, E. H. Kass, *N. Engl. J. Med.* **282**, 1231 (1970).
10. N. I. Krinsky, J. J. Brynes, A. Gordon, Abstracts, Fifth International Congress on Photobiology, Hanover, New Hampshire, August 1968, p. 29.
11. C. S. Foote, Y. C. Chang, R. W. Denny, *J. Amer. Chem. Soc.* **92**, 5216 (1970); *ibid.*, p. 5218.
12. L. L. M. Van Deenen, in *The Molecular Basis of Membrane Function*, D. S. Tosteson, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1969), pp. 47–78.
13. K. Gollnick, *Advan. Photochem.* **6**, 2 (1968). Current research on singlet oxygen is reviewed by D. R. Kearns [*Chem. Rev.* **71**, 395 (1971)].
14. G. O. Schenck, K. Gollnick, A. O. Neumüller, *Justus Liebigs Ann. Chem.* **603**, 46 (1957); G. O. Schenck and O. A. Neumüller, *ibid.* **618**, 194 (1958).
15. L. L. Smith and F. I. Hill, *J. Chromatogr.* **66**, 101 (1972).
16. Prepared according to the procedure of V. T. Marchesi and G. E. Palade [*J. Cell Biol.* **35**, 385 (1967)] except that phosphate buffer was used instead of tris(hydroxymethyl)-aminomethane.
17. K. R. Bruckdorfer, R. A. Demel, J. De Grier, L. L. M. Van Deenan, *Biochim. Biophys. Acta* **183**, 334 (1969).
18. D. Danon, *Brit. J. Haematol.* **13** (Suppl.), 61 (1967).
19. Replacement of cholesterol in RBC by several different steroids (for instance, 7-dehydrocholesterol) leads to a decrease in osmotic fragility (*17*).
20. D. C. Tosteson, in *Cellular Functions of Membrane Transport*, J. F. Hoffman, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1964), pp. 3–22.
21. We thank M. Pathak and J. Cook for stimulating discussions; F. Doleiden, M. Joyner, and C. Castillo for technical assistance; and M. Brin, M. Barker, L. Horn, and G. Reed of the Hoffmann-LaRoche Co., Nutley, N.J., for the use of the Fragiligraph and helpful discussions.

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## Strychnine- and Pentylenetetrazol-Induced Changes of Excitability in Aplysia Neurons

Abstract. *In* Aplysia *neurons isolated from their synaptic input strychnine induces doublet discharges associated in voltage clamp with a decrease in the threshold for the inward current and a reduction and delayed onset of the outward current. Pentylenetetrazol causes oscillations and bursting behavior in normally silent cells together with an increased inactivation of the delayed outward current and induced or enhanced anomalous rectification.*

One experimental approach to the understanding of the neurophysiological basis of epileptic seizures has been the study of action of convulsant drugs (*1*). Unfortunately, there exists a myriad of convulsant agents with different, and possibly multiple, actions on nervous structures. Some of these drugs block inhibitory synaptic transmission, but these drugs constitute only a minority of the known convulsants. At the cellular level, focal epileptic activity is characterized primarily by high-frequency synchronous firing in association with oscillatory membrane potential changes (*1*). The experiments to be reported were undertaken to analyze the action of convulsant drugs responsible for repetitive firing at short intervals. Our hypothesis was that some convulsant may act by directly changing membrane properties related to excitability and stability.

The experiments were performed on neurons of the isolated abdominal ganglion of the marine mollusk *Aplysia californica*. Intracellular voltage recording and voltage and current clamping techniques were used to study the effects of two convulsants, strychnine and pentylenetetrazol (PTZ). The latter was chosen in comparison with strychnine because it does not seem to have a direct effect on synaptic transmission in *Aplysia*, while strychnine blocks acetylcholine, dopamine, and 5-hydroxytryptamine receptors in the ganglion (*2*). Previous investigations have demonstrated, furthermore, that both drugs produce high-frequency discharges with abnormally short interspike intervals and oscillatory membrane potential changes in the *Aplysia* neurons (*3*).

The soma of identified neurons (*4*) were penetrated with single or double microelectrodes (3 to 15 megohms). The drugs were added directly to the artificial seawater perfusate ($pH = 8.0$; 16°C). The effects studied generally took 5 to 8 minutes to become manifest, and all were readily reversible upon washing.