2. Diffusion constants estimated for these cells range from 1.4×10^{-10} to 3.3×10^{-10} cm^2 sec^{-1}. The data scatter about the line but systematic deviation only occurs for $\hat{I} > 0.292$. Data have been evaluated from 46 cells and give a range of values for D from approximately 5×10^{-11} to 5×10^{-10} cm^2 sec^{-1}, with a mean ($\pm$ standard error) of $2.6 \pm 0.16 \times 10^{-10}$. This is about an order of magnitude slower than the value of D for rhodopsin in disks (*5*). It is consistent with the mobilities inferred from other data on protein diffusion in heterokaryons (*3*).

The general application of our method remains to be tested. Preliminary work indicates that many cell types are severely damaged by labeling and cannot be used for direct conjugation with fluorescein. Another approach to such cells is to prepare monovalent labeled ligands such as Fab fragments of antibody, or succinylated concanavalin A. Experiments in our laboratory and those of others (*13*) indicate that this approach is feasible with concanavalin A, although the labeled ligand may itself cause cross-linking of receptors, or may trigger their cross-linking from within the cell.

Whatever the label, our method should be useful for evaluating lateral motions of membrane proteins in a wide variety of cells.

M. EDIDIN, Y. ZAGYANSKY
Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

T. J. LARDNER
Department of Theoretical and Applied Mechanics, University of Illinois, Urbana 61801

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8. Cells to be labeled were plated at high density either on cover slips or into 35-mm petri dishes and cultured overnight in Eagle's minimal essential medium containing 5 percent fetal calf serum and antibiotics. To label the cells, medium was removed and the plates or cover slips were washed three times with Hanks solution free of serum and buffered with HEPES to *p*H 7.2. After the washing, cells were covered with Hanks solution adjusted to *p*H 9.5 with NaOH immediately before use. Fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories) at 1 mg/ml was added to the dishes or slips to give a final FITC concentration of 0.6 to 1.0 mg/ml. Cells were permitted to react with FITC for 10 to 20 minutes at room temperature. They were then washed three times with Hanks solution, *p*H 7.5, and three more times with same solution containing 5 percent fetal calf serum.
9. A 1P28 photomultiplier (RCA) was operated in the photon counting mode as part of an SSR model 1140 (Princeton Applied Research) photon counting system. The discriminator output of the system was fed to a digital counter and could be monitored continuously. The voltage output of the 1140 was fed to a chart recorder (Houston, Omnigraphic) with adequate response time for the measurements. Both measuring light and light for initially scanning the field were supplied by a 200-watt mercury arc, operated from an Oriel d-c power supply. Lamp intensity was reduced to 0.5 to 1 percent for measurement to avoid further bleaching.
10. Freshly prepared 0.5 percent to 5.0 percent paraformaldehyde in phosphate-buffered saline, *p*H 7.4, was applied to cells at 0°C for 1.5 hours.
11. The operational tests were as follows. (i) Isotonic NaCl did not leach fluorescence from labeled cells. (ii) Excess calf serum added during labeling reduced rather than augmented the degree of labeling. Calf serum added after labeling did not displace fluorescence from labeled cells. (iii) Both 1*M* NaCl and 1*M* NaCl plus 0.001*M* EDTA removed about 30 percent of fluorescence from labeled cells previously washed in isotonic saline (four experiments). (iv) Trypsin did not remove label, while papain removed almost all label from the cells.
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Characterization of a Cell-Lethal Product from the Photooxidation of Tryptophan: Hydrogen Peroxide

Abstract. *Near-ultraviolet (300 to 400 nanometers) irradiation of saturated, oxygenated solutions of tryptophan in the absence of added sensitizer gives rise to substances that have various biological effects on isolated cells, including mutagenicity and selective lethality to recombination-deficient bacterial mutants. One of these biologically active products has been identified as H_2O_2, on the basis of spectrometric, chromatographic, chemical, and biological properties. Now H_2O_2 has been shown to account for the biological activities mentioned above.*

Near-ultraviolet to visible irradiation of tryptophan gives rise to substances that have various biological effects on isolated cells, including mutagenicity (*1, 2*), selective lethality to recombination-deficient (*recA*) bacterial mutants (*2, 3*), inhibition of repair of DNA single-strand breaks and of DNA replication gap closure in *Escherichia coli* (*4*), sensitization of bacteria to near-ultraviolet induced DNA strand breaks (*5*), inhibition of growth of cultured mouse embryonic fibroblasts and of fertilized sea urchin eggs (*6*), lethality to cultured mammalian cells (*7*), and binding to a lens γ-crystallin (*8*).

At the same time, several groups (*9*) have examined the physical photochemistry of tryptophan with flash photolysis

Table 1. Purification of tryptophan photoproduct by column chromatography. The purification was begun with 100 ml of a crude photomixture generated by photolysis for 24 hours with four RUL-3500 lamps (Rayonet RPR-202 reactor; Southern New England Ultraviolet Company). The eluents indicated were aqueous solutions. Column chromatography was followed by a selective bacterial killing assay, with *Salmonella typhimurium* strains KSU 2480 (*recA* 7) and the isogenic *recA*$^+$ KSU 9557.

Column*	Dimension	Eluent	Detection method	Elution volume of active peaks (ml)
Sephadex G-10	53 by 2.5 cm	H_2O	Ultraviolet starch-iodide	230–240
Sephadex QAE	15 by 0.9 cm	NH_3/NH_4Cl†	Ultraviolet	10–20
Sephadex G-10	90 by 1.5 cm	$(NH_4)_2SO_4$‡	Refractive index starch-iodide	112–114§

*Listed in sequence used. †0.05*N* Cl. ‡0.1*N*. §Elution volumes of blue dextran and D_2O were 46 and 99 ml, respectively.

Table 2. Thin-layer chromatography of tryptophan photoproduct (TP) and H_2O_2.

Stationary phase	Solvent system	Detection method	R_fTP	$R_f H_2O_2$
Silica gel	Methanol, toluene (3 : 7)	DMDAB*	0.36	0.36
Cellulose	Ether †	DMDAB; $NH_4SCN/FeSO_4$	0.25	0.25
Cellulose	Water, ether, *n*-butanol (1 : 10 : 10)	DMDAB; $NH_4SCN/FeSO_4$	0.53	0.53

*DMDAB, *p*-*N*,*N*-dimethyldiaminobenzene. †Diethyl ether.

techniques, while others (*10*) have investigated some of the many organic products ultimately produced. Their observations have been applied to further studies (*11, 12*) of the photochemistry of tryptophan incorporated in protein and the resulting effects on nucleic acids.

We now report the identification of one of the biologically active products resulting from the photooxidation of tryptophan in aqueous solution as hydrogen peroxide (H_2O_2). This compound accounts for some of the specific biological effects mentioned above: selective killing of *recA* mutants, mutagenicity, and others.

Photolysis of an oxygenated, aqueous solution of tryptophan for 24 hours, with lamps that emit maximally at 350 nm, produces a crude mixture that is selectively toxic to *recA* bacterial mutants. A starch-iodide qualitative test of this mixture demonstrated the presence of an oxidant, and thin-layer chromatography (TLC) indicated that H_2O_2 was present. Chromatographic purification (followed by bacterial killing assay) of the biologically active material [tryptophan photoproduct (TP)] on Sephadex G-10 afforded partially purified TP, which could be further separated from other photoproducts by ion exchange chromatography on Sephadex QAE resin. Final purification was accomplished by chromatography on a second G-10 column (Table 1).

Investigation of the possibility that TP is H_2O_2 was carried out by comparison of the spectrometric, chromatographic, chemical, and biological properties (see Table 2). Thus, both TP and H_2O_2 show no significant ultraviolet absorption other than "end absorption," increasing from about 230 nm. Both behave identically on cellulose TLC plates with two different solvent systems, and on silica gel TLC plates with one system, in addition to having identical Sephadex G-10 elution volumes; both give positive starch-iodide, lead tetraacetate, and *p-N,N*-dimethyldiaminobenzene tests, and both are reduced by sodium borohydride; finally, both are destroyed by catalase and show approximately 64-fold greater toxicity to *recA* mutants than to an isogenic *recA*$^+$ control. These results provide confirmation that the biologically active material TP is H_2O_2.

Semiquantitative analysis indicated that the crude photomixture, after irradiation for 24 hours, has an H_2O_2 concentration of as much as 0.015*M*, compared with an initial tryptophan concentration of 0.05*M*, indicating a 30 percent yield based on organic material. This is in line with the observations of Wood (*13*), who studied H_2O_2 production from the dye-sensitized photooxidation of amino acids, and of Santus (*11*), whose results indicated that the primary photooxidation product of tryptophan (*N*-formylkynurenine) is a good sensitizer. The H_2O_2 detectable by starch-iodide and *p-N,N*-dimethyldiaminobenzene is present after 10 minutes; and H_2O_2 can be detected by a bacterial killing assay after 40 minutes.

In view of these results, the genetic effects of H_2O_2 take on added importance. At least some of the above-mentioned biological properties, in addition to selective *recA* bacterial killing and mutagenicity, presumably are due to H_2O_2. The mechanism giving rise to such relatively large amounts of H_2O_2 is also of considerable interest (*14*).

J. P. McCormick
J. R. Fischer
J. P. Pachlatko
Department of Chemistry, University of Missouri, Columbia 65201

A. Eisenstark
Division of Biological Sciences, University of Missouri

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14. We also have found H_2O_2 to be produced on photooxidation of the tryptophan-rich enzyme lysozyme. Under the same conditions employed for tryptophan photolysis, H_2O_2 can be detected after 18 hours, and when the photolysis was carried out with added *N*-formylkynurenine, H_2O_2 was detected after only 4 minutes.
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Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Implications

Abstract. *The hydration rate of CO_2 catalyzed by human red cell carbonic anhydrase B is 92 percent reduced by the normal concentrations of chloride and bicarbonate in red cells. This reflects a general sensitivity of this reaction to halides and other anions, up to 87 times greater than the effect on red cell carbonic anhydrase C. The catalytic hydration of CO_2 is generally more (up to 24 times) sensitive to inhibition by anions and sulfonamides than the dehydration of HCO_3^-, probably reflecting different mechanisms. The sensitivity of enzyme B to anion inhibition also depends upon the substrate, being much greater for CO_2 than for certain esters. On the basis of the very low catalytic activity of B for CO_2 in the presence of physiological concentration of chloride, and the fact that carbonic anhydrase C is effective for CO_2 hydration (in the presence of chloride) at a rate 340 times greater than that of CO_2 output from tissues, it appears that the biological role of enzyme B is not that of a carbonic anhydrase.*

There are two zinc enzymes of similar size (molecular weight, 30,000), shape (spherical, cleft to active site, single chain, low helix), amino acid sequence, and active site structure in human red cells. Both catalyze the reversible hydration of CO_2 and the hydrolysis of certain esters. Because of the first of these properties, they are both called carbonic anhydrase (carbamate hydrolase, E.C. 4.2.1.1) but their characteristics are quite different (*1–5*).

Separation from blood by electrophoresis yielded 4 g of carbonic anhydrase B per liter of red cells, and 0.7 g of carbonic anhydrase C per liter of red cells. Several significant points have emerged: (i) The turnover number of C for CO_2 or HCO_3^- at *p*H 7.4 is some 20 times greater than that for B. (ii) With certain esters as substrate, B is more active than C (*1*). (iii) The activity and general properties of most tissue carbonic anhydrases closely resemble those of C. Kidney and stomach enzyme are of this high activity type, and are inhibited by $\sim 10^{-8}M$ acetazolamide (*2*). Carbonic anhydrase B and low activity enzyme from the blood of the rat, the rabbit, and the guinea pig, from the uterus and intestinal tissues of some of these species, and from the liver of the male rat required 10 to 1000 times as much acetazolamide (*6, 7*). (iv) Bovine and canine red cells contain but one carbonic anhydrase; each resembles C with regard to high activity and the inhibitory effect of acetazolamide and congeners at 10^{-8} to $10^{-9}M$ (*2, 3*). (v) Chemical differences between B and C suggest differing functions. In the dehydration reaction, the Michaelis constant (K_m) of C rises with *p*H, consistent with HCO_3^-