ing the postnatal rise in plasma bilirubin in the newborn, even with a dose of the metalloporphyrin 50-fold greater than that of an effective dose of tin-protoporphyrin (Fig. 2). It is highly improbable, therefore, that the amounts of zinc-protoporphyrin that might be ultimately released from erythrocytes in individuals with plumbism or iron-deficiency anemia would significantly affect tissue heme oxidation activities or plasma bilirubin levels.

The apparent K_m (K_m, Michaelis constant) for human splenic heme oxygenase (*9*) was determined from six different samples to yield a mean ± standard deviation of 19.04 ± 0.08 μM (Table 1). This value is similar to that previously reported for the human spleen enzyme (*10*), but is three to four times greater than that reported for rat spleen microsomal heme oxygenase (*11*) and nearly 20 times greater than that for the heme oxygenase system reconstituted with its purified enzymic components (*12*). Tin-protoporphyrin was a potent competitive inhibitor of the human spleen enzyme (six different samples), with an average value of the inhibition constant (K_i) of 0.018 ± 0.001 μM (Table 1). This value is similar to the K_i of 0.011 μM previously reported for rat microsomal hepatic and splenic heme oxygenase (*5*). These findings indicate that the degradation of heme to bile pigment in human spleen is several times more sensitive to blockade by tin-protoporphyrin administration than is the comparable tissue in the rat. If tin-protoporphyrin can be safely employed to prevent the development of threatening levels of hyperbilirubinemia in the human newborn, it can probably be used at a much smaller dose than that required to prevent this form of jaundice in the rat. Pharmacokinetic studies with tin-protoporphyrin in humans (*13*) are consistent with this expectation, since significant blood levels of this metalloporphyrin can be achieved with doses of the compound at least two orders of magnitude smaller than those utilized to suppress postnatal jaundice in the rat neonate.

Temporary suppression of heme oxidation would presumably lead to transient heme sequestration in the circulation or in tissues. The consequences of extending the period during which the newborn is normally exposed to high concentrations of unmetabolized heme are not known; in adult humans very large amounts of heme, as hematin, administered therapeutically (100 to 400 mg per infusion intravenously one or two times daily) to patients with hereditary erythroid or hepatic porphyrias have been essentially without detriment (*14*). In neonatal rats, multiple administrations of tin-protoporphyrin in amounts totaling 500 μmole/kg have produced no evident acute or chronic toxicities, and treated animals have matured and reproduced normally (*5*).

The biological properties of tin-protoporphyrin and of related metalloporphyrins that may have a comparable activity should be intensively explored, since a control mechanism of this type may have therapeutic potential in humans.

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Fluorescence Microscopy: Reduced Photobleaching of Rhodamine and Fluorescein Protein Conjugates by *n*-Propyl Gallate

Abstract. *n-Propyl gallate (0.1 to 0.25 molar, in glycerol) reduces by a factor of 10 the rate of fading of fluorescence of cell structures labeled with tetramethylrhodamine or fluorescein-conjugated antibodies. Hence, prolonged photographic exposure of immunofluorescently labeled cells in the fluorescence microscope yields images with increased sensitivity, making feasible multiple data collection, as with serial optical sectioning.*

Fluorescence microscopy is rapidly becoming a powerful tool in cell biology. The method allows identification with high sensitivity of specific fluorescently labeled molecules in biological material. These molecules can be analyzed in their native conformation amidst their natural environment, without interference from neighboring substances or cell structures, even in whole multicellular organs. The main application of fluorescence microscopy is in immunocytology (*1*), but direct labeling of cellular organelles (mitochondria and nuclei) (*2*) and visualization of cell-to-cell interactions (*3*) are possible after uptake of fluorescent dye by cells. Microinjection into cells of fluorescent proteins or peptides has been used to trace their intracellular metabolism (*4*), distribution, organization, and function (*5, 6*) and to elucidate cell lineages (*7*). Additional examples of the extreme specificity of fluorescence microscopy are the characterizations of cell constituents [their subcellular localizations (*8*) and roles in cell function (*9*)] with fluorescently labeled monoclonal antibodies and the analysis of three-dimensional chromosome structure with DNA-specific dyes (*10*).

Tetramethylrhodamine isothiocyanate (rhodamine) and fluorescein isothiocyanate (fluorescein) are the fluorescent dyes most commonly coupled to biological protein probes. A major problem accompanying the use of these dyes in microscopy is light-induced bleaching, apparent as fading of the emitted fluorescent light. Photobleaching is very rapid with microscopy in which epifluorescent illumination is used under conditions of high magnification and resolution because the excitation light beam is extremely intense (proportional to the

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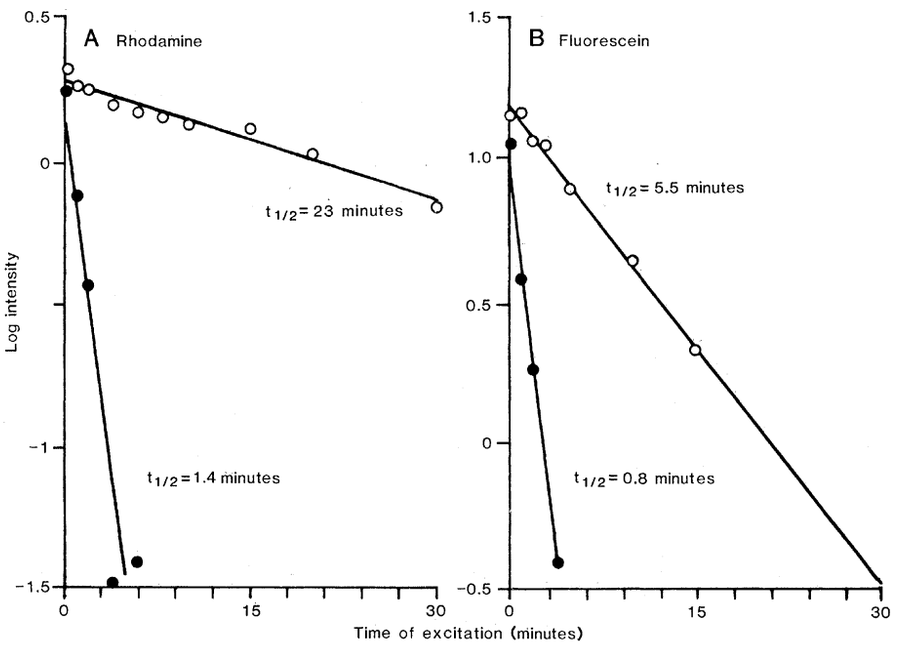
square of the numerical aperture of the objective). In practice, this intensity poses some serious limitations on fluorescence microscopy. Rapid fading of fluorescence limits fine analysis of globally weakly fluorescent objects and dim point sources. Even with brightly fluorescent objects, fine details of the image are frequently lost in photography, because high-speed films with large grain size have to be employed. In addition, it is difficult to accumulate data for longer than a few minutes from individual fluorescent specimens. This limitation precludes serial optical sectioning of biological samples or analysis by tilting samples through multiple angles.

One solution to the problem of fading may be to develop derivatives of fluorescein and rhodamine that are more resistant to photobleaching, or to employ nonbleaching fluorescent dyes, for example, laser dyes (*11*) or dyes that have a reversible or cycling photobleaching, as encountered in nature with rhodopsin. However, these dyes may present new problems, such as difficulties in coupling to proteins and light emission in the near infrared range. We chose therefore to search for a chemical that could be easily added to biological specimens labeled with rhodamine or fluorescein and that would decrease or possibly eliminate photobleaching.

Oxygen and oxygen-induced free radicals have been implicated in the reactions that lead to photobleaching (*12*). Therefore, in our investigation of the subcellular location of antigenic sites in *Drosophila* cell nuclei by immunofluorescence (*8*), we attempted to reduce photobleaching by supplementing the mounting medium of cell samples with antioxidants or free radical scavengers. We followed the fading of fluorescence of rhodamine-labeled cell nuclei in the microscope at high magnification and most intense illumination without resorting to laser light (see legend to Fig. 1). In the presence of the antioxidant *n*-propyl gallate, 2 to 5 percent (weight to volume) in glycerol, we obtained clear photographs of fluorescently labeled cells even after 10 minutes of prior exposure to the incident light; control samples mounted in pure glycerol had completely faded by that time. Butylated hydroxytoluene (a saturated solution in glycerol, about 60 ppm) and commonly used free radical scavengers such as cysteine, dithiothreitol, and imidazole (0.05*M* to 0.2*M* in 90 percent glycerol) had no effect on fading.

To obtain a quantitative estimate of the effect of propyl gallate, we measured the intensity of the fluorescent light emitted from 70 to 100 rhodamine-labeled cells as a function of time of continuous exposure to the incident light. The log of the emitted light was inversely proportional to the duration of sample illumination (Fig. 1A), suggesting a single-hit mechanism for the overall photobleaching reaction. The time required for 50 percent loss of the initial fluorescence intensity ($t_{1/2}$) was calculated from the linear graphs. The $t_{1/2}$ increased proportionally with the concentration of propyl gallate (Fig. 1C). At a 5 percent (weight to volume) propyl gallate concentration, $t_{1/2}$ for rhodamine-labeled cells increased about 15-fold, from 60 to 90 seconds to



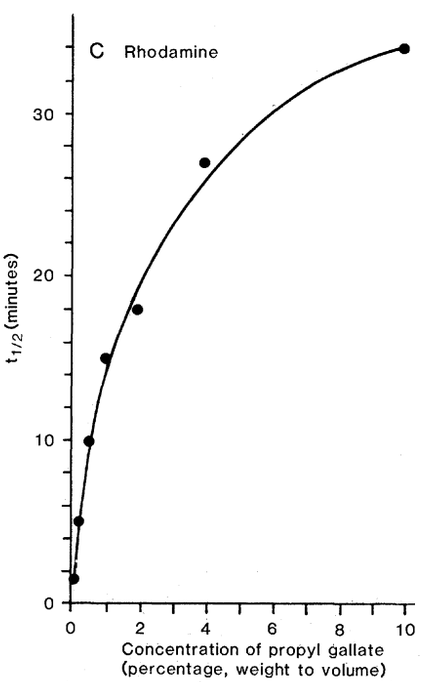


Fig. 1. Half-life ($t_{1/2}$) of fluorescence in cultured cells labeled with rhodamine- or fluorescein-conjugated antibodies and mounted in the presence of *n*-propyl gallate. Cultured *Drosophila* cells (Schneider's line 2) were fixed for 5 minutes in Robb's saline (*17*) on polystyrene cover slips, washed with PBS, permeabilized by incubation in PBS containing 1 percent Triton X-100 and 1 percent sodium deoxycholate, and incubated with monoclonal antinuclear antibody 8-5-F-10 (*8*) and with goat antibody to mouse immunoglobulin G (IgG) conjugated with (A) tetramethylrhodamine isothiocyanate or (B) fluorescein isothiocyanate. Samples were washed in PBS containing 0.1 percent Triton X-100 or with PBS alone, and mounted in glycerol or glycerol containing 5 percent (weight to volume; approximately 0.24*M*) *n*-propyl gallate (Sigma). The cells were observed in a Zeiss Axiomat microscope equipped with a 100×/N.A.1.3 oil immersion objective, an epifluorescence illuminator (Zeiss HBO 100 W/d-c mercury lamp; arc size, 0.25 mm), and a four-lens condenser. The excitation and emission filters were Zeiss filter systems 477715 or 487701. The intensity of the fluorescent light emitted from the cell samples was measured by recording the duration of automatic photographic exposures (5 to 30 seconds) at an ASA setting of 6300; this system gives exposure times proportional to light intensity. Fluorescence intensity was measured—5 seconds after initiation of full power illumination of the cells—as a function of time. The value of $t_{1/2}$ (see text for explanation) was calculated after subtraction, at each time point, of the background fluorescence emitted from areas free of cells. Background fluorescence ranged from 6 to 30 percent of total fluorescence and was not affected by the presence of propyl gallate. (C) The value of $t_{1/2}$ was calculated from data of experiments similar to those shown in (A) except that the propyl gallate concentration in glycerol was varied.

15 to 25 minutes (Fig. 1, A and C), in comparison with the control. This protective effect was noted shortly after the samples were mounted in propyl gallate, but reached a maximum after several hours. The effect of propyl gallate was not significantly altered when the solution was buffered in the *p*H range between 5.5 and 8.3 by citrate, cacodylate, tris, or carbonate buffer (*13*) or when the glycerol concentration was lowered to 80 percent.

Higher concentrations of propyl gallate in glycerol (10 to 20 percent) were more efficient in retarding fading, but were impractical since fluorescence was quenched. From 25 to 50 percent quenching of the fluorescence of rhodamine by 2 to 5 percent propyl gallate solutions in glycerol was occasionally observed, but was prevented by washing the cell samples free of detergent (Triton X-100 and deoxycholate) remaining from the preceding incubations.

Under conditions identical to those described for rhodamine, propyl gallate also showed a protective effect against rapid photobleaching in cells labeled with fluorescein-conjugated antibodies. The relative extent of protection was similar with both dyes, although the $t_{1/2}$ for the fading of fluorescein was only about half that for rhodamine-labeled cells (Fig. 1B). In cells labeled with fluorescein-conjugated antibodies, the intensity of fluorescence was diminished unless the *p*H of the propyl gallate glycerol solution was kept slightly alkaline (*6, 14*).

Among the compounds tested, propyl gallate was the most effective anti-photobleaching agent (*15*). Its stability to spontaneous oxidation in air, when solid or when dissolved in glycerol, made this compound the reagent of choice. Still, propyl gallate should be used with caution. In some whole *Drosophila* tissues, after storage of rhodamine-labeled samples in 5 percent propyl gallate in glycerol for 2 to 3 days, we observed changes in the initial fluorescence pattern resulting from a decrease in fluorescence intensity. This decrease could be reversed by rinsing the samples in phosphate-buffered saline (PBS) containing 0.1 percent Triton X-100 and, finally PBS. Therefore, propyl gallate was added shortly before observation of the samples and when prevention of photobleaching yielded additional information, for example, at high microscopic magnification. After being photographed, the samples were rinsed with PBS and stored in pure glycerol.

The protective effect of propyl gallate in fluorescence microscopy was used for optical sectioning of nuclei in fixed, cultured *Drosophila* cells. These cells were incubated with monoclonal antibody 5-4-D-7, from a library of monoclonal antibodies to *Drosophila melanogaster* embryo nuclei [recently established by Kuo *et al.* (*8*)] and were labeled with a rhodamine-conjugated second antibody. Twenty-two serial photographs of fluorescent nuclei in 5 percent propyl gallate were taken by moving the focal plane by computer control, stepwise, between successive exposures of 30 seconds each (Fig. 2A). The antibody 5-4-D-7 is specific for the nuclear envelope region. The fluorescent intensity decreased less than 50 percent between the first and 22nd step, indicating that it is possible to record multiple view data from fluorescent cellular proteinaceous structures. In addition, the high resolution achieved in modern microscopes can now be preserved in photographs by using slow, fine grain films. The multiple data obtained can first be refined by computer-assisted image processing to remove out-of-focus information and blurring, and can then be further processed to reconstruct three-dimensional images of the fluorescently labeled structures (*10*). Combined with the powerful technique of monoclonal antibodies, this procedure will yield valuable information about the subcellular organization and function of proteins and other cellular antigens.

Reducing the photobleaching of rhodamine and fluorescein adducts, achieved by *n*-propyl gallate, has general and practical implications for fluorescence microscopy. Primarily, reduction of the photobleaching prolongs the flux of photons from the fluorescently labeled molecules in the samples. The sensitivity of fluorescence microscopy can then be increased by appropriate integration methods, for instance, by using long photographic exposure times. As a first approximation, the increased sensitivity achieved is proportional to the reduction in the rate of bleaching; in this case the increase is about 10- to 15-fold. Combination of propyl gallate glycerol, high-power laser beam excitation, and an integration silicon-intensified target television (SIT) or charge-coupled device (CCD) camera could achieve an unprecedented level of sensitivity. We may then approach the goal of sensing single fluorescently labeled molecules.

The mechanisms of photobleaching of

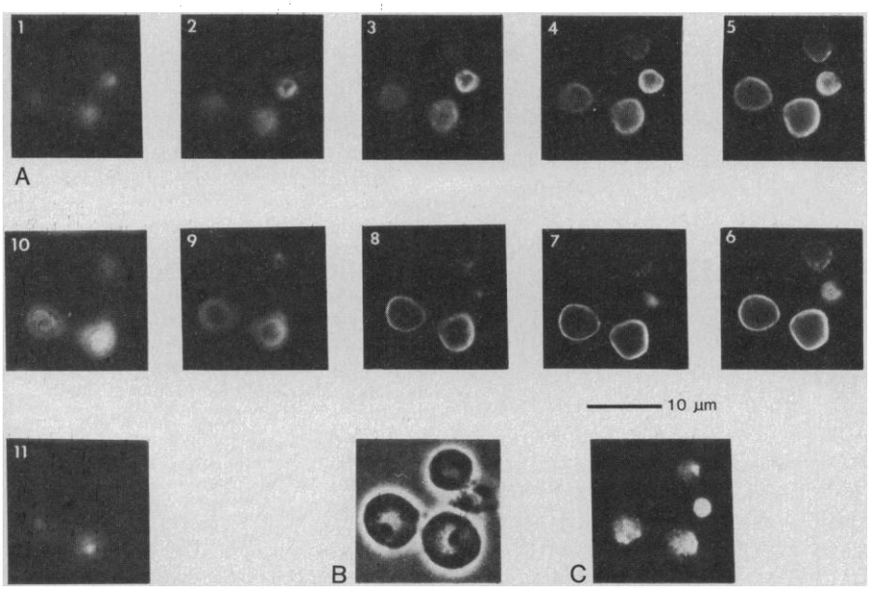


Fig. 2. Serial optical sections through nuclei of cultured *Drosophila* cells, fluorescently labeled with a monoclonal antibody specific for a nuclear envelope antigen. *Drosophila* cells, line Kc (*18*), were fixed and permeabilized as described in the legend to Fig. 1. They were incubated in succession with monoclonal antibody 5-4-D-7, rhodamine-labeled second antibody, and Hoechst 33258 (*8*). Washed cell samples were mounted in 5 percent (weight to volume) *n*-propyl gallate in glycerol for fluorescence microscopy and were photographed as described (*8*). The inclusion of propyl gallate in glycerol did not affect the cellular immunofluorescence pattern. Optical sectioning was accomplished by focusing first on the top of a group of cells, then moving the objective toward the cells in 400-nm steps and exposing a Kodak Technical Pan 2415 film for 30 seconds at each step. Movement, rate of movement, and exposures were controlled by a microcomputer through a precise stepping motor attached to the microscope (*10*). Only every second exposure is shown. The gradual loss of fluorescence intensity (≤ 50 percent) throughout the optical sectioning, which lasted about 30 minutes, was compensated for by adjustment of the final photographic printing. Nonspecific cell fluorescence was below the detection limit [see (*8*)]. (A) Rhodamine fluorescence, (B) phase-contrast image, and (C) Hoechst-DNA fluorescence of the same optical section as shown in (A, 5).

rhodamine- and fluorescein-conjugated antibodies and its retardation by *n*-propyl gallate are still unclear. However, the similar protective effects by three oxygen-reducing compounds, *n*-propyl gallate, ascorbic acid, and *p*-phenylenediamine, and a similar effect of dithionite on fluorescein photobleaching in a model system (*16*) suggest that molecular oxygen is involved in the reactions that lead to the photobleaching.

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Stimulation of Colonic Secretion by Lipoxygenase Metabolites of Arachidonic Acid

Abstract. *Both 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE) increased the short-circuit current (I_{sc}) in rabbit colonic mucosa mounted in vitro in Ussing chambers. Measurements of chlorine-36 fluxes indicated that the I_{sc} response to 5-HPETE is due to stimulation of active chlorine secretion. 9-, 11-, and 12-HPETE's and leukotrienes C_4 and B_4 produced either very small increases in I_{sc} or no increase. In contrast to results in rabbit colon, no HPETE, HETE, or leukotriene was effective in rabbit ileal mucosa. The effects of 5-HPETE in the rabbit colon were unaffected by mepacrine, but could be partially blocked by indomethacin. These results suggest that drugs which block both cyclooxygenase and lipoxygenase may be effective antidiarrheals in patients with colitis.*

Diarrhea frequently develops when there is inflammation in the mucosa of the small or large bowel, whether the inflammation is acute, as in infectious enteritis (*1*), or chronic, as in inflammatory bowel disease (IBD) (*2*). One possible basis for the diarrhea is the action of inflammatory mediators on the intestinal epithelium. We previously showed that kinins, known to be produced in inflammatory lesions, stimulate secretion in both small and large intestine (*3*). Kinin receptors occur on intestinal enterocytes where they stimulate the production of prostaglandin E_2 (PGE_2), which stimulates electrolyte secretion (*3*). Kinin-induced secretion can be inhibited by drugs that inhibit PGE_2 production either by blocking cyclooxygenase (for example, indomethacin) or phospholipase A_2 (for example, mepacrine). In white cells found at inflammatory sites, however, the major route of arachidonic acid metabolism is via the lipoxygenase pathway, resulting in the release of large amounts of hydroperoxyeicosatetraenoic acids (HPETE's), hydroxyeicosatetraenoic acids (HETE's), and leukotrienes (*4*). These products affect many physiological systems including smooth muscle contractions and chemotaxis (*5*).

We report here that 5-HPETE and 5-HETE have powerful secretory effects in rabbit colon, but not ileum, suggesting that lipoxygenase products contribute to diarrhea in patients with inflammatory lesions of the colon. If this is the case, indomethacin, which blocks cyclooxygenase but not lipoxygenase (*6*), should not be an effective antidiarrheal in such patients. Indeed, clinical trials with indomethacin have not been successful (*7*). However, drugs that inhibit lipoxygenase as well as cyclooxygenase or that inhibit arachidonic acid release may be therapeutic, at least with respect to diarrhea. Since lipoxygenase products also induce chemotaxis (*4*), such drugs may also reduce inflammation. Steroids that act indirectly to inhibit phospholipase A_2 and arachidonate release are certainly effective in IBD (*8*). Nonsteroidal antiinflammatory drugs such as eicosatetraynoic acid (ETYA) (*9*) and BW755 (*6*), which inhibit both lipoxygenase and cyclooxygenase may be worth testing in patients with colitis and diarrhea.

Colonic and ileal mucosa from male New Zealand White rabbits was stripped of both muscle layers and mounted in Ussing chambers. Transepithelial potential difference (PD), short-circuit current (I_{sc}), and unidirectional Cl^- fluxes were measured as described (*10*). Serosal addition of either 5-HPETE or 5-HETE (*11*) to distal colon caused a rapid increase in PD which slowly returned to baseline (half-time decline in PD was about 80 minutes). The average tissue conductance also increased and, therefore, the change in I_{sc} was relatively greater than the change in PD. Effects of both 5-HPETE and 5-HETE were observed with concentrations as low as $5 \times 10^{-7} M$. A maximally effective concentration of 5-HPETE ($1.6 \times 10^{-5} M$) increased I_{sc} by about 120 $\mu A/cm^2$, whereas 5-HETE ($1.6 \times 10^{-5} M$) increased I_{sc} by about 90 $\mu A/cm^2$ (Fig. 1).

In contrast neither 5-HPETE nor 5-HETE increased I_{sc} when added to rabbit ileal mucosa. Other HPETE analogs produced little effect in either colon or ileum. Small increases in I_{sc} were observed in the colon with 11-HPETE (20.2 $\mu A/cm^2$ at $3.2 \times 10^{-5} M$) and 12-HPETE (12.2 $\mu A/cm^2$ at $3.2 \times 10^{-5} M$). 9-HPETE ($3.2 \times 10^{-5} M$) and leukotrienes B_4 and C_4 ($10^{-7} M$) had no effect in either colon or ileum. The leukotriene C_4 and D_4 antagonist FPL 55712 did not affect the response to 5-HPETE, indicating that either leukotriene A_4, 5-HETE, or 5-HPETE itself were effecting the change in I_{sc}. To establish the ionic basis of the electrical response to 5-HPETE and 5-HETE we determined the I_{sc} responses in the absence of both Cl^- and HCO_3^- and also measured Cl^- fluxes. Replace-

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