phenomenon of a negative Poisson's ratio consequently does not require a coarse cellular structure or depend on the structure size. In principle, materials with microstructure on a scale smaller than 1 μm could exhibit a negative Poisson's ratio. The theory of elasticity also predicts a variety of unusual phenomena to occur in solids with a negative Poisson's ratio. For example, the top and bottom lateral surfaces of a bent prismatic beam of a conventional material with a positive Poisson's ratio assume a saddle shape: the "anticlastic curvature" of bending, in which the transverse curvature is opposite the principal curvature of bending (*9*). In the case of a negative Poisson's ratio, the theory of elasticity predicts that these surfaces will assume an ellipsoidal shape, or a synclastic curvature. I have observed such synclastic curvature in bent bars of transformed foam. Furthermore, in the indentation of a block of material caused by a localized pressure distribution, the indentation for a given pressure is proportional to $(1 - \nu^2)/E$, in which E is Young's modulus and ν is Poisson's ratio. Consequently, a material with a negative Poisson's ratio approaching the thermodynamic limit $\nu = -1.0$ will be difficult to indent even if the material is compliant. The origin of this predicted phenomenon may be traced to the relation between the shear modulus G, the bulk modulus B (the inverse of the compressibility), and Poisson's ratio ν: $B = 2G(1 + \nu)/(1 - 2\nu)$. When the Poisson's ratio approaches 0.5, as in rubbery solids, the bulk modulus greatly exceeds the shear modulus and the material is referred to as incompressible. When Poisson's ratio approaches -1.0, the material becomes highly compressible; its bulk modulus is much less than its shear modulus. The toughness of a material can also depend on its Poisson's ratio. Specifically, the critical tensile stress (*10*) for fracture of a solid of surface tension T, Young's modulus E, with a plane circular crack of radius r is $[\pi ET/2r(1 - \nu^2)]^{1/2}$. When the Poisson's ratio approaches -1.0, the material is predicted to become very tough.

Applications of novel, reentrant foams with negative Poisson's ratios may be envisaged in view of these properties. An example of the practical application of a particular value of Poisson's ratio is the cork of a wine bottle. The cork must be easily inserted and removed, yet it also must withstand the pressure from within the bottle. Rubber, with a Poisson's ratio of 0.5, could not be used for this purpose because it would expand when compressed into the neck of the bottle and would jam. Cork, by contrast, with a Poisson's ratio of nearly zero, is ideal in this application. It is anticipated that reentrant foams may be used in such applications as sponges, robust shock-absorbing material, air filters, and fasteners.

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Encystation and Expression of Cyst Antigens by *Giardia lamblia* in Vitro

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The cyst form of *Giardia lamblia* is responsible for transmission of giardiasis, a common waterborne intestinal disease. In these studies, encystation of *Giardia lamblia* in vitro was demonstrated by morphologic, immunologic, and biochemical criteria. In the suckling mouse model, the jejunum was shown to be a major site of encystation of the parasite. Small intestinal factors were therefore tested as stimuli of encystation. An antiserum that reacted with cysts, but not with cultured trophozoites was raised in rabbits and used as a sensitive probe for differentiation in vitro. Cultured trophozoites that were exposed to bile salts showed a more than 20-fold increase in the number of oval, refractile cells that reacted strongly with anticyst antibodies, and in the expression of major cyst antigens. Exposure to primary bile salts resulted in higher levels of encystation than exposure to secondary bile salts. These studies will aid in understanding the differentiation of an important protozoan pathogen.

GIARDIASIS, A MAJOR HUMAN INTESTINAL disease worldwide, is transmitted by ingestion of the oval cyst form of *Giardia lamblia* from fecally contaminated water or food (*1*). Exposure of cysts to gastric acid triggers excystation in the duodenum (*2*). Emerging flagellated trophozoites divide and colonize the small intestine where some remain and cause diarrhea, while others encyst and are passed in feces, completing the life cycle (*3*). Although trophozoites can be cultured in vitro (*4*), neither encystation nor expression of cyst antigens in vitro has been reported. The studies presented here were designed to (i) elucidate the process of encystation in vivo; (ii) develop sensitive reagents for the detection and quantitation of differentiation in vitro; and (iii) develop a system for induction of encystation of trophozoites cultured in vitro.

Since little is known about encystation of *G. lamblia*, we studied the location of cysts and trophozoites along the small and large intestine of suckling mice as a function of time after infection. Three-day-old suckling mice (strain CF1) were infected (*5*) with 10^3 axenically cultured *G. lamblia* trophozoites by direct transcutaneous injection into the milk-filled stomach (*6*). Trophozoites of strain WB (ATCC #30957) had been grown to late log phase in supplemented (*6*) Diamond's TYI-S-33 medium (*7*) as described (*8*), washed, and resuspended in 0.2 ml of 0.1*M* phosphate-buffered saline (PBS; *p*H 7.2). We found cysts in every intestinal section, although few were in the duodenal section "D" (Fig. 1). Through day 16, large numbers of cysts were in sections 3 or 4 (mid to lower jejunum). As infection progressed, increasing proportions of cysts were found in the large intestine. Since cysts are not motile and therefore move downstream with the flow of intestinal fluid, sections 3 and 4 appeared to be major sites of encystation. The percentage of parasites in cyst form per mouse averaged 8.6 (from nine determinations, days 4 through 20, range,

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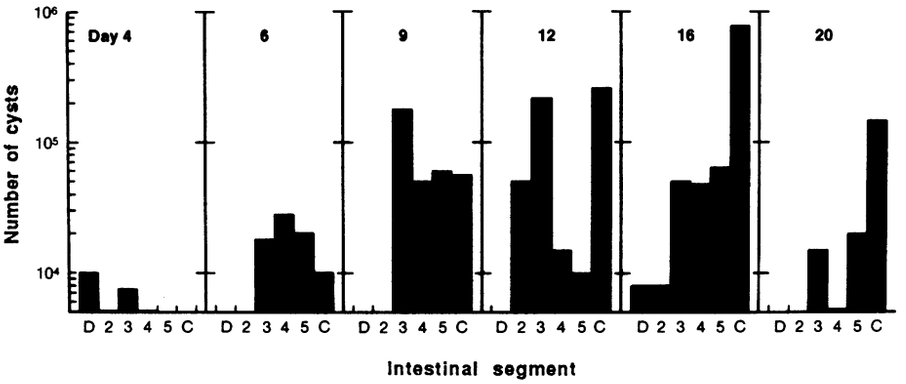

Fig. 1. Location of *G. lamblia* cysts in intestinal tract of suckling mice as a function of time after infection. Mice were killed at 24-hour intervals for the first 9 days after infection and thereafter as indicated. The intestines were removed and cut transversely just below the pylorus and above the cecum. The small intestine was cut into five segments of equal size and labeled as: D, the uppermost segment, including the duodenum, segments 2 through 5; and C, the cecum and large intestine. To recover all the parasites we macerated each section in 0.5 ml of PBS. Cysts and trophozoites were enumerated in hemocytometer chambers by phase-contrast microscopy. The data show the average of duplicates from a single experiment that was repeated four times. No parasites were observed in intestines or feces of uninfected mice.

0.6 to 35%). In this model (*9*) and in adult mice infected with *G. muris* (*10*) the numbers of intestinal or fecal (*10*) cysts declined in parallel with the number of trophozoites, indicating that resolution of infection was not due to terminal parasite differentiation.

While attempting to induce encystation in vitro, we regularly observed oval, refractile, nonmotile cells with cyst morphology (see Fig. 2A). To confirm their identity, we developed cyst-specific antibodies as probes for the quantitation of *G. lamblia* differentiation. To detect as many neoantigens as possible, we prepared a polyclonal antiserum by injecting a rabbit intravenously on days 1, 3, 6, 8, and 13 with 5×10^5 highly purified (*10*) *G. lamblia* cysts from feces of a chronically infected woman. The rabbits were given a boosting injection 9 weeks later (10^6 cysts). The antiserum reacted at dilutions of more than 1:12,000 in assays of indirect immunofluorescence (IIF) and an enzyme-linked immunosorbent assay (ELISA) with *Giardia* cysts from natural infections of humans (Fig. 2B) and dogs, as well as cysts from our experimental infections of suckling mice. Since the serum reacted with fresh, unfixed cysts (Fig. 2B), at least some of the antigenic determinants are on the surface. No reaction was detected with cultured trophozoites (strain WB) by IIF, ELISA (at 1:100), or immunoblots. In contrast, the monospecific antiserum of Rosoff and Stibbs reacted with a 65-kD antigen of cysts and trophozoites (*12*). Moreover, although chitin was recently reported to be a component of the *Giardia* cyst wall (*13*), it is apparently not a major determinant of our cyst antisera since extensive adsorption of four sera with chitin (*13*) did not reduce the IIF titer against human cysts.

The greatest proportion of trophozoite colonization in the suckling mouse occurred in the upper three sections of the intestine; large numbers of mature cysts were found in sections 3 and 4 (jejunum) (Fig. 1). Thus, conditions in the upper intestinal region appeared to be important in inducing encystation. In this area, trophozoites are exposed to high concentrations of bile, as well as lipolytic products. Therefore, we initially exposed parasites (2.5×10^5/ml) to a bile salt [glycodeoxycholate (GdC), 16 m*M*] for 6 days in the presence and absence of oleic acid (100 μ*M*) in medium without the normal bovine bile (*6*) component. Encystation was analyzed by IIF with the cyst antibodies, and a cyst was defined as an oval, refractile cyst-shaped cell that fluoresced brightly. Morphologic details of cysts were observed by phase contrast microscopy (Fig. 2A). The percentage of encystation was 8.2 ± 3.0% in the presence of GdC alone, and 9.1 ± 2.0% with GdC plus oleic acid,

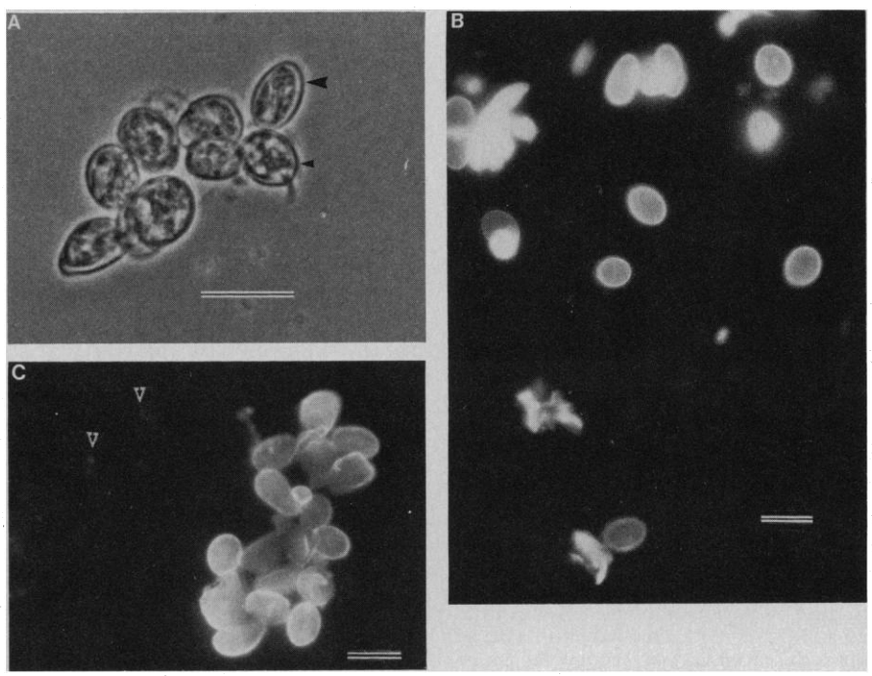

Fig. 2. (**A**) Phase-contrast microscopy of unfixed and unstained cysts induced in vitro, showing typical cyst refractility (see halo around each cyst), size, and morphology. Trophozoites were incubated for 6 days in 4 ml of TYI-S-33 medium lacking bovine bile and supplemented with 16 m*M* glycodeoxycholate and 100 μ*M* oleic acid. Typical cyst nuclei and axostyle can be seen in the cell indicated by large arrowhead. "Claw-hammer" median bodies typical of *G. lamblia* cysts lie across the axostyle of the cell below (small arrowhead). Bar = 10 μm. (**B**) Antigenic reaction of purified cysts from human feces with cyst antiserum raised in rabbits. Intact, unfixed, purified (*11*) cysts from a patient with acute giardiasis were reacted with the rabbit antiserum or preimmune serum [diluted 1:100 in 0.02*M* PBS with 1% bovine serum albumin (BSA) and 1% Tween 20] for 2 hours, washed by centrifugation, and reacted with FITC-conjugated protein A (1:2000). The cysts show fluorescence with the antiserum, while the fecal debris is autofluorescent. (**C**) Encystation of *G. lamblia* in vitro. Trophozoites were incubated for 3 days in TYI-S-33 medium without bovine bile and supplemented with 8 m*M* glycochenodeoxycholate (as in Fig. 3). Samples (20 μl) were spotted onto wells of printed slides, air-dried, and fixed with formaldehyde (1%) and then with acetone (10 minutes). After being washed they were reacted with the rabbit (diluted 1:800 in PBS–BSA–Tween 20), washed again, and incubated with FITC-conjugated goat antiserum to rabbit immunoglobulin G. Ethidium bromide (2 μg/ml) was used as a counterstain. In addition to the large clump of brightly fluorescent oval cells in this field, trophozoites whose paired nuclei appear red in the fluorescence microscope are indicated by the arrowheads. They serve as a control since they do not react with the cyst antiserum. Clumping of cysts is also prominent in preparations from the intestine of infected suckling mice.

whereas the percentage was 0.15 ± 0.17% in the control with no additions ($P < 0.001$, Student's t test). When the trophozoite inoculum was reduced ten times, encystation decreased to 1.6 ± 0.8% in the presence of GdC alone and 2.4 ± 0.5% in the presence of GdC and oleic acid. Others have shown that higher inocula also favored encystation of *Entamoeba invadens* (*14*).

We next compared the ability of the six major bile salts of human bile to stimulate encystation. The primary bile salts (cholate and chenodeoxycholate) are synthesized from cholesterol in the liver and secreted into bile as their glycine and taurine *N*-acyl conjugates. In contrast, deoxycholate, a secondary bile salt, results from bacterial dehydroxylation of cholate and is absorbed in part and conjugated in the liver. Bile salts are actively reabsorbed in the terminal ileum and secreted into the bile where the ratio of primary to secondary bile salts is about 3:1 (*15*).

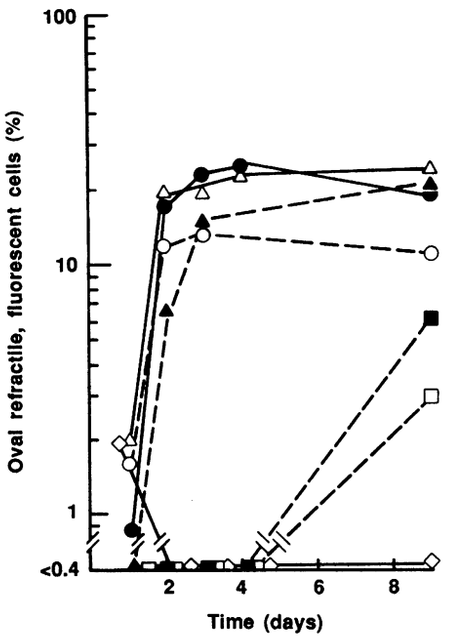

Fig. 3. Effect of primary and secondary bile salts on encystation of *G. lamblia* in vitro. Late log phase cultures were chilled and the cells were harvested by centrifugation (850*g*) and washed twice with Hanks balanced salt solution. Parasites (2.5×10^5/ml, final dilution) were added to TYI-S-33 medium without bile, supplemented with individual bile salts (as sodium salts, Sigma). Piperacillin (Lederle, 500 μg/ml) and Amikacin (Bristol Labs, 125 μg/ml), which do not affect *G. lamblia* growth (*24*) or encystation, were added. Replicate 4-ml aliquots of this mixture were incubated at 37°C. At each time indicated, a set of vials was chilled and inverted ten times to release attached parasites. The contents of each vial were transferred to 4-ml tubes and the parasites sedimented at 850*g* for 10 minutes (8°C), then resuspended in 1 ml of Hanks salts. IIF analysis was performed as above (Fig. 2C). Each data point is the mean of at least five random fields which usually contained 50 to 200 cells. The number of brightly fluorescent, oval, refractile cells (by phase contrast) was divided by the total number of cells in that field. Because of clumping of cysts (as in Fig. 2C), standard deviations were up to 30% of the means, but all values for primary bile salts on days 2 and after were significantly greater than control ($P < 0.01$). The data shown are from the concentration of each bile salt which yielded the greatest percentage encystation. Symbols: ◇, control, no bile salt; ● glycocholate, 16 m*M*; ○, taurocholate, 16 m*M*; ▲, glycochenodeoxycholate, 8 m*M*; △, taurochenodeoxycholate, 8 m*M*; ■, glycodeoxycholate, 16 m*M*; □, taurodeoxycholate, 8 m*M*.

The data in Fig. 3 show that each of the six bile salts tested stimulated encystation (Fig. 2C). However, the kinetics of encystation stimulated by primary and secondary bile salts differed strikingly. In the presence of primary bile salts, the maximum increase in encystation occurred between 1 and 2 days. In contrast, little encystation (less than 0.4%) occurred until after 4 days of incubation with secondary bile salts and the percentage of encystation stimulated by secondary bile salts was lower than with the primary bile salts.

At present we do not understand the differences in encystation-stimulating activity of the primary and secondary bile salts. The primary bile salt chenodeoxycholate (3α,7α-OH) and the secondary deoxycholate (3α,12α-OH) are both dihydroxy and resemble each other in structure, hydrophobicity, and critical micellar concentration [2 to 4 m*M* in 0.15*M* NaCl (*16*)] more than they resemble the primary trihydroxy bile salt cholate (3α,7α,12α-OH). Cholate has a higher critical micellar concentration (~9 m*M*) by virtue of its more hydrophilic nature (*16*). Other properties that might explain the difference are that (i) certain lipases are stimulated by primary, but not secondary, bile salts (*17*) and (ii) primary bile salts bind calcium and iron but conjugates of secondary bile salts do not (*18*).

To analyze cyst antigens expressed in vivo and in vitro, we subjected parasite extracts to electrophoresis on SDS–polyacrylamide gel electrophoresis (*19*), transferred the bands to nitrocellulose (*20*), and reacted them with the rabbit antiserum to cysts. Antigens of cysts purified from human feces are shown in Fig. 4A, lane 2. The major bands are of ~21, ~28, and ~49 kD. Cyst antigens (~21, ~28, and ~36 kD) were also detected in immunoblots of parasites cultured in vitro for 1 to 7 days with glycocholate (Fig. 4B). In contrast, trophozoites from control cultures did not react with the cyst antiserum (Fig. 4A, lane 1). Moreover, neither antibodies to trophozoites nor preimmune serum recognized the cyst-specific antigens.

Some cysts induced with glycocholate had other characteristics that distinguish mature fecal cysts from the binucleate, osmotically

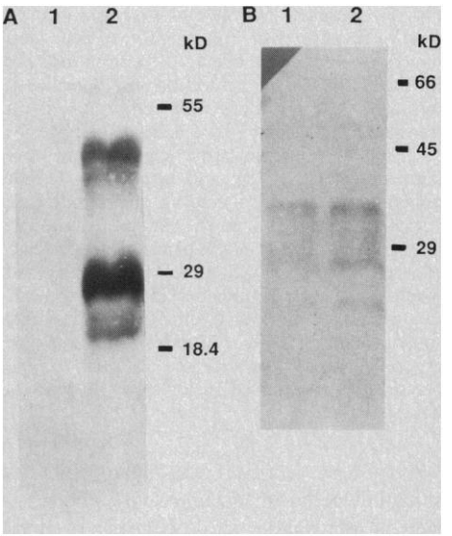

Fig. 4. Cyst-specific antigens of *G. lamblia*. (**A**) Western blots of parasite extracts reacted with rabbit antibody (1:400) against cysts purified from feces and visualized with peroxidase-conjugated protein A. Lane 1, 2.25×10^6 routinely cultured WB trophozoites; lane 2, 1.25×10^6 cysts purified from human feces. (**B**) Expression of *G. lamblia* cyst antigens in vitro. Strain WB parasites were incubated with 8 m*M* glycocholate and then harvested and analyzed by Western blot as in (A). Lane 1, 24-hour incubation, 10^6 parasites; lane 2, 48-hour incubation, 2×10^6 parasites. The patterns of days 3 through 7 were similar. The bands are fainter in (B) than in (A) because not all cells in the culture are expressing cyst antigens.

sensitive trophozoite form, such as four nuclei (revealed by staining with ethidium bromide) and resistance to hypotonic conditions (overnight incubation in double-distilled water) that lyse trophozoites. Since chitin is a prominent component of the *G. lamblia* cyst wall (*13*), we investigated whether conditions that stimulate encystation would also increase chitin synthetase (CS) activity. This enzyme has not been reported in *Giardia* to date. Extracts of parasites incubated for 2 days in encystation medium (the same cells as in Fig. 4B, lane 2) showed a >100-fold increase in CS specific activity: 3.6 ± 0.5 nmol of ^{3}H-labeled *N*-acetylglucosamine was incorporated from uridine diphosphate (UDP)–*N*-acetylglucosamine into trichloroacetic acid–precipitable material (per milligram of extract protein per 5 hours), assay modified from (*21*), compared with control cultures without glycocholate (<0.005 nmol/per milligram of protein). Incorporation was inhibited by Polyoxin D and by Nikkomycin, which are specific CS inhibitors (*22*), and the product was digested by purified chitinase (*23*).

These studies may facilitate attempts to complete the life cycle of *G. lamblia* in vitro. Bingham and co-workers (*2*) reported that

cysts from human feces rapidly (>90% in 7 hours) lose the ability to excyst at 37°C and require 2 to 7 days at low temperature (4° to 8°C) to excyst efficiently. We have not yet determined whether cysts induced in vitro are capable of excystation and are infective for animals. However, the ability to induce encystation in vitro will make it possible to obtain cysts without fecal contamination for studies of their basic biochemistry, of improved methods for disinfection of water, and of possible means of interrupting the life cycle of this parasite.

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Bilirubin Is an Antioxidant of Possible Physiological Importance

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Bilirubin, the end product of heme catabolism in mammals, is generally regarded as a potentially cytotoxic, lipid-soluble waste product that needs to be excreted. However, it is shown here that bilirubin, at micromolar concentrations in vitro, efficiently scavenges peroxyl radicals generated chemically in either homogeneous solution or multilamellar liposomes. The antioxidant activity of bilirubin increases as the experimental concentration of oxygen is decreased from 20% (that of normal air) to 2% (physiologically relevant concentration). Furthermore, under 2% oxygen, in liposomes, bilirubin suppresses the oxidation more than α-tocopherol, which is regarded as the best antioxidant of lipid peroxidation. The data support the idea of a "beneficial" role for bilirubin as a physiological, chain-breaking antioxidant.

ALTHOUGH MOST OF THE MOLECULAR oxygen in aerobic eukaryotic cells and organisms is sequentially reduced to water via the respiratory chain, both the univalent and bivalent reduction of oxygen occur during normal intermediary metabolism to give rise to superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (*1*). These oxygen reduction products have the potential to generate other reactive oxygen species (ROS) such as the hydroxyl radical, which then may initiate a radical chain reaction leading to extensive formation of lipid hydroperoxides. Lipid peroxides and their breakdown products alter the physicochemical properties of biomembranes and can cause damage to membrane-bound enzymes as well as to other macromolecules (*2, 3*). ROS have been implicated in the cause or pathology of cancer, aging, tumor promotion, heart disease, chronic inflammation, and parasitic infections (*2–4*). To prevent the formation of oxidants as well as to repair oxidative damage to tissues and macromolecules, all aerobic living organisms possess a complex armory of enzymatic and nonenzymatic antioxidant defenses. This includes the enzymes superoxide dismutase and catalase, the glutathione cycle, vitamins E and C, and β-carotene (*1–9*).

Work from this laboratory (*10*) has suggested that uric acid, the end product of purine metabolism, which is a powerful antioxidant (*10, 11*), may serve as a protective agent in human plasma. Recently, the antioxidant and membrane-protective properties of taurine, the end product of oxidative metabolism of cysteine, were reviewed (*12*). Another major metabolic pathway in mammalian systems is the degradation of protoheme derived from hemoproteins such as hemoglobin and cytochrome P-450 to bilirubin. As a result of this, approximately 300 mg of bilirubin per day are produced by normal adult humans (*13*). Bilirubin is generally regarded as a toxic compound when accumulated at abnormally high concentrations in biological tissues and is responsible for the clinical symptoms of kernicterus (*13, 14*). However, it has also been suggested that the bile pigments bilirubin and biliverdin may protect vitamin A and linoleic acid from oxidative destruction in the intestinal tract (*15*). Indeed, bilirubin contains an extended system of conjugated double bonds and a reactive hydrogen atom and thus could possess antioxidant properties. Therefore, we examined the antioxidant activity of bilirubin in an in vitro system where lipid was oxidized by a free radical chain mechanism.

The free radical chain oxidation of linoleic acid (LH) gives linoleic acid hydroperoxide (LOOH) quantitatively at the initial stage (*16*). This enabled us to follow the oxidation of linoleic acid simply by measuring the formation of LOOH. To get a constant rate of initiation (reaction 1) and, subsequently, of oxidation (reactions 2 and 3), we used the radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) (*16*).

$$\text{AMVN} \xrightarrow{\Delta, O_2} \xrightarrow{\text{LH}} \text{L}\cdot \quad (1)$$

$$\text{L}\cdot + O_2 \rightarrow \text{LOO}\cdot \quad (2)$$

$$\text{LOO}\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}\cdot \quad (3)$$

The effects of bilirubin and its metabolic precursor biliverdin on the rate of peroxyl

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