effect on the absorbing membranes is shown in Table II and Fig. 2. Levy and Miller have shown that under conditions analogous to those used in the present study (*viz.*, drug concentration in the external medium is very much higher than in the fish tissue and the volume of the external medium is sufficiently large so that drug concentration in this medium is essentially constant throughout the experiment) the absorption of 4-aminoantipyrine follows apparent zero-order kinetics where the first-order absorption rate constant (k_a) may be calculated from the relationship k_a = absorption rate/drug concentration in the external medium. Assuming a passive absorption process, it follows that the absorption rate or uptake of drug per fixed interval of time is directly proportional to drug concentration in the fluids bathing the fish. Evidence of this relationship is shown in the lower portion of Fig. 2. The mean absorption rate constant calculated from these data is 4.9×10^{-3} min.$^{-1}$ which is in excellent agreement with previously obtained values values (8).

The effect of 1 m*M* sodium taurodeoxycholate on the absorption of 4-aminoantipyrine by goldfish immersed in solutions at various drug concentrations is shown in Table III and the upper plot of Fig. 2. Again a linear relationship is noted between drug uptake and 4-aminoantipyrine concentration in the external medium which is suggestive of a passive drug transport process even in the presence of the surfactant. These data provide further evidence that the bile salt acts *via* a direct effect on the permeability characteristics of the external membranes rather than by interacting with drug since the degree of enhancement of 4-aminoantipyrine absorption is independent of drug concentration in the external medium.

The maximum effect of sodium taurodeoxycholate on 4-aminoantipyrine uptake was observed in the bile salt dose-response study (Table II, Fig. 1). Concentrations of 1 and 3 m*M* bile salt resulted in a 1.5 to 1.6-fold increase in drug uptake. A similar degree of enhancement of 4-aminoantipyrine uptake in goldfish was observed in the presence of 0.01% polysorbate 80 (10). The effect of 1 m*M* sodium taurodeoxycholate on the second lot of fish, as noted in Table III, resulted in only a 1.2-fold increase in drug uptake. These findings illustrate the considerable lot-to-lot variations frequently observed in studies utilizing goldfish (8). The differences between lots with respect to the degree of bile salt effect probably reflect differences in membrane susceptibility, as discussed above. It is likely that in the second lot of fish the dose-response curve shown in Fig. 1 is shifted to the right so that a 1 m*M* bile salt concentration does not produce a maximal effect.

The present results provide direct evidence of the influence of sodium taurodeoxycholate on the external membranes of the goldfish and further, support the use of pharmacologic effect endpoints to assess the absorption rate of drugs in goldfish (5, 10, 13).

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Comparative Chemical and Toxicological Evaluation of Residual Ethylene Oxide in Sterilized Plastics

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Abstract □ The use of various gases for the sterilization of nondisposable and disposable plastic medical devices has become firmly established in the past decade and a half. However, the subject of sterilant residues remaining in plastic devices following an ethylene oxide gas sterilization has only recently been investigated. This study correlates ethylene oxide residues as determined by gas chromatography to the ability of these residues in polyolefins to elicit toxicological responses in several biological systems. A comparison of the results from a quantitative hemolysis test, rabbit intramuscular implantations with subsequent pathological investigation, cell culture responses, a series of fish tests, and intradermal irritation studies, to the ethylene oxide desorption data, revealed that within 24 hr. after sterilization, no significant toxicities were produced by ethylene oxide-sterilized polyolefins. The concentrations of the gas had decreased from a high of 3.20 mg./g. of plastic to as little as 0.20 mg./g. of plastic after aeration at room temperature for 24 hr.

Keyphrases □ Plastics, sterilized—ethylene oxide (EtO) residual □ Ethylene oxide residual, plastics—toxicity □ Guppy—toxicity determination, EtO residual □ Cell cultures—toxicity determination, EtO residual □ Intradermal irritation—toxicity determination, EtO residual □ Erythrocyte hemolysis—toxicity determination, EtO residual □ GLC—analysis

The toxicology of ethylene oxide (EtO) in both the liquid and gaseous phases has been well demonstrated in man and other animals (1–4). In addition, it has been shown that this epoxide is an effective sterilant for materials which cannot be sterilized by conventional high temperature methods (5). Recently, several reported studies have indicated that residual ethylene oxide in plastic medical devices may be implicated in toxicities

Table I—Fish Toxicity Data for Ethylene Oxide-Sterilized Plastics

Polymer	Time Aerated at 25°, hr.	Initiation Time, min.[a]	Death Time, min.[a]	Minimum Concn. of EtO in Polymer, mg./g.[b]
Petrothene 270	0	1	68	3.20
Petrothene 270	24	none	none	0.18
Tenite 860E	0	1	56	2.20
Tenite 860E	24	none	none	0.40
Marlex 6009	0	1	95	0.50
Marlex 6009	24	none	none	0.15
All controls	—	none	none	0.00

[a] Average value of three experiments in which the death times varied by less than 5 min. [b] Values based on polymers degassed in a circulating air environment and not upon actual EtO concentration found in polymers submerged in aquarium water.

associated with the use of the freshly sterilized plastic (6–11).

The present study was conducted in order to determine if polyethylene and polypropylene sterilized with ethylene oxide would possess any of the toxic properties of the sterilant.

EXPERIMENTAL

Materials and Method of Sterilization—In a recent study, O'Leary and Giles (12) characterized a series of polyethylene and polypropylene polymers for the purpose of correlating the effects of polymeric structure on the desorption kinetics of ethylene oxide from these materials following gas sterilization. The technique employed a gas chromatograph[1] with a 0.63-cm. (1/4 in.) × 182.8-cm. (6 ft.) glass column packed with 25% plasticizer[2] on 80–100 mesh diaformite aggregate[3] and a helium carrier gas. The ethylene oxide was extracted into chloroform and injected as the chloroform solution into the gas chromatograph. Minimum ethylene oxide concentrations in the polymers reported in this paper at time zero (on removal from the sterilizer) and after 24-hr. aeration are recorded in Table I. The same polymers were utilized in this study for the purpose of relating any observed toxicological properties after sterilization to the minimum concentration of residual epoxide. It was felt that since ethylene oxide is completely water soluble and any gas desorbing from the polymer into an aqueous system would go into solution in this medium, it was not necessary to determine the ethylene oxide content of the comparatively voluminous aqueous system. Reaction products of ethylene oxide are recognized as being possible in this aqueous system, but eventually any toxicity noted would be related back to ethylene oxide content in the polymers.[4]

The test samples were sterilized in a 25.4 × 40.6-cm. (10 × 16-in.) experimental gas sterilizer.[5] The specific sterilization involved prehumidifying the test samples in the chamber at 50% relative humidity, 57°, under 660 mm. of Hg vacuum for 1 hr. A mixture of 88% dichlorofluoromethane (CCl_2F_2) and 12% ethylene oxide was added to the chamber until a concentration of 878 mg. of ethylene oxide/l. was obtained. The plastics used were then sterilized for 5 hr. At the end of this time interval, a vacuum of 660 mm. of Hg was placed on the system in order to remove the gas mixture. The samples were then removed for toxicological evaluation. It is to be noted that this cycle differs from that reported by O'Leary and Giles in that only one evacuation of the chamber was utilized in this study as compared to three in the former investigation. Thus, the ethylene oxide residues actually remaining in the polymers tested for toxicological activity might necessarily be higher than reported by the former authors. The dimensions of the test samples were comparable in both residue studies.

Albino Guppy Toxicity Test—In order to investigate the biological activity of ethylene oxide or any of its water extractable reaction products that might be present in freshly sterilized polyolefins, a hybrid strain of albino guppies (*Poecilia reticulata*) were used. The strain which was chosen for the experiment had been controlled in this laboratory for a 3-yr. period immediately prior to this testing. The advantage of using these fish, as compared to those that might be obtained commercially, is that the age, sex, genetic variability, size (total body mass), and general health of the test organism can be more carefully controlled. The albino hybrid was specifically chosen because experience has shown that the albino guppy is more susceptible to low concentrations of toxicants than the wild gray genotype (13).

One-day-old female fry approximately 7 mm. in length and 4–6 mg. in weight were used throughout the entire study. All the test fish were from the same litter. The sex of the fish was determined by placing the fry in a white-bottomed glass pan that was illuminated with a 100-w. bulb. It was observed that the young male fish have a thicker ray along the lower edge of the anal fin and the female fry possess a dark crescent-shaped marking in the region of the urogenital pore.

The use of the young fish had the advantage of allowing the dimensions of the test chamber to be small. Only 25 ml. of test solution in a 50-ml. test tube was used. The test solutions were prepared by immersing 25 g. of the test plastic in 25 ml. of aquarium water in which the test animals were born. This water had a pH of 7.7, a water hardness of 780 mg./gal. (205.5 p.p.m.), and was maintained at 25 ± 0.5°. The controls consisted of unsterilized polymer immersed in the same water under the same conditions as the test samples. Samples of freshly sterilized plastic and plastic which had been aerated for 24 hr. at room temperature were immersed for 24 hr. in the aqueous system and held at 25°. At the end of the 24-hr. extraction period, the plastic was removed from the water and the test animals were placed into the test tubes. Since it is difficult to distinguish individual fish over a long period of time, only one fish was placed in each of the test tubes. In order to avoid faulty interpretation of the results due to individual differences and temperature differences, each experiment was repeated in triplicate and all tubes were maintained at 25°. The behavior of the test and control fish were recorded from the time of their immersion in the extract until death. The result of the test was considered negative when no symptoms were observed within 24 hr. The control fish can continue to live in this microenvironment for as long as 4 days without food and water purification.

The behavior of the test animal was judged according to the work of Klingler (14), considering the following points: (*a*) Initiation Time—this indicates the time interval between the first contact with the potential poison and the appearance of the first disturbances (reeling, lying sideways, turning over, jumping, swimming upside down, alteration of body color, and body swelling).

(*b*) Time of Death—the time interval from the beginning of the experiment to the death of the test animal. Death was considered as the cessation of body motions accompanying the fish floating at the surface on its dorsal side. No motion of the pectoral fins and gills must be noted.

Table I presents the initiation and death time recorded for the freshly sterilized and 24-hr.-aerated plastics. The minimum EtO concentration which could have desorbed into the test solutions has also been presented. These latter values were obtained from the EtO desorption plots presented for these polymers by O'Leary and Giles (12). No consideration has been given to the possible reaction products of ethylene oxide and the desorbing aqueous solution, since ethylene glycol or 2-chloroethanol, if formed in sufficient quantities, had to come from desorbing ethylene oxide.

Cell-Culture Evaluations—The unsterilized, sterilized, and 24-hr.-aerated sterilized plastics were evaluated on cell cultures using the NCTC-929, strain "L" mouse fibroblast cell line (L-929) and 10-day chick embryo cells. Confluent monolayers of each cell line were

[1] F & M model 400 biomedical.
[2] Flexol 8N8, Union Carbide Corp., New York, N. Y.
[3] Chromosorb S, Johns-Manville, New York, N. Y.
[4] The polymers were designated as: Petrothene 270—polyethylene (U.S. Industrial Chemicals Co., Tuscola, Ill.); Tenite 860E—polyethylene (Eastman Chemical Co., Kingsport, Tenn.); Marlex 6009—polyethylene (Phillips Chemical Co., Bartlesville, Okla.); Tenite 425DA—polypropylene (Eastman).
[5] Cryotherm.

Table II—Cell Culture Toxicity Data for EtO-Sterilized Plastics

Polymer	Aeration, hr.	Chick Cells Observations, hr. 24	48	"L" Cells Observations, hr. 24	48
Petrothene 270	0	+[a]	+	+	+
Petrothene 270	24	−[a]	−	−	−
Tenite 860E	0	+	+	+	+
Tenite 860E	24	−	−	−	−
Marlex 6009	0	−	−	+	+
Marlex 6009	24	−	−	−	−
Tenite 425	0	−	−	+	+
Tenite 425	24	−	−	−	−

[a] + = positive toxic response; − = negative toxic response.

overlaid with nutrient agar and stained with a vital dye according to the method of Guess *et al.* (15). The plastics were evaluated on the surface of the agar. The cultures were incubated 24 hr. at 37° in a 95% air–5% CO_2 atmosphere prior to making the first visual evaluations for any toxic responses. A second evaluation was made at 48 hr. after the samples were placed on the agar. Toxicity was indicated as an all-or-none response, where the killed cells released the vital stain to create clear zones around the test material. Questionable areas were examined microscopically for evidence of cell death. Known positive and negative controls were used on each plate to insure viability of test cells. All samples were tested in triplicate. The results have been summarized in Table II.

Intradermal Irritation—Twenty-four-hour extracts of the unsterilized, sterilized, and 24-hr.-aerated sterilized plastics were made with 0.85% saline at a ratio of 1 g. of plastic/ml. of saline at 25°. The extracts were injected into two sites each into the cleanly shaved backs of two albino New Zealand rabbits at a dose level of 0.2 ml. per site. Visualization of the area and intensity of irritation was accomplished within 30 min. by the aid of a 1% trypan blue suspension administered *via* the marginal ear vein at a dose level of 1 ml./kg. body weight. The positive control was a 20% ethanol solution and the saline solution used to extract the unsterilized polymers was the negative control. The degree of erythema, edema, or necrosis was evaluated after 24 hr. in animals receiving only the intradermal injections but no i.v. trypan blue. Since none of the aforementioned test samples produced any signs of irritation, the results were not tabulated.

Quantitative Determination of Percent Hemolysis—The method used to determine the degree of hemolysis of freshly drawn whole human blood in ACD anticoagulant (type O positive) was essentially that of Hunter (16). It is based on the fact that the oxyhemoglobin liberated by a hypotonic solution is a direct function of the number of cells hemolyzed. Furthermore, as complete hemolysis results from the laking of the erythrocytes in a 0.1% sodium carbonate solution, it is possible to express the hemolysis in terms of total hemolysis. A quantitative determination of partial hemolysis can be made by centrifuging off the unhemolyzed cells and determining the oxyhemoglobin in the supernatant fluid by means of a spectrophotometer.

The present method consisted of placing 25 g. of the unsterilized, sterilized, and 24-hr.-aerated sterilized plastic into 50-ml. test tubes. By means of a volumetric pipet, 25 ml. of a 0.4-ml. blood–25 ml. 0.85% saline suspension was added to each of the test tubes. The tubes were stoppered and then placed in a water bath at 35 ± 1° for 10 hr. At the end of this period, the suspensions were decanted off the plastics and centrifuged for 3 min. at approximately 1,000 r.p.m. Readings of the light transmission in the supernatant liquid were made in a spectrophotometer[6] at 545 mμ. Any hemolysis noted was related to ethylene oxide desorption since any reaction product, such as 2-chloroethanol from the chloride of the saline solution, had to come from the original ethylene oxide.

The complete hemolysis of the blood was obtained by placing 0.4 ml. of the blood in 25 ml. of 0.1% sodium carbonate and mixing immediately. The reading obtained for the sodium carbonate–blood mixture in the spectrophotometer represented 100% hemolysis. The reading obtained for the unsterilized plastics was taken as 0% hemolysis. Five separate readings were made for each blood sample and averaged. Each test plastic was evaluated in triplicate.

The percent hemolysis occurring in each tube was calculated by subtracting the control reading from the test readings and then dividing this value by the reading for the complete laking with sodium carbonate. These values were multiplied by 100 to get the final result. Table III summarizes the mean values found for the percent hemolysis obtained on the aerated and nonaerated sterilized plastics. The percent of the original EtO concentration which could be anticipated to desorb from the polymers after 24 hr. of aeration at room temperature has also been calculated.

Interfascial Implantation—Strips of the plastic to be evaluated were cut into sections measuring 6.35 × 2.54 × 0.165 cm. (2.5 × 1 × 0.065 in.) and sterilized as previously described. Two groups of rabbits were anesthetized and an incision made through the skin to gain access to the middle region of the latissimus dorsi muscle and the distal portion of the trapezius muscle. An incision was made through these muscles to expose the fascia covering the paravertebral muscle. The plastics to be implanted were those removed from the ethylene sterilizer and implanted immediately, or those which had been aerated at 25° for 24 hr. The incision was closed and the animal caged for a period of 1 or 2 weeks. At the end of the time period, the animals were sacrificed and the middle area of the implanted plastic examined for macroscopic evidence of tissue damage. Sections of skin, latissimus dorsi, and paravertebral muscle were preserved in 10% formalin (buffered) for histological examination after sectioning and staining with H and E stain. Control plastics were identical in physical size and time of implant, but were not ethylene oxide sterilized. This method did not prove fruitful in detecting any toxic responses from ethylene oxide-sterilized polymers.

RESULTS AND DISCUSSION

The toxicology of these ethylene oxide-sterilized polyolefins correlates well with the observed rates of desorption of the sterilant from the polymer. As could be expected, the initial concentrations of gas entrapped in the polyethylene samples decreased from 3.20 mg./g. to 0.50 mg./g. as the polymer density increased from 0.91 to 0.96 g./ml. (12). These concentrations represent only minimum values for the EtO residues present in those polymers tested for toxicological properties. This is a reflection of the fact that those polymers used in the residue analysis received three gas evacuations as compared to the one gas evacuation placed on the chamber containing the biological test samples. It is important to note that many of the present hospital sterilization cycles use this latter pro-

Table III—Percent Hemolysis from Ethylene Oxide-Sterilized Plastics

Polymer	Crystallinity, %	Aeration, hr.	Hemolysis, %	% of Original EtO Concn. Desorbed After 24 hr.
Petrothene 270	44	0	76.2	—
		24	0.0	94.4
Tenite 860E	52	0	65.7	—
		24	0.0	81.8
Marlex 6009	90	0	74.3	—
		24	0.0	60.0
Tenite 425	62	0	80.0	—
		24	0.0	50.0

[6] Bausch & Lomb Spectronic 20.

cedure, and that all of the nonaerated sterilized samples produced positive evidence of toxicity that could be related to the sterilization process. As Table I indicates, the albino guppy fish test is extremely sensitive to the irritating effects of the gas residue desorbing from the plastics. This was further confirmed in Table II by the high percentages of hemolysis produced by the same test samples. Because the cell culture technique allows the desorbing gas to escape rapidly from the surface of the plastic, only the more amorphous polymers having the highest gas concentrations were able to transmit the toxicant through the agar overlay to the cell surface. The "L" cell strain is known to be more sensitive than the chick cell line and the toxicity results in Table II reflect this fact.

The lack of a toxic response in the fish test by one polyethylene[7] is in excellent agreement with the known desorption kinetics of ethylene oxide from this polymer. After 24 hr. of aeration at 23° the rate of desorption has almost reached equilibrium, *i.e.*, the slope of the concentration *versus* time plot approaches zero. Thus there is little or no contribution of ethylene oxide being made by this polymer to the fish. However, another polyethylene[8] is still rapidly desorbing its ethylene oxide to the aquarium water after 24 hr. of aeration time. The resulting toxicity data thus appear to have a very narrow cut-off concentration when the two polymers are compared, but in reality, this is not true. What is actually being reported is a toxicity difference directly attributable to the different kinetics of EtO desorption from the two polymers.

The results of the interfascial implantation were not fruitful. The trauma of insertion of such a large section of rigid plastic was such that it overwhelmed any discernible toxicity that may have been elicited by the ethylene oxide leaching from the plastic sample. In addition, at the particular locus of implantation chosen, there is a good and rapid exchange of liquids so that a concentration of ethylene oxide high enough to elicit a toxic response was not achieved. This method of evaluating the tissue damage from residual ethylene oxide is not recommended for future investigations.

One of the more significant results from this study is that, depending upon percent crystallinity and the conformation of the polymer chain, the percentage of the original concentration of EtO desorbed in the first 24 hr. after sterilization varies from 50 to 94%, which brings these particular polymers below the toxicity level by the test systems used in this report. Although ethylene oxide analyses were not conducted on the test solutions, as in the fish test or the blood hemolysis test, the desorption data and complete solubility of ethylene oxide in water render this unnecessary for the purposes of this report. This high rate of ethylene oxide desorption is consistent with the values for the diffusion coefficient and permeability constant of ethylene oxide for polyethylene reported by Yasuda (17). Although the results of these preliminary investigations suggest that a 24-hr. minimum quarantine period may be sufficiently safe for these particular polymers, it is notable that recent studies have shown that plasticized polymers and some certain elastomer formulations will require longer aeration times (7, 8). In certain instances, the production of nonvolatile reaction products as the result of the sterilization procedure will produce materials which will require safety evaluations beyond volatile residue analysis.

[7] Tenite 860E.
[8] Marlex 6009.

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