ruled out a role for the acetylcholine receptor in inducing or aiding sprouting, as single daily or twice daily injections of α-bgt are not sufficient to bind with all the extrajunctional receptors that appear within 12 or 24 hours. However, consideration of other research is relevant. Crushed motor axons will regenerate perfectly to end plates completely blocked by continuously infused α-bgt (*13*). In the light of the recent research of McMahan *et al.* (*14*), this finding should not be too surprising, as it appears that the factor recognized by nerves lies within the basement membrane separating the nerve terminal from the lipid plasma membrane. Presumably any local stimulus to nerve growth, if such exists, may lie here too, although its positioning in the basement membrane might initially depend on underlying acetylcholine receptors. The appearance of the newly formed presynaptic nerve endings in Jansen and Van Essen's (*13*) fully paralyzed preparations, however, shows that the axons not only fill out the old synaptic site but also sprout beyond it over the muscle surface into regions where presumably no underlying acetylcholine receptors remained unblocked.

Mammalian skeletal muscle may be unique in being so trophically dependent on activity for its normal biochemical constitution. Other muscles and tissues may depend more on ill-understood agents delivered by nerves. Thus it may not be possible in other tissues to cause sprouting by blocking impulse activity (*15*). The common factor in evoking sprouting in a wide variety of denervated tissues, whether the denervation primarily affects the target organ by withdrawing activity or by withdrawing nerve-borne chemical agents, seems likely to be the resulting biochemical change in the tissues that causes them to manufacture a sprouting agent or, alternatively, stops them from manufacturing a growth-inhibiting agent.

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8. (i) Under sodium dodecyl sulfate gel filtration it runs to the same point as bungarotoxin prepared elsewhere, having a molecular weight ~ 8000; (ii) it precipitates with ^{125}I-labeled acetylcholine receptors isolated from *Electrophorus electricus*; (iii) it competes with bungarotoxin prepared elsewhere for rabbit antibody to α-toxin; (iv) it blocks the cholinergic synapse between the cercal nerve and the giant interneuron in the cockroach; and (v) it blocks the mouse soleus neuromuscular junction.
9. The measurement of the length of the sprouts from an end plate was the length of a straight line in the long axis of the muscle fiber whose ends encompassed the sprouting, minus the length in that axis of the end plate. This measure takes no account of the branching or curvature of the sprouts.
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Angiotoxicity of Oxygenated Sterols and Possible Precursors

Abstract. *Cell death, inflammation, and repair in rabbits' aortas and pulmonary arteries were observed at 3-, 7-, and 10-day periods after the intravenous injection of oxygenated sterols. Thus, oxygenated sterols, not cholesterol, may play the primary role in arterial wall injury and lesion development.*

Atherosclerosis induced by diets containing cholesterol has been studied since the report by Anitschkow in 1913 (*1*). However, investigators conducting experiments on the effects of diet on the circulatory system have often not taken into consideration the ability of cholesterol to undergo spontaneous oxidation (*2*). In some experiments workers have increased the effectiveness of the diet by heating it in air, thereby causing oxidation of the cholesterol (*3*).

We reported previously that pure cholesterol was relatively ineffective in inducing cell death and lesions compared to the cholesterol prepared according to *U.S. Pharmacopeia* standards (*4*). We have now tested the effects of sterols known to be produced by autoxidation (*2*). We have also assayed two presumptive biogenetic precursors of 24- and 25-hydroxycholesterol. Kandutsch *et al.* (*5*) implicated oxygenated sterols as key regulators of sterol metabolism, and enzyme and isolation studies suggest that oxygenated sterols may occur in vivo (*6–10*).

In initial experiments the test material was administered orally (*4*), but this route led to uncertainties associated with biotransformation, retention, excretion, and absorption. We therefore used the intravenous route in subsequent experiments. A concentrate of impurities obtained from the recrystallization of impure cholesterol (*4*) was separated by thin-layer chromatography into three bands. The compounds in each band were eluted with ethanol and emulsified with physiological saline, and then injected into New Zealand White (NZW) rabbits. The aortas of the rabbits were examined 24 hours after the injection.

Only the components of the middle band, identified as sterol hydroperoxides, showed no angiotoxicity. Synthesis and bioassay of the components confirmed this result. We had in-

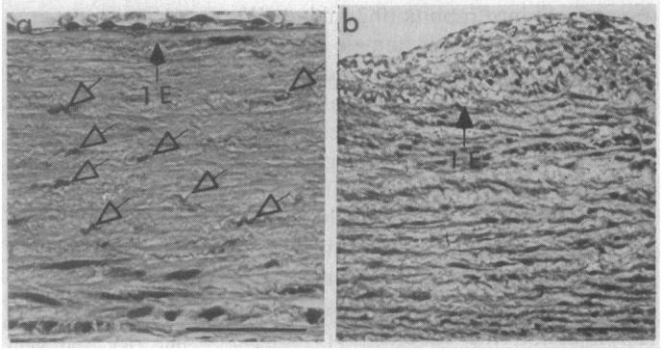

Fig. 1. (a) Transmural necrosis of the thoracic aorta of a JW-NIBS rabbit. A few viable smooth muscle cells are in the outer media. Hollow arrows indicate dead cells. Solid arrows point to the internal elastica (*IE*) demarcating the thin intima (×400). (b) Intimal plaque of thoracic aorta of a JW-NIBS rabbit injected intravenously with concentrate (40 mg/kg on each of 3 days) and killed 10 weeks after the third injection. The inner and mid media under the plaque are hypocellular (×200).

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Table 1. Response of the major and minor branches of the pulmonary artery of NZW rabbits to injections of squalene or squalene epoxides. The branches were examined 3 days or 10 days after the third injection. To rate the major branches we examined transverse slices of fixed lungs (see Figs. 1 and 2) and compared the diameter of the arterial lumen to the diameter of the vessel and the wall thickness. The pulmonary vein in the same section was used as a guide since, normally, the vein and artery are approximately the same in size. Symbols: +++, severe wall thickening and marked lumen narrowing (see Fig. 2b); ++, a moderate response; +, a minimal, but clearly discernible response; 0, no response. For the minor branches we examined four to six sections of each lung. When a majority of the vessels had responded as described in the text, a branch was rated as ++; when a minority of vessels were positive, a branch was rated as +.

Substance and dosage (per kilogram of body weight)	Number of rabbits		Major branch		Minor branch	
	3 days	10 days	3 days	10 days	3 days	10 days
Olive oil (0.2 ml)*	2	2	0	0	0	0
Squalene (40 mg)	3	3	0	0	0	0
Squalene epoxide (40 mg)	3	3	0	0	0	0
Squalene bisepoxide (40 mg)	6	6	0	+	+	++
Lanosterol-24,25-epoxide (5 mg)	7	7	0	+	+	++

*Control.

sufficient material to complete the assays of angiotoxicity of the other bands, although we found that the band containing the least polar constituents contained cholesterol. However, the high probability of the presence of oxygenated sterols (*2*) in the whole concentrate, the demonstrated potency of oxygenated sterols in inhibiting cholesterol biosynthesis (*5*), and the cytotoxicity of these compounds in vitro (*5, 11*) warranted their assay for angiotoxicity. We therefore prepared a concentrate as described earlier (*4*), and used this, and a variety of synthetic compounds known to be present in the concentrate, in bioassays with rabbit aortas.

Cholestan-3β,5α,6β-triol, 25-hydroxycholesterol, cholesterol-5α,6α-epoxide, and all the other compounds we tested were synthesized, and they conformed to recognized standards of purity (*12*). The crystalline agents were suspended in physiological saline (*13*). The particle size of purified cholesterol was estimated to be less than 2 μm by transmission electron microscopy. Other agents yielded particles of less than 1 μm. Oily agents (squalene and its epoxides) were diluted with refined olive oil (200 mg/ml).

The test substances were injected intravenously into rabbits (0.2 ml/kg). We used 78 young adult female or male NZW rabbits (1.8 to 2.5 kg) and 27 Japanese White-Nippon Institute of Biological Sciences (JW-NIBS) (*14*) female rabbits (1.5 to 2.2 kg) that were given free access to commercial food pellets. The test suspensions were injected once daily for 3 days into an ear vein. Controls were given three injections of the vehicle. At 24 hours, 2 weeks, or 10 weeks after the last injection the rabbits were deeply anesthetized with pentobarbital sodium, and the heart, major blood vessels, and viscera were fixed by perfusion at 25 mm-Hg and processed for further study.

The two strains of rabbits that we used showed different patterns and degrees of response to the injections of oxygenated sterols in suspension. In the NZW rabbits, injections of the concentrate increased smooth muscle cell death in the aorta, and the effect was quantifiable by electron microscopy at 24 hours after the third injection (*4*). In JW-NIBS rabbits, however, the aorta became extensively necrotic at 24 hours, as demonstrated by light microscopy (Fig. 1). Six JW-NIBS rabbits were killed at 2 weeks and nine at 10 weeks after the third injection of concentrate. At 2 weeks their aortas contained areas of hypocellularity and cellular debris. Repair, in the form of partial restoration of the media and intimal fibromuscular thickening, was demonstrable at 10 weeks (Fig. 1b). An average of three mound-like fibromuscular plaques was found in 24 whole cross sections, which were obtained at regular intervals from the entire descending thoracic aorta of each rabbit. These plaques (Fig. 1b) were similar to those induced earlier by the concentrate given by stomach tube (*4*). Unlike the usual cholesterol-induced lesion in the rabbit, these plaques had no foam cells or stainable lipids.

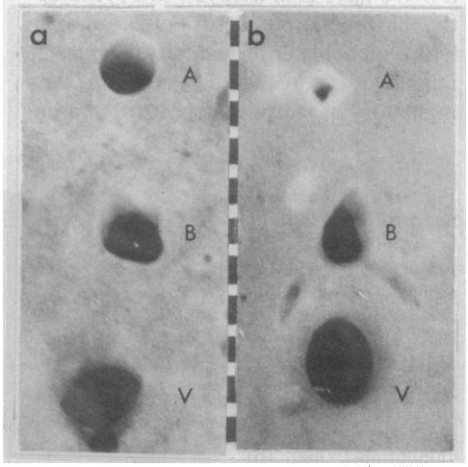

Fig. 2. (a) Transverse slice of control (NZW) lung, showing pulmonary artery (*A*), bronchus (*B*), and vein (*V*). (b) A comparable slice showing grossly visible thickening of the artery (*A*). The vein (*V*) is normal.

We also studied the response of the pulmonary artery. Major branches of the pulmonary artery of both strains of rabbits responded to intravenously injected sterols by undergoing grossly visible, segmental thickening (Fig. 2) at 24 hours after the third injection. Severely inflamed segments were not fully expanded under the perfusion pressure of 25 mm-Hg. The thickening was due to a series of changes ranging from the initial inflammation to subsequent repair by fibromuscular thickening. Similar microscopic changes occurred in minor branches of the pulmonary artery. These pulmonary arterial responses provided a rapid bioassay procedure for arterial wall injury and repair.

Because of a limited supply of JW-NIBS rabbits, 78 NZW rabbits were used in our subsequent bioassays. The maximum dose of any of the test materials was limited to 10 mg per kilogram per day, because even purified cholesterol at this dose caused immediate death (*15*) at the last of the three injections in one of five rabbits.

Neither freshly purified cholesterol nor the vehicles induced changes in the major or minor branches of the pulmonary arteries. The responsivity of the major and minor branches to the various sterols differed markedly and provided a titration endpoint by which we could determine the comparative potencies of the compounds. The endpoint was reached when a dose producing a grossly visible response in the major branches as well as maximum microscopic changes in the minor branches was reduced to one-half, resulting in no response in the major branches but still maximum changes in the minor branches. The concentrate, cholestantriol, and 25-hydroxycholesterol were equipotent; that is, the major branches responded at doses of 5 mg/kg but not 2.5 mg/kg. The 5,6-epoxide and 7-ketocholesterol did not affect the major branches at doses of 10 mg/kg, although at 5 mg/kg the minor branches reacted.

Oxygenated sterols, including 25-hydroxycholesterol, can modulate cholesterol biosynthesis (*5*). Smith *et al.* (*6*) and Brooks *et al.* (*7*) found 24-hydroxycholesterol in the brain and 25- and 26-hydroxycholesterol in the arteries of ba-

boon and man; Corey *et al.* (*8*) showed that the bisepoxide of squalene can be enzymatically converted into the 24,25-epoxide of lanosterol; and Shishibori *et al.* (*9*) and Nelson *et al.* (*10*) demonstrated that inhibition of cyclization of squalene epoxide induced accumulation of the bisepoxide. These reports suggest that squalene bisepoxide may be a normal by-product of cholesterol biosynthesis. Lanosterol epoxide is probably converted to either 24- or 25-hydroxycholesterol.

Both squalene bisepoxide (40 mg/kg) and lanosterol-24,25-epoxide (5 mg/kg) (*16*) affected the minor branches of the pulmonary artery but not the major branches. In view of a possible delayed effect due to biogenesis, we examined the pulmonary arteries of animals killed 10 days after the third injection of either squalene bisepoxide or lanosterol epoxide. Both compounds caused a delayed response that was grossly visible at 10 days in the major branches; microscopic changes in the minor branches had progressed as shown in Table 1.

Kandutsch *et al.* (*5*) suggest that oxygenated sterols, not cholesterol, modulate the rate of biosynthesis of cholesterol. The data presented here show that oxygenated sterols can be potent angiotoxins and indicate that 25-hydroxycholesterol may occur normally in vivo (*17*).

Our data support the hypothesis that oxygenated sterols have an important role in causing injury of the arterial wall and initiating atherosclerotic lesions. They also indicate that feeding experiments with cholesterol should be evaluated with caution when the purity of the dietary cholesterol has not been controlled. Further studies are warranted to evaluate additional factors, such as high plasma lipids, on the progression of basic lesions induced by oxygenated sterols.

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15. Pulmonary arteries develop lesions in conventional cholesterol feeding experiments [C. H. Bailey, *J. Exp. Med.* **23**, 69 (1916); M. Chuma, *Virchow's Arch.* **242**, 275 (1923); R. Schonheimer, *ibid.* **249**, 1 (1924); G. V. Child, *Arch. Pathol.* **98**, 47 (1974)]. Pulmonary arteriosclerosis can be selectively induced by repeated intravenous injections of blood clots; this method is more efficient with heterogeneous than with homologous or autologous blood [C. V. Harrison, *J. Pathol. Bacteriol.* **60**, 289 (1948); B. E. Heard, *ibid.* **64**, 13 (1952); W. A. Thomas, R. M. O'Neal, K. T. Lee, *Arch. Pathol.* **61**, 380 (1956)]. Lesions thus induced are not grossly visible and are confined to small-caliber pulmonary arteries and arterioles. Pulmonary arterioles can be extensively blocked by injecting 75-μm nonthrombotic plastic particles [W. A. Thomas, R. M. O'Neal, K. T. Lee, *ibid.* **62**, 56 (1956); R. Perez-Tamayo, H. Brandt, H. Medellin, J. Doria, *Am. Heart J.* **61**, 515 (1961)]. A single dose of such particles (200 mg/kg) was well tolerated by the rabbit. No pulmonary arterial lesions were induced despite demonstrable right ventricular hypertrophy after repeated injections. No cause was found for the sudden death after the injection of 10 mg/kg. Extensive thromboembolism with associated physiological disturbance is a possibility.
16. The squalene bisepoxide and lanosterol-24,25-epoxide were shown to be potent inhibitors of cholesterol biosynthesis by J. A. Watson (personal communication).
17. In the absence of direct data on the biosynthesis of 25-hydroxycholesterol, this pathway requires substantiation. The observed effects could be direct; that is, without involving the sterol biosynthesis system.
18. We thank A. T. James and P. Dunphy (Unilever Research, England) for assistance with the chromatography and hydroperoxide synthesis. This work was supported by NIH grants HL-14177 and HL-20993; a special grant to Visiting Researcher, Tokyo Metropolitan Institute of Gerontology; and grants N00014-76-C-0027 and N00014-77-C-0339 from the Office of Naval Research, U.S. Navy.

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γ-Glutamyl Transpeptidase in Isolated Brain Endothelial Cells: Induction by Glial Cells in vitro

Abstract. *The endothelia of microvessels isolated from mouse brain by mechanical means are rich in γ-glutamyl transpeptidase; however, the enzyme often disappers when the cells migrate or proliferate from the microvessel isolates. In an endothelial cell line derived from similar isolates and negative for γ-glutamyl transpeptidase, the enzyme could be induced in the endothelial cells when they were cocultured with glial cells. Thus there may be a requirement for continuous induction of γ-glutamyl transpeptidase in brain microvessels by adjacent glial cells.*

γ-Glutamyl transpeptidase (γ-GTP) (E.C. 2.3.2.2) has been demonstrated histochemically in brain capillary endothelium, choroid plexus epithelium, and in several extracranial locations, such as the epithelial brush border of the proximal convoluted tubules of the kidney and portions of intestinal epithelium (*1–4*). We have found a similar distribution of γ-GTP-rich areas in the mouse; however, cerebral vascular endothelium is the only endothelium in this animal that contains histochemically detectable concentrations of γ-GTP.

When mouse brain capillaries are isolated by a mechanical dispersion and filtration technique (*5, 6*), they retain high concentrations of γ-GTP in the endothelium (Fig. 1a), yet the enzyme is often lost from the endothelial cells as they migrate or proliferate from the microvessel isolates. An endothelial cell line ME-2 (Fig. 1b), derived from similar mouse isolates (*6*), is also negative for γ-GTP (Fig. 1c); however, the enzyme can be induced in these endothelial cells by coculturing them with C_6 rat glioma cells (Fig. 1, d to f). The enzyme induction depends on the density of C_6 cells. Similar γ-GTP induction cannot be accomplished with cell-free conditioned medium derived from C_6 cells.

The possible role of γ-GTP in the differentiated function of blood-brain barrier endothelium is of interest because the enzyme is implicated in amino acid transport: high concentrations of γ-GTP are associated with areas where high

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