goons are intermittent, and selection favors salamanders with the ability to metamorphose early. The larvae inhabiting these natural lagoons characteristically metamorphose at a small size around 6 months of age (*7*). The sewage lagoon, in contrast, has a constant inflow of sewage water, and although the water level fluctuates somewhat with seasonal rainfall, the need for rapid metamorphosis to survive has been eliminated (*7*). Thus the fact that no tumors were found on the thousands of larvae examined from nonsewage lagoons has little value, since the two types of populations are not readily comparable. For the same reasons, it has been impossible to compare animals transferred from one environment to another. When transfers are made from the sewage lagoon to an unpolluted environment, individuals metamorphose in 6 to 9 months and papillomas regress.

The possibility of either an exogenous or endogenous tumor virus being activated is being studied by electron microscopy. So far, several epidermal papillomas have been examined but no virus has been discovered.

Regardless of the factor or factors ultimately determined to be responsible for the etiology, tiger salamanders appear to be sensitive indicators for at least certain types of environmental carcinogens. This suggests they have value for tropical screening of potential carcinogens. Since they can live in sewage, by virtue of having both lungs and gills, perhaps sewage ponds could be stocked as a monitor for carcinogens. Finally, it also suggests that other larval forms of vertebrate and invertebrate animals could be developed for screening carcinogens by hormonally prolonging the time spent in the immature stages.

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Niemann-Pick Disease Experimental Model: Sphingomyelinase Reduction Induced by AY-9944

Abstract. *Organs of rats treated with the drug AY-9944 for 5 days showed a significant reduction in sphingomyelinase activity. Evidence is presented which suggests that the reduction is due to impaired enzyme synthesis.*

The heritable metabolic disorder known as Niemann-Pick disease is characterized by a deficiency of sphingomyelinase activity resulting in the accumulation of sphingomyelin in organs and tissues of afflicted individuals (*1*). Studies with AY-9944 (*trans*-1,4-bis[2-chlorobenzylaminomethyl]cyclohexane dihydrochloride), a compound that causes the accumulation of 7-dehydrocholesterol through its inhibitory effect on 7-dehydrocholesterol reductase (*2*), have revealed lamellated cytoplasmic inclusions and pathological degeneration in oligodendroglial cells (*3*). It recently has been found that cytoplasmic inclusion bodies appear in the retina and optic nerves of rats treated with AY-9944 and that they are similar to those in patients with Niemann-Pick disease (*4*). We now report a significant reduction in sphingomyelinase activity in various organs of rats that received AY-9944 over relatively short periods of time. This reduction in enzymatic activity was accompanied by an accumulation of sphingomyelin in the livers of the treated rats. We present evidence here that suggests that the reduction of sphingomyelinase is due to impaired synthesis of the enzyme. Animals treated with AY-9944 may be useful as experimental models of Niemann-Pick disease.

Albino rats of the Sprague-Dawley strain received daily intraperitoneal injections of 50 mg of AY-9944 per kilogram of body weight, beginning on day 2 after birth. Individual animals were killed from 3 to 25 days after the first injection. Chemical and enzymatic analyses were carried out on liver, kidney, spleen, brain, retina, and lens obtained from injected rats and age-matched controls.

After the administration of AY-9944, lamellar inclusion bodies appeared in the retina, lens, and various ocular cells. Prolonged administration of the compound caused degeneration of the retina and cataractous changes in the lens. Similar lamellar inclusion bodies were found in both glial and neuronal cells in the brain, in reticuloendothelial cells in the spleen, and in the Kupffer cells in the liver. The inclusion bodies consisted of concentrically arranged membranes measuring 75 Å. Many appeared as conglomerate large masses. The inclusion bodies are similar to those that occur in sphingolipidoses such as Niemann-Pick disease, GM_1 and GM_2 gangliosidosis, and metachromatic leukodystrophy.

There was a 30 percent reduction in

Table 1. Sphingomyelin in the livers of rats treated with AY-9944 from day 2 after birth. Phospholipids were extracted and analyzed according to Marinetti (*10*). Sphingomyelin was quantified as described (*11*) after separation into the faster (SP-L) and slower (SP-C) migrating molecular species. The values represent the mean and standard deviation of four determinations.

Item (wet weight)	Age of rats					
	7 days		12 days		19 days	
	Control	Treated	Control	Treated	Control	Treated
Total phospholipids (mg/g)	20.6 ± 1.0	23.9 ± 0.88	23.8 ± 0.6	22. ± 0.5	28.7 ± 1.1	43.9 ± 2.3
Total sphingomyelin (mg/g)	1.5 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	1.6 ± 0.2	3.0 ± 0.3
SP-L (mg/g)	1.14 ± 0.14	1.4 ± 0.09	1.22 ± 0.08	1.35 ± 0.05	1.3 ± 0.18	2.04 ± 0.13
SP-C (μg/g)	402. ± 7	568. ± 60	380. ± 62	669. ± 83	346. ± 71	1011 ± 147

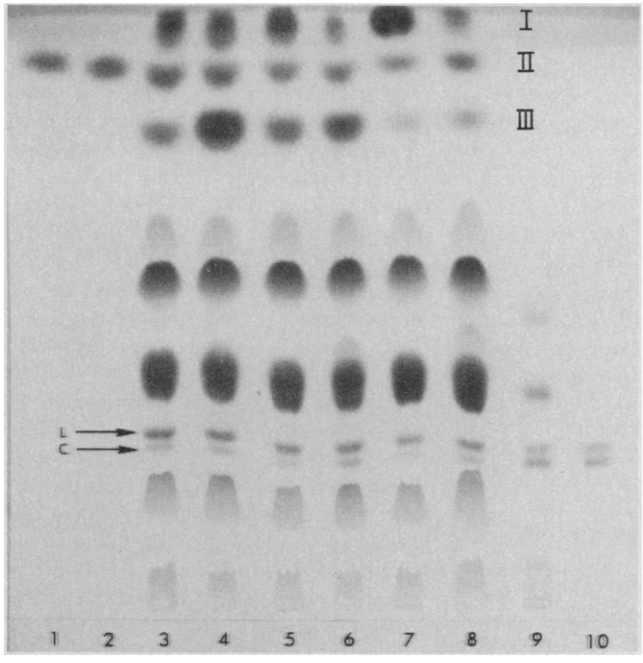

Fig. 1. Thin-layer chromatogram of lipids extracted from the livers of rats treated with AY-9944 and from age-matched controls. The solvent system consisted of chloroform methanol and water (72 : 28 : 4.5) (*5*). The plates were charred with ammonium bisulfate at 200°C for 20 minutes (*13*). Lane 1, 7-dehydrocholesterol standard; lane 2, cholesterol standard; lane 3, control; lane 4, treated liver from 7-day-old rats; lane 5, control; lane 6, treated liver from 12-day-old rats; lane 7, control; and lane 8, treated liver from 19-day-old rats; lane 9, mixture of phospholipids, from the origin to the top, phosphatidyl serine, sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine; lane 10, standard of sphingomyelin. Abbreviations: *C*, sphingomyelin-C; *L*, sphingomyelin-L.

the body weight of the animals injected with AY-9944 over a period of 5 days and a 60 percent reduction in 19-day-old experimental rats. In the livers of the animals injected with AY-9944 for 17 days, there was an 80 percent reduction in the wet weight and moderate increase of phospholipids. Sphingomyelin was separated by thin-layer chromatography (*5*) into slow- and fast-migrating components called SP-C and SP-L, respectively (*6*). Total liver sphingomyelin in the animals treated for 17 days increased gradually to 188 percent over that in age-matched controls. The increase of SP-C was more striking (292 percent of the age-matched controls) than that of SP-L (157 percent of controls) (Table 1). One-dimensional thin-layer chromatography (Fig. 1) revealed that lipid migrating at position I in lane 8 (probably cholesterol esters and triglycerides) decreased in rats on prolonged administration of AY-9944. Lipids in area II, representing cholesterol and 7-dehydrocholesterol, are slightly increased. The lipid at area III (possibly free fatty acids) increased initially (lane 4), and thereafter gradually decreased (lane 8). In addition to the increase in sphingomyelin there was a 20 percent elevation in phosphatidyl inositol in the livers of the treated rats. The distribution of other phospholipids such as phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine was unchanged in animals that received AY-9944.

There was an increase in β-galactosidase, α-galactosidase, α-glucosidase, β-glucuronidase, α-mannosidase, β-*N*-acetylhexosaminidase, acid phosphatase, and glucocerebrosidase activity in the various organs and tissues of the experimental animals. These findings are consistent with the report of increased activity of 13 lysosomal hydrolases in the brain of rats treated with AY-9944 (*3*). In contrast, sphingomyelinase activity was significantly reduced in the tissues of the animals that received the drug (Table 2). Phosphodiesterase activity was not changed by administration of AY-9944.

Experiments were undertaken to examine whether the decrease of sphingomyelinase activity was due to inhibition of the catalytic activity of the enzyme by AY-9944 or the presence of an inhibitor of sphingomyelinase in vivo. Addition of AY-9944 to purified placental sphingomyelinase in vitro did not cause a reduction in the activity of this enzyme. Furthermore, when homogenates of liver tissue from treated and control rats were prepared and assayed for sphingomyelinase separately and in admixtures from the two groups of animals, there was no convincing indication that an inhibitor of sphingomyelinase had accumulated in the tissues of the animals that received AY-9944.

Table 2. Sphingomyelinase and glucocerebrosidase activity in tissues of rats treated for 5 days with AY-9944 and in age-matched controls. Enzymatic activity was measured as described (*12*). The reported values are the means of six separate determinations. Extracts of retina and lens tissue were prepared from six to nine combined tissue samples. Abbreviations: C, controls; E, experimental.

Tissue	Enzymatic activity (nanomoles per milligram of protein per hour)			
	Sphingomyelinase		Glucocerebrosidase	
	C	E	C	E
Liver	100	11	49	53
Kidney	30	15	76	83
Spleen	20	7.7	70	90
Brain	42	23	116	139
Retina	69	23	146	204
Lens	1.1	0.3	2.9	3.5

It has been known for a number of years that there is an intimate relation between the metabolism of sphingomyelin and cholesterol (*7*). Patients with Niemann-Pick disease have a significant elevation of tissue cholesterol. The molecular basis of this interaction has not been established. It has been shown that the amount of ^{32}P incorporated into sphingomyelin-containing fatty acids that are less than 20 carbon atoms in length (SP-C) is 1.3 to 1.4 times greater than that in sphingomyelin with fatty acids longer than 20 carbon atoms (SP-L) (*6*). Furthermore, the increased sphingomyelin in the livers of patients with Niemann-Pick disease is predominantly 18 carbon atoms in length (*8*). It is clear from the present investigation that, in addition to interfering with cholesterol metabolism, AY-9944 causes a decrease in tissue sphingomyelinase activity and an accumulation of sphingomyelin, which was particularly apparent with regard to rapidly turning over sphingomyelin (SP-C). Since there was no significant inhibition of sphingomyelinase activity in vitro by AY-9944 and there was no indication of an accumulation of an inhibitor of sphingomyelinase activity in vivo, the most logical explanation of the present results

is that sphingomyelinase synthesis is impaired in animals receiving AY-9944. An experimental animal analog of Niemann-Pick disease produced in this fashion has distinct advantages over attempts to mimic lipid storage diseases by the use of compounds that inhibit the activity of sphingolipid hydrolases (*9*).

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The Heart: A Target Organ for Estradiol

Abstract. *Autoradiographic studies of rat heart reveal that tritiated estradiol concentrates in cell nuclei of the myocardium of the atria and auricles, similar to the myometrium of the uterus. This suggests that estrogen has a direct effect on atrial myocytes through which its "protective" action may be mediated. Cardiac glycosides that are known to exert estrogen-like effects on classical estrogen target tissues, such as uterine muscle, endometrium, vagina, and mammary gland, probably act on atrial muscle through a genomic, steroid hormone-like mechanism of action.*

Cardiovascular diseases are more frequent in men than in women. This has been ascribed to stress in males as well as protective effects of sex hormones in females. Clinical and experimental evidence suggest that estrogen mitigates or delays the occurrence of hypertension, coronary artery disease, paroxymal tachycardia, myocardial ischemia, and certain pathologic changes in the electrocardiogram (*1*). Whether in some of these conditions estrogen plays a role by a primary—that is, direct—action on heart muscle is not known. The sites of action of estrogen on the cardiovascular system have not been clearly identified, and the mechanisms of action are little understood. Autoradiographic studies with [^{3}H]estradiol, by means of special techniques developed in our laboratory (*2*), demonstrated target sites for estrogen not only in such target tissues as uterine muscle but also in a number of presumed nontarget tissues, as well as in the walls of blood vessels and in heart muscle (*3*). In the past, biochemists have used "heart muscle" as a control nontarget tissue in their investigations of estradiol receptors (*4*). In our study, serial section autoradiograms of rat heart were prepared 1 hour after the intravenous injection of [^{3}H]estradiol in order to identify the types of target cells for the hormone and to determine their topographic distribution.

Two 26-day-old intact and six 26-day-old female Holtzman rats, ovariectomized for 9 days, were each injected intravenously with 0.5 μg per 100 g of body weight of 17β-[2,4,6,7-^{3}H]estradiol (specific activity 91 c/mmole), dissolved in 10 percent ethanol in isotonic saline. The specificity of estradiol localization was tested by injecting 50 μg of unlabeled 17β-estradiol into two immature ovariectomized rats 5 minutes prior to the injection of 17β-[^{3}H]estradiol. One additional male rat was injected with [^{3}H]estradiol in order to ascertain whether estradiol target cells exist in the male as well as in the female. One hour after injection of the 17β-[^{3}H]estradiol the rats were killed. The heart was removed, mounted on tissue holder, and frozen in −180°C liquefied propane. Serial frozen sections (4 μm) were cut (wide range cryostat, Harris Manufacturing Co., Inc., North Billerica, Mass.) and thaw-mounted or dry-mounted on photographic emulsion (Kodak NTB-3) coated slides. After autoradiographic exposure for several months, the slides were photographically processed and stained with methyl green pyronin as described (*2*).

After the injection of [^{3}H]estradiol, muscle cells of the left and right auricles and atria (Fig. 1, a and b) in both the female and male rat show concentration and retention of radioactivity in nuclei (Fig. 2 a, b, and d). The muscle cells of the ventricles (Fig. 2c) do not show such nuclear concentration, even after autoradiographic exposure times of more than 1 year. The concentration of radioactivity in nuclei of atrial myocytes can be prevented if unlabeled 17β-estradiol is injected prior to 17β-[^{3}H]estradiol (Fig. 2e). Muscle cells of the auricles concentrate more radioactivity than those of the atria. In the myoctes of the atria, including the auricles, nuclear labeling is not uniform. Variations exist not only among cells, but also within the same cell: that is, in some myocytes, labeled and unlabeled nuclei are seen side by side.

In our preparations, the sino-atrial node and the atrial conducting system could not be identified. Since, throughout the atrium, labeled muscle cells are found, it can be assumed that the cells of the sino-atrial node and the atrial conducting system do not behave differently regarding [^{3}H]estradiol uptake, compared to the other cells. In the ventricles, the easily recognizable Hiss bundle and Purkinje fibers, similar to other ventricular cells, do not concentrate radioactivity. Cells in the tunica media of the arteria pulmonalis and the aorta show weak nuclear labeling as do some of the connective tissue cells at the base of the tricuspid and mitral valves. Radioactively labeled fibroblasts are also found in the extramyocardial area at the base of the heart in the vicinity of the unlabeled autonomic ganglion cells. The separation between labeled atrial and unlabeled ventricular myocytes is abrupt in the area of the auricles, but somewhat transitional in the centrally located atrioventricular contact zone.

The autoradiographic studies reveal that the myocardium cranial to the atrioventricular skeleton contains target cells for estradiol. This atrio-auricular part of the heart is derived embryologically from the primitive paired atrium and sinus venosus, which are located extrapericardially at the eight-somite stage