min for 30 minutes to remove the large particulate material, and 2 ml of the supernatant was filtered through a membrane filter that had a pore diameter of 0.2 μ. The sterile filtrate was activated with 1 percent trypsin according to the method of Duff *et al.* (*3*). One-half milliliter of 1 : 10 and 1 : 100 dilutions in gelatin phosphate buffer, *p*H 6.2, was injected intraperitoneally into each of two mice (Swiss Webster strain). The animals were observed closely for 48 hours for typical symptoms of botulism and for death; survivors were discarded after 4 days.

The toxin was identified by repeating the injections of diluted trypsin-activated filtrate into mice that had been individually protected with antitoxin to *Cl. botulinum*, types A, B, C, D, or E or with tetanus antitoxin. The toxic filtrate was also tested by mixing it with a combination of the five botulinum antitoxins in vitro, as well as with these five antitoxins plus tetanus antitoxin. Four mice were injected with the latter combination. The heat-labile character of the toxin was demonstrated by heating a portion of the culture filtrate to 100°C for 10 minutes and then injecting 0.5 ml of a 1 : 10 dilution of this filtrate intraperitoneally into mice. The results of tests for protection by antitoxins are shown in Table 1. Neutralization of the toxin was achieved only by type F antitoxin.

After typing the toxin, the original mixed culture was treated with alcohol as described by Johnson, Harmon, and Kautter (*4*) and then streaked on plates of liver-veal agar with egg yolk and on plates of agar containing 5 percent of blood. All plates were incubated in an anaerobic jar, and botulinum-type colonies were transferred to TPG medium; these were incubated for 5 days, and the toxin that was produced was typed by the method given above.

Animals protected with *Cl. botulinum* type F antitoxin were the only ones that survived. The titer of type F antitoxin was lower than that of the other types, which necessitated the use of 1:200 and 1:500 dilutions of filtrate, but these dilutions killed all mice except those protected by type F antitoxin. Identity of the culture has been confirmed by repetition of the typing procedure.

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Myotonic Response Induced by Inhibitors of Cholesterol Biosynthesis

Abstract. *Steroid inhibitors of cholesterogenesis containing nitrogen-substituted side chains induced electromyographic myotonia in rats. Cholesterol reduction or desmosterol accumulation, per se, did not cause myotonia, and cholestrol feeding prevented drug-induced myotonia. Desmosterol accumulation in combination with a specific drug effect may cause the observed myotonia.*

True myotonia is characterized by an abnormally delayed relaxation of skeletal muscle fibers which follows voluntary or induced contraction and is associated with repetitive depolarization of the muscle-cell membrane. Such a contraction, as recorded by an electromyograph (EMG), initiates a burst of action potentials of increased amplitude and high frequency which decreases over a period of seconds and is audible as a "dive-bomber" sound. The myotonic phenomenon has been observed in man (*1, 2*) and in the goat (*3*) after administration of 20,25-diazacholesterol, an inhibitor of cholesterol synthesis. Analyses of the sterol composition of plasma, erythrocyte stroma, and muscle showed a decrease in cholesterol and the appearance of its immediate precursor, desmosterol.

We now report the induction of EMG myotonia in rats by several cholesterol analogs and an androstene derivative and describe studies undertaken to determine the properties required for such agents to induce myotonia.

The agents (Figs. 1 and 2) were administered by daily subcutaneous injection to 20-day-old white, male Wistar rats, maintained on either a standard Purina rat diet or a 2 percent cholesterol diet. Electromyography (TECA electromyograph, Model B) was performed at weekly intervals with monopolar electrodes inserted into the thigh muscles of anesthetized rats. Animals were killed when definite myo-

Table 1. Electromyograph myotonia and mean concentrations of plasma sterol in rats. The number of animals treated is shown within the parentheses.

Agent	Treatment (wk)	Dose (mg kg^{-1} day^{-1})	EMG myotonia*	Plasma (mg/100 ml ± S.E.)	
				Cholesterol	Desmosterol
Control† (6)	6		0	64.6 ± 7.4	0.0
25-azacholesterol (4)	2	0.1	+	22.6 ± 4.9	28.2 ± 4.9
24-azacholesterol (2)	3	5	+		
20-azacholesterol (6)	6	10	0	59.0 ± 1.5	0.0
22, 25-diazacholestanol (5)	3	30	+	7.3 ± 2.1	43.9 ± 4.5
20, 25-diazacholesterol (5)	3	10	+	14.4 ± 2.4	40.7 ± 3.9
SC-13820 (3)	4	20	+	19.3 ± 1.6	24.3 ± 1.6
SC-12998 (3)	2	10	+	10.0 ± 2.8	37.8 ± 12.3
3β-(β-diethylaminoethoxy)-androst-5-en-17-one-methoxime (5)	4	15	+	3.7 ± 0.9	54.4 ± 11.3
Triparanol (6)	6	100	0	12.6 ± 2.8	29.5 ± 3.5
W-398 (3)	6	20	0	45.5 ± 2.5	0.0
W-398 + 20, 25-diazacholesterol (2)	6	20	+	13.6 ± 0.9	38.2 ± 2.2
2% cholesterol diet + 20, 25-diazacholesterol (6)	6	10	0	83.0 ± 2.8	6.7 ± 1.6
2% cholesterol diet + 20,25-diazacholesterol (2)	6	100	0	76.1 ± 6.3	2.8 ± 0.4

* Plus sign indicates presence of myotonia; zero indicates absence of myotonia. † Twenty percent methyl cellulose was used in place of the sterol.

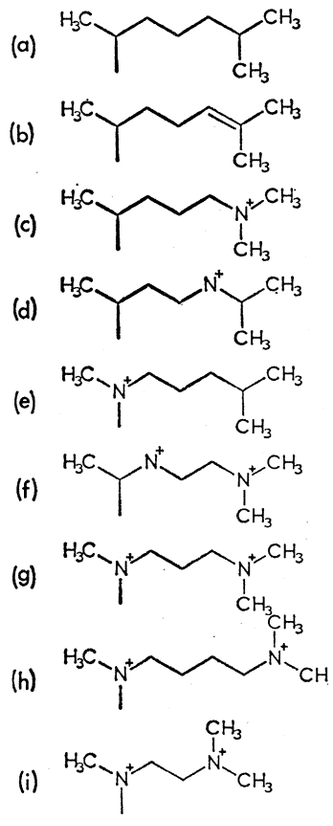

Fig. 1. The cholesterol analogs differ in the structure of their side chains. Shown in comparison to the side chains of cholesterol (*a*) and desmosterol (*b*) are those of 25-azacholesterol (*c*), 24-azacholesterol (*d*), 20-azacholesterol (*e*), 22,25-diazacholestanol (*f*), 20,25-diazacholesterol (*g*), SC-13820 (*h*), and SC-12998 (*i*). With the exception of 20-azacholesterol, all the analogs caused desmosterol accumulation and EMG myotonia.

tonia appeared, or after 6 weeks if it did not. Plasma and muscle specimens were frozen for subsequent sterol analysis by the gas-liquid chromatographic method of Burns *et al.* (*4*) and the chemical method of Zak (*5*).

The results of treatment are shown in Table 1. Analogs of cholesterol whose primary action is the inhibition of the conversion of desmosterol to cholesterol consistently induced EMG myotonia within 2 to 4 weeks of treatment. The evolution of the myotonia followed a characteristic pattern, consisting of slight prolongation of the insertion potential after 1 to 2 weeks, runs of positive waves by 2 to 3 weeks, and the appearance of overt myotonia shortly therafter. In addition, 3-β(β-diethylaminoethoxy)androst-5-en-17-one-methoxime (Fig. 2*a*) also induced myotonia. Administration of each of these agents was associated with the accumulation of a large quantity of desmosterol in the plasma and a significant reduction in the mean concentration of cholesterol in the plasma compared with the control group. Similar alterations of cholesterol-desmosterol ratios were found in muscle specimens of treated rats. 20-Azacholesterol was the only cholesterol analog tested that was not associated with either myotonia or desmosterol accumulation.

To assess the role of cholesterol reduction in the pathogenesis of the observed myotonia, benzyl-*N*-benzylcarbethoxyhydroxamate (W-398) (Fig. 2) was administered. This agent, which limits the conversion of hydroxymethylglutaryl coenzyme A (HMG CoA) to mevalonic acid (*6*), reduced the plasma-cholesterol concentration (mean) to about 30 percent of that of the control group; it did not cause desmosterol accumulation or myotonia, an indication that the fall in cholesterol, by itself, is not a cause of the myotonia.

Triparanol (Fig. 2), a nonsteroid inhibitor of cholesterol biosynthesis, caused the accumulation of large quantities of desmosterol in both plasma and muscle, but failed to induce myotonia. Therefore, it would appear that desmosterol accumulation, per se, is not responsible for the myotonia.

To ascertain whether the myotonia-inducing effect of these agents represented a direct myotoxic action independent of altered sterol synthesis, either W-398 or a high cholesterol diet was administered during treatment with 20,25-diazacholesterol. Administration of W-398 or cholesterol feeding (*7*) might be expected to inhibit the conversion of HMG-CoA to mevalonic acid and thereby prevent the accumulation of significant quantities of desmosterol. However, addition of W-398 exerted little effect on sterol synthesis, compared with that of 20,25-diazacholesterol alone, and did not affect the development of myotonia. In contrast, exogenous cholesterol administration effectively diminished desmosterol formation and prevented the development of myotonia. Moreover, a tenfold increase in 20,25-diazacholesterol dosage failed to overcome the preventative effect of cholesterol feeding, an indication that in the absence of significant concentrations of desmosterol, even large quantities of the drug were insufficient to induce myotonia.

Thus, the observed myotonia might result from the combined effect of desmosterol accumulation and agents

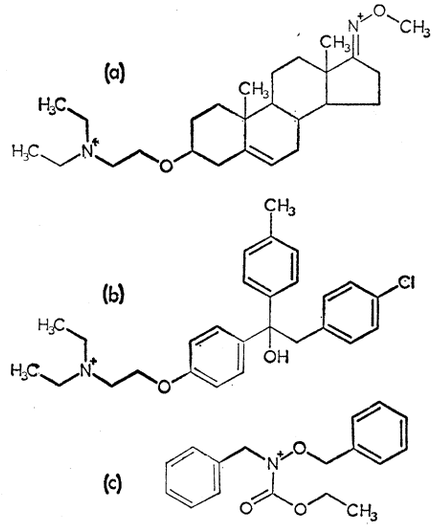

Fig. 2. Both the androstene derivative (*a*) and triparanol (*b*) produced significant concentrations of desmosterol, a property of their identical side chains, but only the steroid agent (*a*) induced EMG myotonia. W-398 (*c*) reduced cholesterol concentrations in the plasma, but did not induce myotonia.

with specific structural features, namely, a steroid nucleus with a side chain containing a nitrogen atom at or near a terminal dimethyl or diethyl group. Since myotonia probably results from a defect in the muscle-cell membrane and sterols are important in membrane structure (*8*), it is of interest that desmosterol was found to represent a major proportion of the total sterol content of erythrocyte stroma during treatment with 20,25-diazacholesterol (*2, 3*). Whether desmosterol or the myotonia-inducing sterols are present in the muscle-cell membrane of treated animals is not yet known.

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