Table 1. Incorporation of labeled precursors into lincomycin and a new compound, the *S*-ethyl homolog of lincomycin. The labeled compound and unlabeled ethionine (100 μg/ml) were added 48 hours after inoculation. The compounds had the following specific activities: L-methionine-C^{14}-methyl, 20 mc/mmole; L-ethionine-ethyl-l-C^{14}, 1.2 mc/mmole; and DL-methionine-2-C^{14}, 0.6 mc/mmole.

Product	Specific activity (μc/mg)
L-Methionine-methyl-C^{14}	
Lincomycin	5.3
New compound	2.6
L-Ethionine-ethyl-1-C^{14}	
Lincomycin	0
New compound	1.4
DL-Methionine-2-C^{14}	
Lincomycin	0
New compound	0

neutralized to *p*H 4.5 with Amberlite IR-45 (Rohm and Haas Co.) and lyophilized. The residue was dissolved in methanol (200 mg/ml), and five volumes of acetone were added. After storage overnight at 4°C, the crystalline hydrochloride salt was collected and recrystallized twice in the same way. It had the following properties: m.p. 160° to 162°C; $[\alpha]_D$ + 141 (*c* 1.02, H_2O); *pK* 7.3 (neutralization equivalent 438); and no characteristic absorption in ultraviolet and visible light. Analysis (percentage): C, 49.16; H, 8.23; N, 6.40; S, 7.27; Cl, 8.01; O, 21.16; *O*—CH_3, 0.5; *N*—CH_3, 2.6; *C*—CH_3, 5.8; acetyl, 0.7. Calculated (percentages) for $C_{19}H_{36}N_2O_6S \cdot HCl$: C, 49.91; H, 8.17; N, 6.13; S, 7.02; Cl, 7.76; O, 21.01.

The nuclear magnetic resonance spectra of the two antibiotics, as the hydrochloride salts in deuterium oxide, were essentially the same except for one mutually exclusive feature in each not shared by the other. In both spectra, determined at 60 Mc/sec, there was a signal (3H) at 2.99δ which was assigned to a protonated *N*-methyl (*N*–CH_3) function (*7*). This is consistent with the chemical functional group analyses which indicate that each antibiotic probably contains 1 mole of *N*-methyl. Lincomycin had a singlet peak (3H) at 2.17δ corresponding to *S*-methyl (*8*). This signal was absent in the new compound, and instead there was a quartet at 2.65δ (2H, J = 6 cy/sec, J being the spin coupling constant) (*7*) and a triplet at 1.27δ (3H, J = 6 cy/sec) which was interpreted as *S*-ethyl (*S*–CH_2–CH_3) (*8*). These data suggest that lincomycin contains a methyl thioether group and that the new compound is the homologous ethyl thioether compound. This interpretation is permissible on the basis of the elemental analyses. If this constitutes the only difference between the two antibiotics, then the new compound should also contain one more *C*-methyl group than lincomycin. This was substantiated by functional group analyses (*9*). The structure of lincomycin was recently reported (*10*), and the foregoing conclusions concerning the functional groups in it are correct.

In a study of the radioactive-labeling pattern in the two antibiotics produced in the presence of certain radioactive precursors, C^{14} from L-methionine-methyl-C^{14} was incorporated into both antibiotics (Table 1). However, the amount found in the new compound was one-half that in lincomycin. This is the proportion expected if the conclusions previously made concerning the relation between the structures of the antibiotics are correct. The groups labeled would be one *N*-methyl and one *S*-methyl in lincomycin and one *N*-methyl in the new compound. Neither antibiotic contains any *O*-methyl. Radioactivity (C^{14}) from L-ethionine-ethyl-1-C^{14} was incorporated into the new compound but not into the lincomycin, suggesting the presence of an ethyl group in the new compound and its absence in lincomycin. Label from DL-methionine-2-C^{14} was incorporated into neither antibiotic. The last observation rules out the possibility that methionine was incorporated as a unit into the antibiotics, thus restricting the reactions to alkylation.

These data suggest that the *S*-ethyl homolog of lincomycin is formed by a process of *S*-transethylation with ethionine replacing methionine as alkyl donor. However, label from S^{35}-methionine was incorporated into both lincomycin and the new compound, and the possibility that the new compound arises by a process of ethylthiolation has not been excluded. To our knowledge, neither biological *S*-transethylation nor ethylthiolation have previously been reported.

The production of the new compound was dependent upon the presence of ethionine as a specific source of *S*-ethyl groups. Neither *S*-ethyl-L-cysteine nor L-rhamnose diethylmercaptal could serve as a source of *S*-ethyl groups. Out of 32 compounds tested only D-, L-, and DL-ethionine caused the formation of the new compound. High concentrations of L-methionine (0.5 mg/ml) prevented the ethionine-stimulated production of the new compound.

After the completion of this work, the isolation of an *S*-ethyl homolog of lincomycin was reported from the culture filtrate of *Streptomyces lincolnensis* var. *lincolnensis* fed DL-ethionine (*11*).

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5. We thank M. Dann, A. C. Dornbush, Dr. J. S. Schultz, Dr. S. O. Thomas and staffs, and the organic chemical section of our laboratories, for large-scale extractions, microbiological assays, large-scale fermentations, preliminary microbiological work, and micro and spectroscopic analyses, respectively.
6. A direct comparison was made to a sample of lincomycin hydrochloride kindly supplied by Dr. G. M. Savage, The Upjohn Company, Kalamazoo, Mich.
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9. The new compound contained 0.3 mole more *C*-methyl per mole of antibiotic than lincomycin. This compares to the value of 0.4 mole of *C*-methyl per mole of ethionine determined under similar conditions of analysis.
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28 September 1964

Nervous Control of Ciliary Activity

Abstract. *Electrical stimulation of the visceral ganglion of the mussel* Mytilus edulis *caused cilia on the lateral epithelium of the gill to beat faster. This effect was blocked by cocaine, physostigmine, hyoscyamine, and acetylcholine but not by tubocurarine. These agents did not block the cilioexcitatory effect of serotonin.*

The beating of cilia on the lateral epithelium of the gill of the mussel *Mytilus edulis* provides the force for propelling water through the gills. This

water brings oxygen and food particles to the mussel and takes gametes and excretory products away. The rate of water flow is determined by the activity of the cilia, the resistance to the flow of water through the gills, and the positioning of the valves and mantle edges (*1*). Water propulsion is virtually continuous when the valves are open and is influenced by physical and chemical factors in the same way that these factors influence ciliary activity on isolated gills (*1*). Lucas (*2*) reported that the branchial nerve, which arises from the visceral ganglion and enters the root of the gill, did not innervate the ciliated gill filaments. However, it has also been reported that transecting the branchial nerve close to the visceral ganglion caused ciliary activity to diminish (*3*). Because this finding implies that the branchial nerve does go to the ciliated filaments we have studied this nerve in more detail and found that electrical stimulation of one branch of the branchial nerve caused a marked increase in the rate of ciliary beating. Furthermore, by using stains not employed by Lucas, we have been able to trace nerve fibers from the visceral ganglion into the ciliated filaments (*4*).

In animals that were intact except for having the shell and mantle removed, stimulation of the cerebro-visceral connective, which runs from the cerebral to the visceral ganglion, or stimulation of the visceral ganglion itself, caused an increase in the rate of ciliary beating (three experiments). The rate of beating was estimated by synchronization with stroboscopic light as described earlier (*3*). Isolated gill-nerve-ganglion preparations were made by removing one gill with its associated branchial nerve and visceral ganglion still attached and supported by a small piece of adductor muscle. The preparation was immersed in sea water at 22° to 25°C, *p*H 7.7 to 7.9, and remained viable and responsive for up to 72 hours provided the water was changed and the temperature lowered to 5°C overnight. Electrical stimulation (2 to 25 volts, 10 pulses per second, 1-msec biphasic pulse) for 5 to 300 seconds caused an increase in the rate of ciliary beating in 45 of the 50 preparations studied so far.

The basal rate of beating in the isolated preparation was usually 5 to 12 beats per second. Stimulation of the visceral ganglion increased the rate to 17 to 25 beats per second for as long as stimulation continued. When the stimulus was turned off ciliary beating slowly returned to the basal rate (Fig. 1). A similar response was obtained by stimulating that branch of the branchial nerve which runs anteriorly in the root of the gill. This nerve arises from the visceral ganglion in the form of many fine fibers that can be seen only with the microscope. Cutting this nerve prevented the response in those filaments peripheral (anterior) to the cut but did not influence the response in filaments originating between the ganglion and the cut (six experiments). Stimulation of nonnervous tissue or of the main branch of the branchial nerve, which runs posteriorly in the root of the gill, did not cause a response.

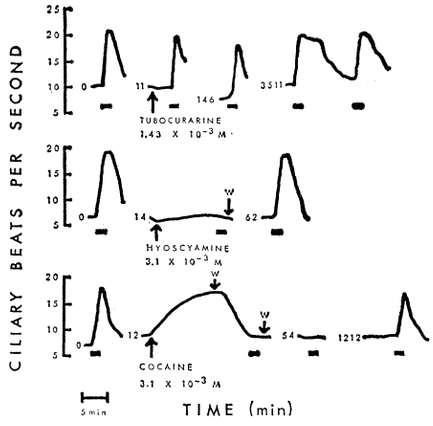

Fig. 1. The increase in rate of beating of cilia in response to electrical stimulation of the visceral ganglion. The period of stimulation is indicated by a heavy bar below each graph. An estimation of the rate of beating was made about every minute. The time in minutes from the beginning of the experiments is indicated at the beginning of each series of measurements. Each of the three graphs refers to one point on the lateral epithelium of a different mussel but is representative of the activity of cilia on the rest of that gill. Stimulation for 90 seconds with biphasic d-c pulses at 10 per second, 1 msec duration, voltage set for maximum response (10.5 v in the top graph, 25 v in the other two). Top graph: d-tubocurarine added at 12 minutes and left in throughout experiment. Middle graph: *l*-hyoscyamine added at 15 minutes and washed out (*W*) at 30 minutes after stimulus failed to elicit response. Bottom graph: cocaine added at 13 minutes, washed out at 25 minutes to restore basal rate and washed again at 34 minutes, after stimulus failed to elicit response.

The addition of cocaine (3.1×10^{-3} *M*) caused a temporary increase in the rate of beating. After washing with sea water the basal rate was restored but the response to electrical stimulation was still blocked. The response was restored only by prolonged washing (five out of five experiments). The addition of serotonin ($10^{-6}M$), which is considered to be the naturally occurring cilio-excitatory substance in the mussel gill (*5*), caused an increase in the rate of beating to 22 to 27 beats per second in both untreated gills and gills treated with cocaine. Physostigmine ($1.43 \times 10^{-3}M$), 1-hyoscyamine ($1.43 \times 10^{-3}M$), and acetylcholine ($5.5 \times 10^{-3}M$) blocked the response to electrical stimulation of the visceral ganglion or branchial nerve without affecting the basal rate or the cilioexcitatory action of serotonin. Tubocurarine ($1.43 \times 10^{-3}M$) had no effects. Synthesis, storage, and degradation of acetylcholine in gill tissue has already been demonstrated (*6*), and the present findings suggest that the acetylcholine system is involved in the nervous control of ciliary activity. Serotonin, on the other hand, probably acts directly on the ciliated cell because its action is not modified by blockade of the neural pathway.

The importance of nervous control of ciliary activity in the regulation of water propulsion has not yet been determined. There is a peripheral chemoreceptor, the osphradium, which lies in the path of water flow and which sends a nerve to the visceral ganglion. Bailey and Laverack (*7*) have reported that chemical stimulation of the osphradium in the snail evokes action potentials in the branchial nerve. If this also occurs in the mussel it provides a physiological basis for the regulation of water propulsion in response to chemical sensing of the water flowing through the mussel.

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