7. Certain combinations of neomycin and streptomycin showed considerably greater bacteriostatic and bactericidal effects than the corresponding concentrations of either antibiotic alone or concentrations of streptomycin equal to the total number of units of both antibiotics. Such antibacterial effects were never greater, however, than concentrations of neomycin equal to the total units of the two antibiotics. Very small amounts of neomycin in synergistic mixtures with streptomycin had a much greater effect than streptomycin alone.

8. No marked increase in the resistance of *E. coli* cells which survived exposure to neomycin and streptomycin was observed toward either antibiotic or combinations of the two.

9. The neomycin-resistant strain of *E. coli* showed some resistance to streptomycin after several weeks on media containing neither antibiotic.

10. Neomycin appears to be about four times as active, on a unit basis, as streptomycin against many bacteria. In view of the fact that the most potent neomycin preparations so far obtained have 250 units per milligram, the antibacterial potency of the two antibiotics is about similar on a gram basis.

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[1] Waksman, S. A., Lechevalier, H. A., and Harris, D. A., *J. Clin. Invest.*, **28**, 934–939 (1949).

[2] The neomycin was a crude preparation containing 100 u/mg.; the streptomycin was in the form of sulfate assaying 650 μg./mg.

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*ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. VII. CLITOCYBE ILLUDENS**

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Clitocybe illudens[1] was reported earlier from this laboratory[2] to evidence antibacterial activity. In later tests the antibacterial action of this fungus was found to be substantially greater on a corn steep agar medium than on the original thiamine-peptone agar or on a potato-dextrose agar. Grown on corn steep agar and tested by the agar disc method,[2, 3] zones of inhibition up to 25 mm. in diameter were observed for *Staphylococcus aureus*

and up to 40 mm. in diameter for *Mycobacterium smegma*. There was little or no effect on *Escherichia coli*.

Culture liquids with antibacterial activity were produced by growing the fungus at 25°C. on a corn steep medium in Fernbach flasks as previously described.[4] After three to four weeks the mycelial mats covered the surface and the culture liquids had an activity of from 64 to 256 dilution units per ml. against *Staph. aureus, Klebsiella pneumoniae* and *Myco. smegma*. Little inhibitory action was noted on *Bacillus mycoides, Bacillus subtilis, E. coli* or *Pseudomonas aeruginosa*. It was possible to obtain active liquid two weeks after reflooding such mats with fresh corn steep medium. Mats were successfully reflooded several successive times.

Isolation of Crystalline Substances.—Two antibacterial substances and one substance inactive for the organisms tested were isolated in crystalline form from culture liquids of *C. illudens*.

Extraction of the active material from the culture liquid with organic solvents was incomplete. However, the active material was adsorbed practically quantitatively by charcoal from which it could be eluted by aqueous acetone.

Bioassay of the material extracted by organic solvents and that left in the aqueous phase suggested the presence of more than one active component. Thus after extraction of culture liquid with chloroform the activity for *Myco. smegma*, compared to that for *Staph. aureus*, was increased in the organic solvent and decreased in the aqueous phase. By countercurrent distribution between chloroform and water, of the material eluted from the charcoal, three distinct components were separated.

Batches of culture liquid at their original pH of from 4.2 to 4.5 were stirred for an hour with 20 g. of Norit A (Pfanstiehl) per liter, using an efficient mechanical stirrer. The solution was usually kept in a cold room at 8° to 10°C. overnight, and the charcoal allowed to settle. After the charcoal had been filtered off by suction, through a thin layer of celite, a perfectly clear solution was obtained which had only a small percentage of the original activity; this was discarded. The Norit A, on which the active material was adsorbed, was stirred for three half-hour periods with 80 per cent acetone, the first time with 10 per cent of the volume of culture liquid used, then twice more with 5 per cent each time. The eluate was concentrated under reduced pressure, in a water bath at about 60°C. to remove the acetone, leaving about a liter of aqueous concentrate. This contained essentially all the active material originally present in the culture liquid. Fractionation was accomplished by countercurrent distribution. The distribution was carried out in ten funnels, using equal volumes of chloroform and water.

By concentration of the extract to a small volume under reduced pressure, and finally to a sirup under a stream of nitrogen, crystalline ma-

terial was usually obtained from "Chloroform 1" (the first chloroform extract which passed through the series of funnels) and sometimes also from "Chloroform 2." This material was over a hundred times as active against *Myco. smegma* as against *Staph. aureus;* it is designated *illudin M.* Chloroforms 4 to 10 on evaporation yielded inactive crystalline material.

"Water 1" (the original eluate after being washed with ten portions of chloroform) and "water 10" (the tenth water washing of the chloroform) were of low antibacterial potency and accounted for only a small fraction of the activity of the original culture liquid. These extracts were discarded. Waters 2 to 9 were extracted with two and one-half times their volume of ethyl acetate in five equal portions. On evaporation of the ethyl acetate, a crystalline compound highly active against both *Staph. aureus* and *Myco. smegma* was obtained. This substance is referred to as *illudin S.*

The yield of crystalline illudin S per liter of culture fluid (average of ten batches) was 0.33 g.; of crystalline illudin M, 0.04 g. About one-third of the activity of the original culture liquid against *Staph. aureus* was recovered in the crystalline material and about one-half of the activity against *Myco. smegma.* The average results per liter for ten batches of culture liquid are given below:

	WEIGHT, G.	*Staph. aureus* DILUTION UNITS	*Myco. smegma* DILUTION, UNITS
Illudin S	0.33	75,460	20,650
Illudin M	0.04	260	30,140
Total	0.37	75,720	50,790
Culture liquid	...	230,000	101,000

By redistribution of mother liquors from which crystals had been obtained, more of each of the compounds was crystallized. The chloroform mother liquors yielded varying amounts of crystals of the inactive compound in addition to illudin M. Average results of redistribution of three batches of chloroform mother liquors and four batches of ethyl acetate mother liquors are given below:

		ACTIVITY	
	WEIGHT, G.	*Staph. aureus* DILUTION UNITS	*Myco. smegma* DILUTION UNITS
No. 1, Solids in $CHCl_3$ mother liquors	29.00	238,000	4,500,000
Illudin M from No. 1	1.01	6,200	907,000
Inactive crystals from No. 1	0.50	...	...
No. 2, Solids in ethyl acetate mother liquors	30.00	3,900,000	1,400,000
Illudin S from No. 2	5.51	718,000	804,000
Total illudin M and illudin S	6.52	724,200	1,711,000

If the additional material obtained by redistribution is taken into account the percentage of the activity of the original culture liquid against

Staph. aureus which was recovered in crystalline form was about 45 per cent; against *Myco. smegma*, about 80 per cent. While it is possible that other antibiotic substances may have been present, it is believed that illudin M and illudin S account for the major part of the activity of the culture liquids.

Chemical Characteristics of Illudin M and Illudin S.—The antibacterial activity of the culture liquid, or of the active crystalline compounds dissolved in water, was not reduced by bringing the solution to a quick boil. This method of sterilization was used in preparing samples for bioassay. The activity of the culture liquid was not affected by incubation for one hour at 37°C. and pH levels of from 3 to 11. While no systematic studies of stability have been made, it has been observed that the crystalline compounds are unstable under somewhat more stringent conditions than those mentioned.

Both active compounds are fairly soluble in organic solvents, less so in water. Illudin M is soluble in water to the extent of at least 1 mg. per ml.; illudin S is somewhat more soluble.

The three crystalline compounds have been further purified and characterized; the data given below suggest that they are probably of related molecular structure. The molecular formula of illudin M differs from that of illudin S by the elements of one molecule of water.

Compound	Illudin M	Illudin S	Inactive crystals
Recrystallized from	Aqueous ethanol	Acetone	Chloroform
Melting point (uncorr.)	130–131°	124–125°	72–74°
Absorption maxima (mμ) in 95% ethanol	230, 320	235, 328	None between 215 and 360
$(\alpha)_D^{25}$ in absolute ethanol	−126	−165	−107
Analytical values, C, H[5]	C, 72.81 H, 8.05	67.78 8.16	60.39 8.29
Empirical formula, and calculated C, H	$C_{15}H_{20}O_3$ C, 72.53 H, 8.12	$C_{15}H_{22}O_4$ 67.62 8.33	$C_8H_8O_2$ 59.98 8.06
Molecular weight (found)[5]	241	264	204
Molecular formula	$C_{15}H_{20}O_3$ (mol. wt. 248)	$C_{15}H_{22}O_4$ (mol. wt. 266)	$C_{16}H_{16}O_4$ (mol. wt. 200)[6] or $C_{15}H_{24}O_6$ (mol. wt. 300)

All three compounds are neutral. A further similarity is their behavior in acid solution, in which characteristic changes occur in their absorption spectra. The products resulting from this change, and their possible bearing on the structure of the antibiotic compounds, will be reported separately.

Antibacterial and Antifungal Activity.—The potency of the active crystalline compounds, after recrystallization, was determined for a

number of bacteria by serial dilution[7] and is given in the following table. The activity is expressed as the minimum inhibitory concentration in μg. per ml. (p = partial inhibition)

Bacterium	Illudin S	Illudin M
Bacillus mycoides	> 500	> 500
Bacillus subtilis	31 (16p)	500
Escherichia coli	> 500	> 500
Klebsiella pneumoniae	4	16 (8p)
Mycobacterium smegma	4 (2p)	1 (0.5p)
Mycobacterium tuberculosis[8] H37R	6	1
Pseudomonas aeruginosa	500 (125p)	> 500
Staphylococcus aureus	4	250

The potency of the antibacterial compounds against *Staph. aureus*, *Myco. smegma* and *K. pneumoniae* was not reduced after incubation at 37°C. for three hours with 5 per cent human blood in 0.9 per cent saline with beef extract.

The antifungal activity of illudin S and illudin M was measured by serial dilution in a peptone medium at pH 6. Spore suspensions of the fungi were used as inoculum.[9] *Trichophyton* was incubated at 30°C.; the others at 25°C. The fungi tested included *Aspergillus niger*, *Chaetomium globosum* (USDA 1042.4), *Gliomastix convoluta* (PQMD4c), *Memnoniella echinata* (PQMD1c), *Myrothecium verrucaria* (USDA 1334.2), *Penicillium notatum*, *Phycomyces Blakesleeanus* (plus strain), *Saccharomyces cerevisiae*, *Stemphylium consortiale* (PQMD41b), and *Trichophyton mentagrophytes*. As much as 250 μg. per ml. of illudin S did not inhibit the growth of these fungi.

The antifungal activity of illudin M was somewhat greater, though the difference in response between fungi was marked. A wide range of partial inhibition (p) was noted for some species. The minimum inhibitory concentration of illudin M in μg. per ml. was 16 for *Memnoniella echinata*, 16 (2p) for *Penicillium notatum*, 32 (1p) for *Chaetomium globosum*, 64 (32p) for *Trichophyton mentagrophytes*, 125 (8p) for *Myrothecium verrucaria*, 250 (32p) for *Aspergillus niger*, 250p to 32p for *Stemphylium consortiale*, 250p to 125p for *Saccharomyces cerevisiae*, 250p for *Phycomyces Blakesleeanus* and more than 250 for *Gliomastix convoluta*.

Toxicity.—In preliminary tests, using Carworth male white mice weighing on an average, 16 g., all mice were killed by illudin S at 15.6 mg. per kilo in from seven to twenty-two hours, and by illudin M within forty-four hours. Four groups of five mice each were used in the tests: one control group, and three groups receiving 0.25, 0.50 and 1.0 mg. per mouse, respectively. The test compound was dissolved in 0.5 ml. of 0.9 per cent saline solution, and injected into a tail vein. The controls received only saline solution.

Persons handling *Clitocybe illudens* or its products showed some sensitivity on contact, which manifested itself as a dermatitis of varying degrees of severity.

Hollande obtained an antibacterial substance, clitocybine, from the sporophores and mycelium of *Clitocybe gigantea* (*Fr. ex. Sow.*) *Quel. var. candida* (*Bres.*) *Heim.* The chemical properties given for clitocybine[10] distinguish it from illudin M and illudin S.

The sporophores of *Clitocybe illudens* are poisonous when eaten.[11] Clark and Smith[12] concluded from chemical and biological evidence that *C. illudens* had a muscarin-like effect. They were able to overcome the toxic action on frogs' hearts by atropin. Illudin M and S do not appear to be related to muscarin; it is, however, possible that the compounds found in sporophores of the fungus differ from those produced in the culture liquid by the mycelium.

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[1] We are indebted to Ross W. Davidson, Division of Forest Pathology, U. S. Department of Agriculture for the culture of *Clitocybe illudens*, No. 72027-S in his collection.

[2] Robbins, W. J., Hervey, A., Davidson, R. W., Ma, R., and Robbins, W. C., *Bull. Torrey Botan. Club*, **72**, 165–190 (1945).

[3] Hervey, A., *Ibid.*, **74**, 476–503 (1947).

[4] Robbins, W. J., Kavanagh, F., and Hervey, A., these PROCEEDINGS, **33**, 171–176 (1947).

[5] The carbon and hydrogen analyses and molecular weight determinations were carried out by Joseph Alicino, Metuchen, N. J.

[6] The molecular weight of this compound is uncertain since it shows some signs of decomposition during the camphor melt.

[7] Kavanagh, F., *Bull. Torrey Botan. Club*, **74**, 303–320 (1947).

[8] Determinations for *M. tuberculosis* were made through the courtesy of Dr. Walsh McDermott and Dr. W. C. Robbins of the Cornell Medical College.

[9] Kavanagh, F., Hervey, A., and Robbins, W. J., these PROCEEDINGS, **35**, 343–349 (1949).

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[12] Clark, Ernest D., and Smith, Clayton, S., *Mycol.*, **5**, 222–232 (1913).