two limbs and followed by death. A small number of paralyzed hamsters survived, but developed atrophy and contractures of the affected muscles. The pathology in the infected hamsters is typical of poliomyelitis. The hamster strain is infectious for mice. Human convalescent poliomyelitis serum neutralized the hamster strain. Hamster convalescent serum neutralized the hamster strain as well as the mouse strain. The hamster strain inoculated intracerebrally into 4 monkeys produced no paralysis. Three monkeys, subsequently tested with virulent monkey cord virus, proved to be immune.

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13858

Antimicrobial Action of Pyocyanine, Hemipyocyanine, Pyocyanase, and Tyrothricin.

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The isolation of substances from various bacteria, fungi and actinomycetes which in extremely small amounts inhibit or destroy many types of pathogenic bacteria, has aroused considerable interest in recent years. These include such substances as pyocyanase,[1] pyocyanine,[1] hemipyocyanine,[1] tyrothricin[2] (and its components, gramicidin and tyrocidine), actinomycin,[3] streptothricin,[4] gliotoxin,[5] and penicillin.[6]

In view of the present marked interest in antibiotic substances of microbial origin and because of the availability of pyocyanase, pyocyanine and a number of synthetic pyocyanine salts as well as hemipyocyanine, it appeared to be of some interest to compare the antimicrobial activities of these substances with each other and to some extent with tyrothricin.

Hemipyocyanine* (α-hydroxyphenazine), pyocyanine hydrochloride and pyocyanine methosulfate (monomethyl sulfuric acid salt of pyocyanine) were prepared by synthetic methods.[7] Pyocyanine was also isolated as the phenazonium compound and as the hydrochloride and as the perchlorate from cultures of *Ps. aeruginosa*. Pyocyanase which is a crude ether-soluble fatty material composed of a mixture of substances, probably including some pyocyanine, was isolated from the same cultures. Tyrothricin was prepared according to Dubos and Cattaneo.[8]

Bacteriostatic activity was determined by thoroughly mixing varying amounts of each substance with 10 ml of melted brain-heart infusion agar in Petri dishes. After the agar had solidified, the surface was streaked with a loopful of an 18-24 hr broth culture of each test organism. The presence or absence of growth was noted after the plates had been incubated at 37°C for 24 hr. The inhibitory values given in Table I are probably somewhat low since, in most cases, the next dilution used at which growth occurred was twice the inhibitory dilution. It is evi-

1 Kramer, H., *Z. Immunitäts.*, 1935, **84**, 505.

2 Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

3 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1941, **42**, 231.

4 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1942, **43**, 9.

5 Dutcher, J. D., *J. Bact.*, 1941, **42**, 815.

6 Chain, E., *et al., Lancet*, 1940, **239**, 226.

* We are indebted to Dr. M. Tishler of Merck & Co., Inc., for supplies of hemipyocyanine and pyocyanine perchlorate.

7 Wrede, F., and Strack, E., *Ber.*, 1929, **62**, 2053; *Z. physiol. Chem.*, 1928, **177**; 1929, **181**, 58; also Hilleman, H., *Ber.*, 1938, **71**, 34. See also Einhorn, A., *et al., Ber.*, 1904, **37**, 106.

8 Dubos, R. J., and Cattaneo, C. J., *J. Exp. Med.*, 1939, **70**, 249.

TABLE I.
Comparative Bacteriostatic Activity of Pyocyanines, Hemipyocyanine, Pyocyanase and Tyrothricin.

Organisms	Natural pyocyanine (perchlorate)	Synthetic pyocyanine (hydrochloride)	Synthetic pyocyanine (methosulfate)	Synthetic hemi-pyocyanine	Pyocyanase	Tyrothricin
	Dilution at which growth is inhibited × 1000					
S. aureus	40	40	40	10	10	40
S. albus	20	20	20	5	5	40
S. viridans	20	20	20	5	5	1000
S. lactis	20	20	20	20	5	—
S. hemolyticus	100	100	100	10	10	1000
N. catarrhalis	100	100	100	20	10	1.6
S. aertrycke	10	10	10	<5	<5	—
S. paratyphi	20	20	20	5	10	1.2
S. schottmuelleri	20	20	20	<5	5	—
E. coli	10	10	10	5	5	1.2
E. typhosa	20	20	20	5	5	1.2

dent that a dilution of 1:10,000 or greater of natural or synthetic pyocyanine inhibited the growth of all bacteria tested; the Gram negative rods were about as sensitive as the Gram positive cocci. *S. hemolyticus* and *N. catarrhalis* were highly susceptible to the action of pyocyanine as shown by the complete inhibition of growth at a dilution of 1:100,000. Synthetic pyocyanine and the pyocyanine isolated from natural sources were identical in activity, as would be expected. Pyocyanine methosulfate, pyocyanine hydrochloride and perchlorate had the same activity on a molar basis, which indicates that the bacteriostatic properties of these compounds are due entirely to the pyocyanine moiety.

Hemipyocyanine has considerably less inhibitory action than pyocyanine. Most of the bacteria were inhibited at a dilution of 1:5,000 or less. The difference between the activities of pyocyanine and hemipyocyanine is brought out sharply in the case of *N. catarrhalis* and *S. hemolyticus*. While pyocyanine inhibited these organisms at a dilution of 1:100,000, it was necessary to use 5 to 10 times as much of hemipyocyanine to stop their growth.

The bacteriostatic potency of pyocyanase closely paralleled that of hemipyocyanine and was, therefore, considerably less than that of pyocyanine.

The specificity of the bacteriostatic properties of tyrothricin is clearly indicated. Although *S. viridans* and *S. hemolyticus* were inhibited at a 1:1,000,000 dilution and the staphylococci at 1:40,000, the Gram negative rods, *E. coli, E. typhosa* and *S. paratyphi* failed to grow only at a dilution of 1:1200 and *N. catarrhalis* at 1:1600.

All of these substances have much greater bacteriostatic potency than the sulfonamides. Sulfanilamide, sulfapyridine and sulfathiazole when tested in the same manner failed to inhibit the growth of any of the bacteria at a dilution of 1:1000.

An interesting reversal of order of activity is found in Table II which contains results obtained with a number of different yeasts. Pyocyanine perchlorate and pyocyanine hydrochloride were unable to inhibit the growth of the yeasts at a dilution of 1:5000; pyocyanine methosulfate was somewhat more active since most of the yeasts were inhibited at a dilution of 1:10,000. Hemipyocyanine which had been relatively inactive toward bacteria was the most active inhibitory compound towards the yeasts. A dilution of

TABLE II.
Inhibition of Yeasts by Pyocyanines, Hemipyocyanine and Pyocyanase.

Yeasts	Natural pyocyanine perchlorate	Synthetic pyocyanine hydrochloride	Synthetic pyocyanine methosulfate	Synthetic hemi-pyocyanine	Pyocyanase
	Dilution at which growth is inhibited × 1000				
Torulopsis kefir	<5	<5	40	100	40
Zygosaccharomyces Marxianus	<5	<5	10	100	5
Saccharomyces fragilis	<5	<5	10	40	5
Schwanniomyces occidentalis	<5	<5	10	40	40
Saccharomyces cerevisiæ	<5	<5	10	100	40
Saccharomyces ellipsoideus	<5	<5	10	100	40

1:100,000 of hemipyocyanine was bacteriostatic for most of the yeasts. These results emphasize strongly the specificity of action of antibiotic substances of microbial origin.

Pyocyanase inhibited the growth of the yeasts at higher dilutions than the pyocyanines but was not as active as hemipyocyanine.

Studies on the activity of antibiotic agents of microbial origin have been almost entirely confined to their action on bacteria. We, therefore, have very little information concerning their effects on fungi. Many of these agents in their present form cannot be used intravenously because of hemolytic properties, toxicity or because they are ineffective by this route. Their therapeutic use is thus limited to surface or localized infections which are directly accessible. Fungus infections in man are predominantly of this type and may represent an important field of application for microbial therapeutic substances.

The fungistatic properties of pyocyanine (perchlorate), hemipyocyanine and tyrothricin towards the pathogenic fungi: *Achorion schoenleinii, Microsporium gypseum, Trichophyton gypseum* and *Candida albicans,* were determined.† Sterile water dilutions of the microbial agents were added to 5 cc of sterile Sabouraud's broth contained in 25 cc Erlenmeyer flasks. The fungi were cultured on Sabouraud's agar for 1-2 weeks. Suspensions of the fungi were made by scraping off a small amount of growth and suspending it in sterile water. Each flask of medium was inoculated with 0.1 cc of suspension. All cultures were incubated in 28°C for 7 days after which the presence or absence of growth was recorded. The results are given in Table III. It is evident that both hemipyocyanine and tyrothricin, in relatively high dilutions, completely inhibited the growth of the pathogenic fungi while pyocyanine was considerably less effective. Tyrothricin completely inhibited all of the pathogenic fungi at dilutions of 1:5000 to 1:20,000; pyocyanine from 1:2000 to 1:5000; and hemipyocyanine from 1:20,000 to 1:60,000. *Achorion schoenleinii* was the most sensitive of the fungi to the agents while *Candida albicans* was the most resistant fungus. No attempt was made to equalize the amount of inoculum used and this may be a factor in the difference in resistivity of the fungi. It is interesting to note that hemipyocyanine which was most active against yeasts was also the most active compound against fungi.

Although it is difficult to compare data of this type with that of other investigators because of differences in medium, strain of

† We are indebted to Dr. N. F. Conant, Medical School, Duke University, for strains of *Monilia albicans, Achorion schoenleinii,* and *Trichophyton gypseum,* and to Dr. R. Benham, Dept. of Bacteriology, Columbia College of Physicians and Surgeons, for the strain of *Microsporium.*

TABLE III.
Fungistatic Properties of Tyrothricin, Pyocyanine and Hemipyocyanine.

Dilution	*Achorion schoenleinii*	*Microsporium gypseum*	*Trichophyton gypseum*	*Candida albicans*
		Tyrothricin.		
1:2000	—	—	—	—
1:5000	—	—	—	—
1:10,000	—	—	—	++++
1:20,000	—	+	+	++++
1:40,000	+	++	+	++++
1:60,000	+	++	+	++++
1:80,000	++++	+++	+++	++++
1:100,000	++++	+++	+++	++++
Control	++++	++++	++++	++++
		Pyocyanine.*		
1:2000	—	—	—	++
1:5000	—	+	+	+++
1:10,000	++	++	++	+++
1:20,000	++	++	++	++++
1:40,000	++	+++	++++	++++
1:60,000	++++	+++	++++	++++
1:80,000	++++	++++	++++	++++
1:100,000	++++	++++	++++	++++
Control	++++	++++	++++	++++
		Hemipyocyanine.		
1:2000	—	—	—	—
1:5000	—	—	—	—
1:10,000	—	—	—	—
1:20,000	—	—	—	—
1:40,000	—	—	—	++
1:60,000	—	++	+	++++
1:80,000	++	++++	+	++++
1:100,000	+++	++++	++	++++
Control	++++	++++	++++	++++

*Pyocyanine perchlorate.
— No growth.
+ to ++++ Increasing amount of growth.

fungus, size of inoculum, etc., which greatly influence the results obtained, nevertheless it appears that the *in vitro* fungistatic potency of tyrothricin, and to a considerable extent of pyocyanine, is equal to or superior to that of such common disinfectants as mercuric chloride, phenol, cresol, salicylic acid, mercurochrome, etc. These inhibit the growth of various pathogenic fungi under similar conditions at dilutions generally less than 1:10,000.[9-10] The activity of hemipyocyanine is definitely superior to that of the commonly used disinfectants with the possible exceptions of crystal and gentian violet. All three antibiotic agents appear to be more effective than sulfanilamide, sulfapyridine, sulfathiazole and their sodium salts.[11]

Discussion. There has been some tendency to describe antagonistic substances derived from microörganisms as being sharply differential in their effectiveness towards Gram positive and Gram negative bacteria. Such a demarcation is a useful one in many instances. Actually, however, as our results indicate and as is evident in the literature on penicillin, streptothricin and actinomycin, we are dealing, in the case of each substance, with a "spectrum" of activity ranging from low to high values depending upon the particular bacterium or type of microörganism involved. The greatest contrast in sensitivity, based on Gram straining properties was obtained with tyrothricin since, without exception, the Gram positive bacteria were about 40 to 1000 times as susceptible to this agent as members of the Gram negative group. However, even here the differentiation is not an absolute one since the Gram negative bacteria were also

9 Schamberg, J. F., and Kolmer, J. A., *Arch. Dermat. Syphilol.*, 1922, **6**, 746.

10 Gomez-Vega, P., *Ibid.*, 1935, **32**, 49.

11 Lewis, G. M., and Hopper, M. E., *Ibid.*, 1941, **44**, 1101.

inhibited in the presence of a sufficiently high concentration of tyrothricin. On the contrary, there was little or no correlation between Gram staining ability and the bacteriostatic potency of the pyocyanines, hemipyocyanine and pyocyanase.

The inhibitory action of the antibiotic substances studied is not confined to bacteria but extends to other groups of microorganisms such as yeasts and fungi. High inhibitory activity towards one type of microorganism does not necessarily signify similar potency towards other types: hemipyocyanine was much more inhibitory for yeasts and fungi than for bacteria.

Summary. The inhibition of growth of bacteria, yeasts and pathogenic fungi by natural and synthetic pyocyanine, synthetic hemipyocyanine, and natural pyocyanase and tyrothricin has been investigated. Natural and synthetic pyocyanine have equal activities which surpass those of pyocyanase. Tyrothricin is particularly inhibitory for Gram positive bacteria, especially streptococci. The considerable fungistatic potency of tyrothricin and hemipyocyanine suggests that these substances may be of value in the treatment of fungus infections.

13859

Protein Content of Rabbits' Aqueous Humor Following Intravenous Injection of *E. coli* toxin.*

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It has been shown[1,2] that culture filtrates of Gram-negative microörganisms capable of producing the Shwartzman phenomenon[3] (such as meningococcus, coli, typhoid, Shiga, proteus, and choleræ toxins) produce in rabbits a primary reaction characterized by iridoconjunctival hyperemia, and, upon removal, coagulation of the aqueous humor. This reaction appears several minutes after intravenous administration of toxin and reaches a maximum intensity in a few hours. The ocular symptoms are bilateral, usually of equal intensity, and disappear in the ensuing 24 hr.

In view of the possible bearing of this reaction on the problem of uveitis and other intraocular conditions, such as epidemic dropsy, the influence of intravenous injection of *E. coli* toxin on the protein content of the aqueous humor of rabbits has been investigated.

Methods and Material. A strain of *E. coli communior,* freshly isolated from a case of purulent cholecystitis, was inoculated in nutrient agar slants, of pH 7.0. After 24 hr at 37°C, the growth of each slant was washed into 100 ml of nutrient broth, of pH 7.2. The 24 hr broth cultures, tested aerobically and anaerobically for contaminants, were centrifuged, and the supernatants filtered through Mandler candles No. 9. Filtrates, controlled for sterility, were stored at 4°C. Male albino rabbits, weighing about 2 kg, received *E. coli* toxin in saline dilutions ranging from 1:5 to 1:500 in volume of 1 ml per kg of body weight.

Rabbits' aqueous humor, placed into small stoppered tubes, was weighed. The presence or absence of coagulation was noted

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1 Ayo (Ajo), C., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 500.

2 Ayo, C., accepted for publication by the *Journal of Immunology.*

3 Shwartzman, G., *Phenomenon of Local Tissue Reactivity,* Paul B. Hoeber, N.Y., 1937.