purified testis hyaluronidase, or saline and supernatant fluid of heated normal semen containing hyaluronidase, or in saline and semen of vasectomized buck containing no hyaluronidase. Thirty-two does were inseminated at the same time with the same number of sperms collected from the same rabbit but suspended in saline, serving as parallel controls. It was found that it was the seminal fluid, not hyaluronidase, which really increased the fertilizing capacity of spermatozoa.

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Range of Antibiotic Activity of Protoanemonin.*†

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Extracts of buttercups and *Anemone pulsatilla* exert an inhibitory action on the growth of a number of pathogenic bacteria.[1,2] The active principle extracted from *A. pulsatilla* proved to be protoanemonin.[3] The present studies deal with the titration of the antibiotic activity of protoanemonin when tested against a variety of bacteria and fungi, as well as a few representatives among the viruses and protozoa.

Methods of testing the bacteria. The protoanemonin used in these experiments was extracted from dried ground *A. pulsatilla* or was prepared synthetically, as described previously.[3] The stock was a 1-100 dilution by volume in sterile distilled water from which further dilutions in the test media were prepared.

The *in vitro* susceptibility of the bacteria, with the exception of the Mycobacteria, was determined by inoculating 0.5 cc of a 10^{-3} dilution of a 24- or 48-hour culture of the test organisms into 4.5 cc of media containing decreasing concentrations of the antibiotic. The media chosen, as indicated in the tables, were those which provided favorable growth conditions for the bacteria under investigation. In some cases the organisms were tested when grown in each of 2 media. After a period of incubation at 37°C, sufficient for optimum growth of the control tubes, containing no protoanemonin, the cultures were examined for the presence of visible turbidity or other evidence of growth, such as the production of a pellicle, pigment or gas. The maximum dilution of protoanemonin capable of preventing the appearance of growth in the period of time specified is recorded in the tables.

Testing of the Mycobacteria required modifications in technique. A loopful of pellicle 1 cm in diameter from a 25-day-old culture was used to inoculate 100 cc volumes of

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1 Seegal, B. C., and Holden, M., *Science*, 1945, **101**, 413.

2 Carlson, H. J., Bissell, H. D., and Mueller, M. G., *J. Bact.*, 1946, **52**, 155.

3 Baer, H., Holden, M., and Seegal, B. C., *J. Biol. Chem.*, 1946, **162**, 65.

TABLE I.
The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-positive Aerobic and Anaerobic Bacteria.*

Organism	Dilution of protoanemonin × 1000	Media used	Time of incubation
Strep. hem. Group A (3 strains)	52-66	1% chicken serum meat infusion broth	Overnight (approx. 16 hr)
Strep. hem. Group D (2 strains)	16-20	" " " " " "	"
Strep. viridans (2 strains)	33-55	" " " " " "	"
D. pneumoniæ Types I, II, III, VII	55	" " " " " "	"
Staph. Oxford H	60-83	Meat infusion broth	"
Staph. Oxford H	100-150	Casein hydrolysate broth	"
Staph. albus	66	Meat infusion broth	"
M. lysodeikticus	44	" " "	"
C. diphtheriæ	75-100	0.5% glucose meat infusion broth	"
C. hoffmanni	75-100	" " " " "	"
C. xerosis	6-12	" " " " "	"
C. xerosis	16-30	Casein hydrolysate broth	"
My. tuberculosis hominis	166	Sauton's	1 month
My. tuberculosis hominis	100-330	Dubos'	4 days
My. tuberculosis bovis	100-250	"	"
My. tuberculosis avium	250-450	"	"
B. subtilis (contains spores)	20-50	Meat infusion broth	Overnight (approx. 16 hr)
B. anthracis (contains spores)	20-50	" " "	"
Cl. histolyticus	30-350	0.1% agar in 1% glucose meat infusion broth	48 hours
Cl. tetani	100-120	" " "	"
Cl. novyi	30-60	" " "	"
Cl. welchii	30-350	" " "	"
Cl. oedematiens	50-100	" " "	"
Cl. sporogenes	30-350	" " "	"

* All organisms were tested repeatedly. When results varied from day to day the extremes of variation are given.

Sauton's media containing the appropriate dilutions of protoanemonin. When Dubos'[4] medium was used 0.1 cc of a 5-day-old culture was the inoculum employed to seed the 5 cc of test medium.

All tests were repeated at least once and usually several times. If the concentration of protoanemonin causing inhibition of growth varied on different days the range of activity is indicated in the tables.

Results with bacteria. In Tables I and II it may be seen that all the bacteria tested possessed some degree of sensitivity to the antibiotic action of protoanemonin. However, the maximum inhibiting dilution varied widely. One strain of *Corynebacterium xerosis* required a 1-6000 dilution to prevent growth, while the Mycobacteria were inhibited by approximately one-thirtieth of this amount of protoanemonin. It is interesting also that many of the Gram-positive bacteria were somewhat less sensitive that the Gram-negative organisms. Those organisms tested in both broth and casein hydrolysate medium[3] showed greated sensitivity to protoanemonin when grown in the semi-synthetic medium.

Experiments were undertaken to determine whether the acidity of the medium, the size of the inoculum and the age of the culture might be contributing factors in the susceptibility of bacteria to protoanemonin. First, the possibility that the acidity of the medium might influence the inhibiting action of protoanemonin was investigated, using *Escherichia coli* as the test organism and hydrogen ion values ranging from pH 6.7 to pH 7.8. In neither meat infusion broth nor casein hydrolysate broth, with or without the addition of 1% glucose, was there a change in the end titer of the protoanemonin.

The size of the inoculum was investigated, using 4 organisms, *Staphylococcus oxford* H, *Corynebacterium xerosis, Escherichia coli* and

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE II.
The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-negative Aerobic Bacteria. All Tests Were Incubated Overnight (Approximately 16 Hours).*

Organism	Dilution of protoanemonin × 1000	Media used
K. pneumoniæ	40	1% chicken serum meat infusion broth
N. catarrhalis	166	Glucose broth
Ps. aerugenosa (3 strains)	30-100	Meat infusion broth
" " " "	40-200	Casein hydrolysate broth
Ser. marcescens	80-180	Meat infusion broth
V. choleræ	60-100	" " "
" "	200	Casein hydrolysate broth
P. vulgaris	80	Meat infusion broth
P. OX19	120	" " "
Alk. fecalis (2 strains)	60-80	" " "
Es. communis	50	" " "
Es. communior	50	" " "
" "	100	Casein hydrolysate broth
Eb. typhi "O" and "H"	250-330	Meat infusion broth
S. paratyphii	166	" " "
S. Schottmülleri	166	" " "
Sh. dysenteriæ Shiga	250	" " "
" " Flexner	166	" " "
" " Sonne	166	" " "

* See footnote Table I.

Vibrio cholerae. Sixteen-hour cultures, undiluted and diluted 10^{-1}, 10^{-3} and 10^{-7}, were added to casein hydrolysate broth and to meat infusion broth, both media containing varying amounts of the antibiotic. The findings indicated that the antibacterial activity was independent of the size of the inoculum when the inoculum was diluted 10^{-1}, 10^{-3} or 10^{-7}, however the undiluted inoculum was not consistently inhibited by similar dilutions of protoanemonin.

The effect of the age of the inocula upon the susceptibility of the same 4 organisms was determined by using 2-, 4-, 6-, 16-, and 48-hour cultures. Each culture was diluted until the turbidity approximated that of the 2-hour culture, and 0.5 cc served as the inoculum. The number of viable organisms was determined by pouring plates from each tube and counting colonies. The variation in number of viable organisms was well within the limits of what was found to be without effect on the outcome of the test. The results showed that the end titer of protoanemonin was the same irrespective of the age of the inoculum used.

Methods and results with fungi. Three yeasts, a non-pathogenic *Saccharomyces cerevisiae* and the pathogenic *Candida albicans* and *Cryptococcus neoformans,* were grown in 1% glucose broth for 2 to 5 days. Five-tenths cubic centimeters of a 10^{-3} dilution of the culture was added to 4.5 cc of the glucose broth containing varying amounts of the antibiotic. The dermatophytes or ringworm fungi were cultivated in honey broth for 10 days until a luxuriant mycelium was formed. The mycelium and spores were triturated in a mortar with sterile saline and one drop of the suspension added to the glucose broth tubes. In the case of *Coccidioides immitis,* broth was added to cover a honey agar slant culture and, after repeated pipettings to free the culture, 2 drops of the broth were added to each of the tubes of glucose broth containing the dilutions of protoanemonin. The last 3 fungi tested—*Allescheria boydii* (*Monosporium apiospermum*), an unidentified mold from a box of strawberries, and another from an old orange—were inoculated into glucose broth by simply touching the mycelial growth with a platinum loop and transferring it to the test media. When the growth in the control tubes was abundant, the amount of protoanemonin required to give inhibition of growth of the fungi was noted. The time of incubation varied with the different species (Table III).

These experiments show that protoanemonin inhibits the growth of fungi, which proved to be as sensitive as the bacteria. The inhibit-

TABLE III.
Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Fungi Grown in Glucose Broth.*

Organism	Dilution of protoanemonin, × 1000	Time of reading, days
Saccharomyces cerevisiæ	50-166	2
Candida albicans (2 strains)	100-200	2
Cryptococcus neoformans (4 strains)	83-300	2
Trichophyton mentagrophyles (gypseum)	125	5
Microsporium canis	125	5
Microsporium audouini	62-83	5
Trichophyton purpureum	83	5
Coccidioides immitis	125	2 ?
Allescheria boydii	166	5
Unidentified mold from strawberry plant	62	1
Unidentified mold from orange	62	2

* See footnote Table I.

ing concentration of protoanemonin varied from 1-50,000, in the case of *Saccharomyces cerevisiae,* to 1-300,000 for *Cryptococcus neoformans.*

Methods and results with protozoa. Tetrahymena geleii[5] and *Trypanosome gambiense* were two protozoa tested for their sensitivity to protoanemonin. Four-day cultures of *Tetrahymena,* grown in 2% bactopeptone or proteose peptone at room temperature (26°-28°C), were added in 0.5 cc amounts of 4.5 cc of peptone broth containing dilutions of protoanemonin varying from 1-200,000 to 1-500,000. In the tube containing 1-200,000 dilution of protoanemonin only a very occasional sluggish, round, small organism with many fine granules might be seen after one day, while in 2 days none were discernible. The 1-300,000 dilution produced a markedly modified growth but did not usually kill. At the end of 2 days the organisms were still small and far less active. Morphologically the stoma was hard to distinguish and the granules appeared smaller and more numerous than in the control organisms. The protozoa in the tubes containing protoanemonin diluted 1-400,000 and 1-500,000 showed increasingly less variation from the normal growth, which was distinguished by the appearance of many actively mobile organisms with several undergoing fission in each field.

The sensitivity of *Trypanosome gambiense* was investigated by inoculating 2 drops of citrated infected guinea pig blood into 1 or 2 cc amounts of Weinman's semi-solid cell free medium[6] containing different concentrations of protoanemonin. The cultures were incubated at room temperature (26°-28°C). The trypanosomes in the control tubes had grown in 5 days after inoculation. No trypanosomes were demonstrable in dilutions of protoanemonin 1-200,000 at the end of 5 days, whereas active organisms were present in the 1-400,000 dilutions which appeared similar to those organisms in the control tubes (Table IV).

In other tests the inoculum was a drop from a 9-day culture of *T. gambiense.* Growth of trypanosomes was absent in the 1-1,600,000 dilution after 5 days of observation.

Methods and results with bacteriophages. An investigation was undertaken to determine the effects of protoanemonin on coli and on staphylococcus bacteriophage. Two sets of tubes containing protoanemonin diluted 1-2000 in bacteriophage were prepared. One set was incubated at 37° C for 1½ hours, the other set remained at room temperature over night. Serial dilutions in broth from 10^{-1} to 10^{-9} were then seeded with their respective organisms. In the controls distilled water was substituted for protoanemonin. The bacteriophage titer was the same in all the tests, the ones in which the bacteriophages were first incubated with the antibiotic and those in which distilled water was substituted for pro-

[5] Furgason, W. H., *Arch. Protistenkunde,* 1940, **94**, 224.

[6] Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 82.

TABLE IV.
Inhibition of Growth of Two Protozoa by Varying Dilutions of Protoanemonin.

Organism	Dilution of Protoanemonin 200,000	300,000	400,000	500,000	1,600,000	3,200,000	Controls	Media used	Time of reading, days
Tetrahymena geleii (3 strains)	0	+	+++	+++			+++++	2% protease peptone or bactopeptone	2
Tryp. gambiense guinea pig blood	0		++++				+++++	Weinman's	5
Tryp. gambiense culture					0	++++	+++++	"	5

toanemonin. The experiments, thus, failed to show any inhibitory effect of protoanemonin on either coli or staphylococcus bacteriophage.

Methods and results of testing influenza virus grown in chick embryos. In order to study the action of protoanemonin on the growth of influenza virus in fertile eggs, it was necessary, first, to determine the amount of protoanemonin which could be tolerated by the chick embryo. The tests showed that 0.2 cc of a 1-1000, 1-2000 or 1-4000 dilution of protoanemonin was not toxic when injected 2 days in succession in 11- or 12-day-old fertile eggs and the latter 2 concentrations were harmless to 10-day-old eggs.

The treatment of influenza infected eggs with protoanemonin in dilutions non-injurious to the chick embryo then was attempted. The combination of 1-2000 dilution of protoanemonin and 10^{-5} or 10^{-6} dilution of influenza virus killed the embryo, while the same dilution of protoanemonin with 10^{-7} dilution of virus resulted in a viable embryo with a concentration of virus comparable to that in the control eggs. The growth and titer of the virus in the allantoic fluid was determined by an Hirst[7] agglutination test. The eggs treated with 1-4000 protoanemonin and infected with the same dilutions of virus survived, and it was evident that there was a multiplication of the virus as demonstrated by the agglutination titer. These experiments would indicate that a combination of protoanemonin, non-toxic by itself, and influenza virus in adequate concentration is lethal to the embryo. Furthermore, where conditions are such that the embryo survives there is no evidence of inhibition by protoanemonin of the growth of the virus.

Tissue culture tests. The effect of protoanemonin on tissue cells was tested through the courtesy of Dr. Mary Parshley. Whole thicknesses of chicken skin were planted in chicken plasma diluted one-third with two solutions, one of which was optimal for fibroblasts and the other optimal for epithelial cells. These two solutions contained protoanemonin in concentrations of 1-1 million and 1-5 million. When compared with the

[7] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

growth of the controls, it appeared that protoanemonin was toxic to both fibroblasts and epithelial cells in 1-1 million dilution and inhibitory to growth in the 1-5 million dilution. However, it seemed to be less inhibitory for both cells when they were cultivated in the solution which favored the growth of epithelial cells.

Discussion. Protoanemonin is of interest because of its wide range of activity. *In vitro* it inhibits the growth not only of Gram-positive and Gram-negative bacteria but also fungi, two protozoa and fowl epithelial and fibroblastic tissue cells.

The action of protoanemonin on bacteria, fungi and the tetrahymena involves two types of effect, an inhibition of the growth of the microorganisms and an actual killing of the organisms. Only the former effect has been considered in the data presented here. In most cases subcultures from the tubes containing the greatest dilution of protoanemonin inhibiting growth, as reported in the tables, would demonstrate the presence of viable organisms. Indeed, this frequently might be demonstrated by the simple procedure of further incubation of the original tubes. Greater concentrations of the protoanemonin, however, actually kill the organisms. This phase of the action of protoanemonin will be described at a later time.

Another factor under investigation which may influence the action of protoanemonin is the composition of the medium. It may be observed that the bacteria tested for sensitivity to protoanemonin in both meat infusion broth and casein hydrolysate broth were inhibited by greater dilutions of protoanemonin in the latter medium. This influence of medium on the sensitivity of an organism to protoanemonin was apparently unrelated to the relative growth of the organisms in the two broths. *Staphylococcus oxford* H. *Vibrio cholerae* and *Pseudomonas aeruginosa* grew better in the infusion broth, while *Escherichia coli* and *Corynebacterium xerosis* grew as well or better in the casein hydrolysate broth. The casein hydrolysate medium was employed because it is apparent, as seen in the tables, that the greatest dilution of protoanemonin causing inhibition of growth of a given organism sometimes varied considerably in different tests. It was thought that the introduction of a semi-synthetic medium, more uniform in its composition than meat infusion broth, might prevent this fluctuation. It has continued, however, even when casein hydrolysate broth was used. The explanation for this is not at present available.

Certain mammalian bloods, when added to culture media, cause a decrease in sensitivity to protoanemonin. For example, guinea pig blood is highly inhibitory. This may contribute to the difference in the titer of activity of protoanemonin against *Trypanosome gambiense* in tubes inoculated with infected guinea pig blood and those inoculated from culture media. There also is the possibility that the diverse forms of trypanosomes found under the two conditions of growth contribute to the variation in sensitivity.

The method of testing the sensitivity of the bacteriophages was such that protoanemonin was in contact only with the resting bacteriophage. Experiments to be reported indicate that protoanemonin is relatively harmless to resting bacteria and is antagonistic mainly during the period of active growth. It is therefore possible that the method was not suitable to indicate the effect of this agent on bacteriophage. The antibiotic nature of protoanemonin makes it difficult to evaluate when the organism tested requires a viable and actively growing substrate. Thus the failure to demonstrate inhibition of the influenza virus may have been due to the fact that such small amounts of protoanemonin were tolerated by the chick embryo that the effective concentration of protoanemonin for the virus was not achieved.

Summary. 1. Protoanemonin inhibited the growth of all the aerobic and anaerobic bacteria, the fungi and the protozoa tested. 2. The maximum dilution of protoanemonin which was effective against the bacteria and fungi varied from 1-6000 to 1-300,000. 3. The anti-bacterial activity of protoanemonin was independent of the acidity of the medium, the size of the inoculum and the age of the culture, within the limits tested. 4. The two

protozoa were prevented from growing in dilutions of protoanemonin ranging from 1-200,000 to 1-1,600,000. 5. No inhibition by protoanemonin of the growth of the two bacteriophages and the influenza virus was demonstrable by the techniques employed. 6. A dilution of 1-1,000,000 protoanemonin was toxic for chicken tissue culture epithelial and fibroblastic cells.

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Determination of O_2 Capacity on 39.3 Cubic Millimeters of Blood.

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In a recent study of bone marrow blood gases[1] in which the use of minimal sample volumes was imperative, estimation of O_2 capacity was necessary. The current procedure used for such determinations involves aeration of blood after which the O_2 content is determined. This technique has been used by Roughton and Scholander[2] and by Lilienthal and Riley.[3] Preliminary aeration was carried out in a separate vessel with 0.5 or more cc of blood although only 39.3 cmm were used for the O_2 determination. When blood is aerated in a flask or syringe, a certain amount of plasma is lost by evaporation as well as by adherence to the sides of the vessel. Should quantities of blood smaller than 0.5 cc be used, concentration of the red cells becomes greater. To avoid the errors inherent in separate aeration and to reduce the amount of blood required, the micro method of Roughton and Scholander has been modified so that the O_2 capacity can be determined on a total blood sample of only 39.3 cmm.

Apparatus. The Roughton-Scholander syringe and pipette (Roughton and Scholander[2]) are employed. A second mark (designated "upper mark") is scratched on the syringe cup at a distance above the existing one such that the volume of the cup as measured between the two marks is approximately 25 cmm. Since the diameter of most cups is 2.5 mm, the upper mark may be made 5 mm above the first. In the estimation of O_2 capacities exceeding 16 vol % the syringe with the 50 unit capillary is necessary.

Reagents. In addition to those listed by Roughton and Scholander 0.9% NaCl is required.

Sampling blood. From a finger prick or a needle inserted in a vessel, blood is sucked directly into the 39.3 cmm pipette which has been previously flushed with anti-coagulant solution (heparin) and dried in a current of air.

Procedure. 1. The syringe is flushed 3 times with separate portions of saline, emptied, and the cup filled to the lower mark with saline.

2. The pipette is filled to its mark with blood; its tip is passed carefully into the cup containing saline and pressed firmly against the bottom. 3. Blood is sucked into the syringe by a smooth withdrawal of the plunger. Once the pipette is emptied it is quickly removed and the saline in the cup is drawn in after the blood.

4. The blood-saline mixture is lowered far enough into the syringe to permit entry of an air volume of approximately 1.0 cc.

5. The syringe is then rotated for 5 minutes on its long axis with an occasional rotation at right angles to spread a small layer of blood over the inside of the syringe and thus ensure

1 Grant, W. C., and Root, W. S., *Fed. Proc.*, 1947, **6**, 114.

2 Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, **148**, 541.

3 Lilienthal, J. L., Jr., and Riley, R. L., *J. Clin. Invest.*, 1944, **23**, 904.