TITLE GROWTH CURVES OF SALMONELLA PULLORUM IN DIFFERENT MEDIA AND SOME OBSERVATIONS ON THE IN VITRO ACTION OF NEOMYCIN

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INTRODUCTION

On more than 85 per cent of the farms in the United States poultry is a partial, if not the chief, source of income (Tucker, 1950); it is used more by the food consumer than it has been in past years. As a result, the control of diseases of poultry is important economically to the farmer, and hygienically to the consumer. Among poultry diseases, according to Simms (1950), three of the more important are: Newcastle disease or pneumoencephalitis, pullorum disease, and avian leucosis. Although pullorum disease is losing ground among our poultry flocks, it is still widespread and potentially very destructive. This dissertation is concerned with the laboratory study of Salmonella pullorum, the organism which causes pullorum disease.

Constantly being searched for is a chemotherapeutic agent which is destructive to the pathogen and harmless to the host. Preliminary investigations by Waksman, Frankel, and Graessle (1949), and Waksman and Lechevalier (1949), have indicated that a new antibiotic, neomycin, is more potent in vivo against S. pullorum than streptomycin, and has little or no toxicity for animals.

It is recognized that there are a number of factors, such as life phase, size of inoculum, hydrogen ion concentration, interfering substances, etc., which may or may not modify the antibacterial action of an antibiotic in vitro. A knowledge of these factors is of great importance in laboratory studies and in the clinical treatment of

infections. Although the same general factors apply to the considerations of the activity of any antibiotic, the relative importance of a given factor may vary from one antibiotic to the next. A review of the literature indicates that there is need for a study in which data are collected about these factors in relation to neomycin, using S. pullorum as the test organism.

Purpose of the study. The purpose of this study was two-fold: first, to determine the bacterial culture cycle of five strains of S. pullorum; second, to study some of the factors which may or may not influence the antibacterial action of neomycin.

The bacterial culture cycle was compared when the organisms were grown in three different culture media; nutrient broth, 1 per cent tryptose-water, and a synthetic medium. The complete culture cycle was not studied, but it was limited to the initial stationary phase, lag phase, logarithmic phase, and part of the stationary phase.

The factors studied that may possibly influence the antibacterial action of neomycin were: the sensitivity of five strains of S. pullorum which had been grown in three different culture media, the cultural or life phase, temperature and time of incubation, size of inoculum, pH of the medium, sodium chloride, and some of the constituents of two culture media.

REVIEW OF LITERATURE

As stated in the problem, one of the factors which is capable of influencing the action of an antibiotic is the cultural or life phase of the test organism. When the literature was reviewed for information about the culture cycle of S. pullorum, little data were found. The only reports were those of Huntington and Winslow (1937), and Mooney and Winslow (1935), who studied the culture cycle of S. pullorum in three different media, i.e., peptone water, peptone-glucose water, and peptone-lactose water. The media chosen for this study were: nutrient broth, 1 per cent tryptose-water, and a synthetic medium designed for the strains being used; therefore, the work that had been done with S. pullorum was useful only as a guide in technique.

The latter medium, a synthetic one, might serve as a useful means of attempting to study the mode of action of an antibiotic. The knowledge of such a medium for S. pullorum is restricted. Davis and Solowey (1950) found that the one strain of S. pullorum with which they were working would not grow in any of the synthetic media used by them. A simple synthetic medium containing glucose, salts, and asparagine, composed by Gray and Tatum (1944), supported the growth of two strains of S. pullorum with which Lederberg (1947) worked when he added the amino acids leucine and cystine for the one strain; and leucine, cystine, and methionine for the other strain. In an extensive study of the nutritional requirements of S. pullorum by Johnson and Rettger (1943), no

attempt was made to devise a simple synthetic medium. They reported that only 2 of the 45 strains studied required a vitamin supplement; that 8 of these strains grew without glucose; that 3 would not grow in the test media used; and that there were definite amino acid requirements, but that these varied with strains. Of interest is the fact that none of the 45 strains studied by these men required tryptophane, and that leucine and aspartic acid were taken, on the whole, as the most important amino acids for 8 of 10 strains for which the amino acid requirements were more thoroughly investigated. Beard and Snow (1936) were able to culture a strain of S. pullorum on Sahyun's medium #12 (Sahyun, Beard, Schultz, Snow, and Cross, 1936), when a supplement of 20 amino acids, asparagine, and creatine were added. Koser's medium (Koser, 1923) with the addition of the salts of lactic, succinic, fumaric, and citric acids would not support the growth of three strains of S. pullorum, according to Hajna (1935). When Weldin and Miller (1932) investigated the utilization of citric acid and sodium citrate by a number of strains of S. pullorum in Koser's synthetic medium, and in Simmons agar medium (Simmons, 1926), they found that the majority of the strains would grow, but that growth was variable.

The foregoing literature covers the research that has been done in relation to part one of the problem; namely, culture cycle and synthetic media. Literature pertinent to part two (factors that may or may not affect the antibacterial action of neomycin) follows.

Neomycin was first described by Waksman and Lechevalier (1949) who isolated it from Streptomyces fradiae. Although neomycin has been extracted from the same genus of actinomycetes as streptomycin,

streptothricin, aureomycin, chloromycetin, and other less well-known antibiotics, it has been defined chemically and biologically as a different antibiotic. Neomycin is a generic term for a "neomycin complex" of antibiotics from which Neomycin A has been isolated, according to Peck, Hoffhine, Jr., Gale, and Folkers (1949).

Waksman and Lechevalier (1949), Waksman, Lechevalier, and Harris (1949), and Swart, Waksman, and Hutchinson (1949) characterized neomycin chemically as a basic compound; soluble in water, slightly soluble in methanol, insoluble in other organic solvents; thermostable in neutral solution and at pH 2.0; and negative to Sagaguchi test for arginine.

A large variety of organisms have been tested in vitro and in vivo with neomycin by Hobby, Lenert, and Dougherty (1949) and Felsenfeld, Volini, Ishihara, Bachman, and Young (1950). Neither of these papers specifically mentioned S. pullorum. Waksman, Frankel, and Graessle (1949) and Waksman and Lechevalier (1949) pointed out that neomycin was far more effective than streptomycin in suppressing the infection of chick embryos with S. pullorum.

Neomycin is now being produced in a homogeneous state, and, as a result, experimentation with human administration is beginning. Duncan, Clancey, Walgamot, and Beidleman (1951) concluded that neomycin is an effective agent in the treatment of human infections, particularly those of the urinary tract, but Waisbren and Spink (1950b) found that neomycin was not always successful in eradicating Pseudomonas aeruginosa from the urinary tract. Oral administration of neomycin to people who were subsequently subjected to operations on the colon has indicated its value in the prevention of post-operative peritonitis or fecal fistulae,

according to Path, Fromm, Wise, and Hsiang (1950).

In a study of the bactericidal activity of neomycin against Escherichia coli, Waisbren and Spink (1950a) reported that 20 µg per ml. is sufficient to kill in 2 hours all the organisms of a 2×10^6 inoculum from an 18 hour culture suspended in saline. Waksman, Frankel, and Graessle (1949) showed that 20 units of neomycin, which is rather crude and has an assay of 30-100 units per mg., incubated with a heavy suspension of S. pullorum was completely bactericidal in 3½ hours.

Using E. coli and beta streptococcus, Worth, Chandler, and Bliss (1950) studied the effect of size of inoculum on the action of neomycin, and found that the bactericidal concentrations of neomycin are affected by this factor. They concluded that the necessary increase in bactericidal concentration of neomycin with increase in inoculum is of the same order of magnitude as that for aureomycin, chloromycetin, and penicillin.

The antibacterial activity of neomycin is favored by a mild alkaline reaction of the medium. The best activity occurs at pH 7.0 - 8.0 according to Waksman, Lechevalier, and Harris (1949).

Cysteine had no marked effect upon the activity of neomycin in the experimental procedure used by Waksman, Lechevalier, and Harris (1949). On the other hand, they reported that the presence of glucose in the test medium reduces the potency of the antibiotic by favoring either acid production or growth of the test organism. Likewise, the potency is reduced by oleic acid (Waksman, Katz, and Lechevalier, 1950).

Although a review of the work concerning the development of resistant strains, synergisms, and toxicity is beyond the scope of this study, it is included because this antibiotic is relatively new and these are subjects of importance to those interested in antibiosis.

Waisbren and Spink (1950a), Waksman, Katz, and Lechevalier (1950), Demerec and Demerec (1950), and Waksman and Lechevalier (1949) all came to the same general conclusion - that development of resistance to neomycin occurs slowly. It resembles the "penicillin" rather than the "streptomycin" type of drug resistance. That is, resistance to neomycin is a step-wise process of development. It is of interest that streptomycin-resistant, streptomycin-sensitive and streptomycin-dependent strains are all sensitive to neomycin; whereas neomycin-resistant strains are slightly more resistant to streptomycin than the original sensitive strain (Demerec and Demerec, 1950). These authors also pointed out that strains resistant to chloromycetin are sensitive to neomycin, and visa versa. Hobby, Lenert, and Dougherty (1949), and Waksman, Lechevalier, and Harris (1949) noted that neomycin is active against streptomycin-sensitive and streptomycin-resistant tubercle bacilli. Neomycin has been used successfully by Duncan, Clancy, Walgamot, and Beidleman (1951) in the treatment of human infections where organisms were completely or moderately resistant to penicillin, aureomycin, chloramphenicol, and streptomycin.

A possible synergistic action of neomycin and streptomycin in the treatment of ten-day-old chick embryos infected with S. pullorum was substantiated by Waksman, Frankel, and Graessle (1949). Having treated

the infected chick embryos with different concentrations of streptomycin only, neomycin only, as well as with a mixture of streptomycin and neomycin, they found that 100 µg of streptomycin had no protective effect; 25 units of neomycin gave 30 per cent protection; 50 units of neomycin gave 70 per cent protection; and a combination of 50 units of streptomycin and 25 units of neomycin raised the protective effect of the latter to 60 per cent, thus pointing to the potential synergistic action of the two antibiotics.

The original work with crude neomycin by Waksman and Lechevalier (1949) led these men to believe that it has either no toxicity or limited toxicity for animals. This was verified by Rake (1949), Hobby, Lenert, and Dougherty (1949), Waksman, Frankel, and Graessle (1949), Spencer, Payne, and Schultz (1950), Path, Fromm, Wise, and Hsiang (1950), and Felsenfeld, Volini, Ishihara, Bachman, and Young (1950); but Waisbren and Spink (1950b) noticed definite nephrotoxic and ototoxic reactions of human beings treated with neomycin.

MATERIALS AND METHODS

Cultures. Three of the 5 strains of S. pullorum used were isolated in 1950 from poultry by the Poultry Pathologist, Department of Bacteriology and Public Health, Michigan State College; number 12 came from a 2-month-old white leghorn, number 13 came from a 4-week-old turkey, and number 17 came from an adult turkey. It should be pointed out that number 17 has produced only acid in dextrose and mannitol since the original isolation; nevertheless, it has been confirmed as being S. pullorum at the Coordinating Bacteriology Section, Michigan Department of Health Laboratory, Lansing. Strains number 11 and 29 (original number 296X) were kindly supplied by R. Gwatkin.* It is interesting to note that number 29 is known as a variant which has the same biochemical reactions in the routine tests as a regular strain but a different antigenic structure. For further information about these strains the reader is referred to Gwatkin (1945) and Younie (1941).

Culture media. All the media were used at pH 7.0 and sterilized by autoclaving for 20 minutes at 15 pounds pressure except where otherwise specified.

Nutrient broth was composed of Difco beef extract, 3 gm.; Difco peptone, 5 gm.; and distilled water, 1 liter.

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*Address: Division of Animal Pathology, Science Service, Dominion Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec, Canada.

Tryptose-water was made up of Difco tryptose, 10 gm.; and distilled water, 1 liter.

The synthetic medium desired was to be as simple as possible but of such a composition that 2.5 ml. would support visible growth after 24 hours incubation at 37°C. when an inoculum of approximately twenty-five thousand organisms was used.

The glucose-asparagine-mineral base of the synthetic medium was of the same composition and concentration as the one utilized by Gray and Tatum (1944). Part of the mineral base was a trace element solution reported by Horowitz and Beadle (1943).

It was necessary to determine what specific amino acids to use; therefore, a synthetic medium was made with the glucose-mineral base (note that asparagine was omitted) and 17 amino acids. (See Appendix, page 89, for their source). The amino acids were: DL-alpha alanine, L(+) arginine monohydrochloride, DL-aspartic acid, L(-) cystine, glycine, L(+) histidine monohydrochloride, L(-) leucine, L(+) lysine monohydrochloride, DL-methionine, DL-phenylalanine, L(-) proline, L(+) glutamic acid, DL-serine, DL-threonine, DL-tryptophane, L(-) tyrosine, and DL-valine. They were used in the concentrations stated by Johnson and Rettger (1943). Threonine, which was not used by these workers, was added to give a M/4,000 concentration. This medium supported luxuriant growth of all strains. One amino acid at a time was then omitted from this synthetic medium. Each medium thus composed was inoculated with approximately twenty-five thousand organisms, incubated at 37°C. for 24 hours, and observed for reduction in visible growth or no growth. If either of these conditions occurred, the amino acid omitted was considered to be of probable nutritional value. The effect on growth was then noted when the organisms were grown in the glucose-mineral base with these acids added

singly and in different combinations.

Whether vitamins and other organic nutrients would hasten the growth of S. pullorum was then tested. A medium composed of cystine, methionine, leucine, glucose, asparagine, and minerals in the concentrations stated above, was prepared. All the strains would grow in this medium, but slowly. Four different mixtures of vitamins and other organic nutrients were employed initially for screening purposes. The four different mixtures screened, and the quantity of each substance used per liter of medium were: (1) thiamine hydrochloride, 1.0 mg.; riboflavin, 0.5 mg.; pyridoxine hydrochloride, 0.5 mg.; calcium pantothenate, 2.0 mg.; (2) para-aminobenzoic acid, 0.5 mg.; nicotinamide, 2.0 mg.; i-inositol, 4.0 mg.; pimelic acid, 4.0 mg.; (3) choline hydrochloride, 2.0 mg.; nucleic acid, 5.0 mg.; folic acid, 0.106 µg; biotin 5.0 µg; (4) all the vitamins and other organic nutrients of the other three mixtures. The source of these substances is given in the Appendix, page 89. The substances of any mixture which had appeared to accelerate the growth were then tested individually.

The final composition of the synthetic medium was: NH_4Cl, 5.0 gm.; NH_4NO_3, 1.0 gm.; Na_2SO_4, 2.0 gm.; $MgSO_4 \cdot 7\ H_2O$, 0.1 gm.; $CaCl_2$, trace; K_2HPO_4, 3.0 gm.; KH_2PO_4, 1.0 gm.; trace element solution, 1 ml.; (salts of boron, 0.01 mg.; molybdenum, 0.02 mg.; iron, 0.2 mg.; copper, 0.1 mg.; manganese, 0.02 mg.; zinc, 2.0 mg.; and distilled water, 1 liter) glucose, 10.0 gm.; asparagine, 1.5 gm.; L(-) cystine, 60 mg.; DL-valine, 78 mg.; L(+) arginine monohydrochloride, 43 mg.; L(+) histidine monohydrochloride, 39 mg.; thiamine hydrochloride, 1.0 mg.; calcium pantothenate, 2.0 mg.; and distilled water, 1 liter.

This medium was prepared as follows. The inorganic substances

were added to 750 ml. of distilled water and autoclaved. Each of the organic components then was added aseptically from the stock solutions described below to give the proper concentrations. The pH was adjusted to 7.0 with 0.2N NaOH and the volume brought up to one liter with sterile distilled water.

One and one-half grams of asparagine was dissolved in 30 ml. of 0.2N HCl; sixty mg. of cystine was added to 10 ml. of 0.2N HCl. They were filter sterilized with a frittered-glass bacteriological filter. A solution of 87 mg. of leucine in 10 ml. of 0.2N HCl was made and autoclaved for 20 minutes at 12 pounds pressure. Ten grams of glucose was dissolved in 20 ml. of distilled water. Valine, arginine, and histidine, in the amounts given above, were dissolved separately in 10 ml. of distilled water. Stock solutions of thiamine hydrochloride, 0.2 mg. per ml., and calcium pantothenate, 0.4 mg. per ml., were made separately in distilled water. Glucose, valine, arginine, histidine, thiamine hydrochloride, and calcium pantothenate were autoclaved for 20 minutes at 12 pounds pressure.

Growth studies. The stock cultures were maintained on tryptose agar slants in the refrigerator at 4°C. and transferred to fresh slants once a month. At least 4 days before the culture cycle was determined, individual tubes containing approximately 10 ml. of the medium being studied, i.e., nutrient broth, 1 per cent tryptose-water, or the synthetic medium, were inoculated with organisms from the stock cultures; and a transfer was made to fresh medium every 24 hours. At the beginning of the growth curve determination, or zero time, a 250 ml. Erlenmeyer

flask containing 100 ml. of medium at the incubation temperature, 37°C., was inoculated with approximately ten thousand organisms that were taken from a 24 hour culture. The actual size of the inoculum was determined with duplicate pour-plates. The inoculated medium was incubated at 37°C.; at 3 hour intervals a sample was taken, diluted properly, if necessary, in physiological saline dilution blanks; and pour-plates were made in duplicate. This procedure was repeated until the organisms reached the stationary phase of growth. At each 3 hour interval, except when the sample was actually being taken, the cultures were maintained at a constant temperature of 37°C. Before a sample was taken from the culture medium, it was mixed by alternating clockwise and counter-clockwise circular motions for one minute. The dilution bottles were shaken 25 times through a two foot arc. With the aid of a Quebec Colony Counter, colonies on the dilution plates were counted approximately 48 hours after being made.

Another procedure that was tried with 1 per cent tryptose-water and nutrient broth was to inoculate several tubes containing exactly 10 ml. of medium at zero time. Each tube was inoculated with approximately one thousand organisms from the same 24 hour culture which had been previously transferred three times. At each 3 hour interval a different one of these tubes was used to determine the number of organisms present. The rest of the procedure was the same as stated above.

Neomycin.* A solution containing 1 mg. of the salt of neomycin,

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*Neomycin used in this study was supplied through the courtesy of The Upjohn Co., Kalamazoo, Michigan.

neomycin sulfate, per ml. of solvent was prepared fresh each time that it was used. The solvent was either the medium in which the tests were made or sterile physiological saline. The results with neomycin are reported in micrograms in this study. It had been assayed by the Bacillus subtilis plate method and the Klebsiella pneumoniae turbidimetric methods and found to contain 200 Waksman units per milligram. Paper chromatography, using several solvent systems, had showed that this material is homogeneous.

Sensitivity to neomycin. The sensitivity of S. pullorum to neomycin was determined by the tube dilution method. Five-tenths ml. of a 1:100 dilution of a 24 hour culture was added to 10 X 75 mm. rimless culture tubes containing 0.5 ml. of two-fold serial dilutions of neomycin. The solvent for the antibiotic, the diluent for the bacterial suspension, and the diluent for the serial dilutions were the same, and were varied with the medium in which the organisms had been growing. That is, if the sensitivity of the organisms grown in 1 per cent tryptose-water was being checked, then 1 per cent tryptose-water was used as the solvent for the antibiotic, and as the diluent for both the bacterial suspension and the serial dilution. A control tube contained 0.5 ml. of the test medium and 0.5 ml. of the 1:100 dilution of the 24 hour culture. The tubes were incubated at 37°C. and observed macroscopically at 24 hour intervals for 72 hours and, again, after one week to see whether growth had occurred.

Factors that may or may not influence antibacterial action of neomycin. The cultures for all of the tests that are described under this

heading were grown in 1 per cent tryptose-water.

In order to study the effect of pH on the activity of neomycin, 5 lots of 1 per cent tryptose-water with different pH values, pH 5, 6, 7, 8, and 9, were prepared by adding 1N NaOH or 1N HCl to the medium. The pH values were limited to a range of pH 5 to 9 because it had been shown by Chang, Kuan-how and Stafseth (1950) that S. pullorum would not grow at pH values beyond these extremes. The measurement of the pH value was made with a Beckman pH meter. The same procedure as stated in the description of the sensitivity tests was followed with these media.

The effect of some of the constituents of the different media and sodium chloride was determined with two sets of serial dilutions of neomycin. One set was made with 1 per cent tryptose-water to which had been added a quantity of a constituent of a medium to give the same final concentration as that of this particular constituent in its regular medium. When sodium chloride was tested, a quantity of it sufficient to give a final concentration of 0.85 per cent was added to 1 per cent tryptose-water. The other set of the serial dilutions of neomycin was made with 1 per cent tryptose-water itself. This was the control set. In addition, a control tube, as stated in the description of the sensitivity test, was included. An inoculum of 0.5 ml. of a 1:100 dilution of a 24 hour culture was added to each tube. The tubes were incubated at 37°C. and observed macroscopically at 24 hour intervals for 72 hours and, again, after one week to see whether growth had occurred.

In the experiments to test the effect of temperature, size of inoculum, and growth phase on the antibacterial activity of neomycin, the size of the sample taken from the experimental tubes to determine the

number of organisms present was limited to 0.1 ml. because of the lack of knowledge of a neutralizer for neomycin. It was assumed that 15 to 20 ml. of tryptose agar, used to make a pour-plate, would dilute the neomycin removed with 0.1 ml. of a sample to a point below its bactericidal concentration. To check this assumption two samples were taken at the same time. One was plated out directly and the other one was washed and centrifuged three times with saline before plating. It was found that when plates made with a direct inoculum were free of growth so were those made with the washed inoculum. Also, at the same time that a sample was taken for a pour-plate, another one was taken and inoculated into 20 ml. of 1 per cent tryptose-water. It was found that when plates made with a direct inoculum were free of growth so were the tubes of 1 per cent tryptose-water, except on rare occasions.

To test whether temperature would influence the action of neomycin, undiluted 12 hour cultures were divided into 6 equal portions of 10 ml. each. To each of 3 samples, 0.1 ml. of a solution of neomycin was added, giving a final concentration of 0.5 μg. per ml. To each of the remaining 3, which were the control tubes, 0.1 ml. of 1 per cent tryptose-water was added. One tube from each set was incubated at 4°, 20°, and 37°C. It should be noted that before neomycin or 1 per cent tryptose-water was added to the cultures they were either cooled or heated to the temperature at which they were to be incubated. The number of organisms per ml. was determined at the beginning and at the end of 18 hours' incubation by a colony count of pour-plates.

To observe the effect of the size of inoculum, 6 different sizes of inocula with 3 different concentrations of neomycin were employed.

One and 0.1 ml. quantities of an undiluted 24 hour culture; and 1 and 0.1 ml. quantities of a 1-100, and 1-10,000 dilution of a 24 hour culture were used for the inoculum. The final concentration of organisms per ml. of medium was of a range of approximately 3.5×10^7 to 3.5×10^2 with a 10-fold increment. Four sets of 6 tubes were prepared in this manner. Neomycin was added to three sets of 6 tubes in the amount of 0.5, 5.0, and 50.0 µg respectively, giving a final concentration of 0.0495, 0.495, and 4.95 µg. per ml., and the cultures were incubated at 37°C. For the control, a set of 6 tubes without neomycin were incubated simultaneously. The number of organisms per ml. at 0, 30, 60, 120, and 240 minutes was ascertained by colony counts in pour-plates.

To find out whether the action of neomycin was affected by the phase of growth, organisms were tested in the logarithmic and stationary phases. Nine tubes, each containing 9.9 ml. of 1 per cent tryptose-water, were inoculated with approximately a thousand organisms from a 24 hour culture and incubated at 37°C. This procedure was repeated for each strain. After 12 hours (logarithmic phase), the number of organisms in 5 of the 9 tubes was determined by taking a sample from each one and making pour-plates. Then, to 2 of the 5 tubes enough neomycin was added to give a final concentration of 1.09 µg. per ml., and to the other 2 a quantity of it was added to give a final concentration of 3.2 µg. per ml. No neomycin was added to tube 5, which was the control. These 5 tubes were incubated for 18 hours and then the number of organisms per ml. was determined again. After 30 hours (stationary phase), the number of organisms in the other 4 tubes of the set of 9, plus tube

5 which was the control, was determined, and neomycin added as above. These 5 tubes were incubated for 18 hours and then the number of organisms per ml. was determined again. All plates were counted approximately 48 hours after inoculation and incubation. The data were handled by computing the percentage of organisms that was killed with different concentrations of neomycin in each phase of growth.

RESULTS AND DISCUSSION

Growth studies. The results of the growth studies that are described first were accomplished by taking samples from the same culture of 1 per cent tryptose-water or nutrient broth for a period of at least 24 hours and, in the case of the synthetic medium, for a period of 60 hours.

Washing the inoculum and adapting organisms to a culture medium are two of several things which can influence the bacterial culture cycle. Chesney (1916) made the interesting observation that an inoculum of washed cells showed a greater lag than one of unwashed cells. Adapting bacteria to enhance reactions, normally but slowly performed, or to grow in media previously insufficient to support growth has been reported by many workers (Knight, 1936; Dubos, 1940; and Rahn, 1938). Graham-Smith (1920) demonstrated that the initial stationary phase and lag phase were shorter when the inoculum was from bacteria which had been frequently subcultured than from those in which few previous subcultures had been made.

In this study 5 strains of S. pullorum were subcultured for at least 4 days in the culture medium being tested prior to the growth determination. Strain 11 was subcultured for at least 6 days when grown in the synthetic medium and each subculture was inoculated with two loopfuls rather than one. The inoculum was not washed but simply diluted with physiological saline to give the desired quantity of organisms. This procedure was used in order to abolish the initial

stationary phase and to reduce the lag phase time.

The results of the growth determinations are given in tables I, III, V, (pages 22, 25, 28) and in figures 1, 2, 3 (pages 23, 26, 29). All strains, except 11, had a lag phase of approximately 3 hours when they were grown in 1 per cent tryptose-water. Strain 11 remained in this phase for 3 to 6 hours. A lag phase of 3 hours occurred for all strains when they were grown in nutrient broth. Three of the 5 strains had a noticeable lag phase when grown in the synthetic medium. Strains 13 and 29 were in this phase for about 6 hours, whereas strain 12 was in it for about 12 hours. The absence of a lag phase with the other two strains was probably due to the fact that they were inoculated with organisms which were in the logarithmic phase. Barber (1908) showed that when a transfer was made from a culture in the logarithmic phase to the same medium and under the same conditions, the new culture multiplied at once at a logarithmic rate.

The pattern of the logarithmic phase was not consistent among all 5 strains when grown in the same medium. Strains 12, 13, and 17, grown in 1 per cent tryptose-water, were in the logarithmic phase of growth from about the third hour to the eighteenth hour of incubation or for a period of 15 hours. These strains had an average generation time of 52 to 53 minutes which was shorter than that of strains 11 and 29. Table II (p. 24) gives the reproductive rate of all strains growing in 1 per cent tryptose-water. Strain 11 was dividing at a maximum rate from about the sixth hour to the twenty-fourth hour of incubation or for a period of 18 hours. It had an average generation time of 66 minutes. Strain 29 was in this phase from the third to the twenty-fourth hour of incubation or for a period of 21 hours. This extended logarithmic phase was

accompanied by a long generation time. It had an average generation time of 74 minutes which was 21 to 22 minutes longer than those of strains 12, 13, and 17.

In nutrient broth strains 12, 13, 17, and 29 were in the logarithmic phase from the third hour to the fifteenth hour of incubation or for a period of 12 hours. The average generation time for these strains was 42 to 45 minutes. Strain 11 was in this phase from the third to the eighteenth hour of incubation or for a period of 15 hours. It had an average generation time of 53 minutes. Table IV (page 27) gives the reproductive rate of all strains growing in nutrient broth.

The length of the logarithmic phase varied with each strain when all strains were grown in the synthetic medium. Strains 11 and 17 were in this phase from the time of inoculation to about the forty-eighth and sixtieth hours respectively. Strain 11 had an average generation time of 138 minutes; strain 17 had an average generation time of 160 minutes which was the longest one of all the strains. Strains 12 and 29 had the shortest over-all logarithmic phase, which was 30 hours. Strain 12 was in this phase from the twelfth to the forty-second hour of incubation, and it had an average generation time of 114 minutes. Strain 29 was in the logarithmic phase from the sixth to the thirty-sixth hour of incubation, and it had an average generation time of 107 minutes which was the shortest one of all the strains. Strain 13 was in this phase from the sixth hour to the forty-eighth hour of incubation or for a period of 42 hours. It had an average generation time of 133 minutes. Table VI (page 30) gives the reproductive rate of all strains growing in the synthetic medium.

TABLE I

MEAN POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN 1 PER CENT TRYPTOSE-WATER

Hours	Strains				
	11	12	13	17	29
0	65	105	115	124	91
3	203	275	347	394	256
6	760	3,255	4,333	5,560	1,270
9	6,297	35,580	41,183	52,866	5,983
12	32,113	481,000	469,333	658,000	24,966
15	238,666	5,686,666	6,346,666	7,103,333	130,000
18	2,866,666	48,766,666	60,266,666	73,366,666	1,043,000
21	12,055,000	*	*	*	3,930,000
24	58,433,333	159,500,000	199,166,666	270,000,000	62,666,666

Figures are based on the average of the populations of 3 different growth determinations and represent organisms per ml. The populations of the 3 individual growth determinations are given in the Appendix, Tables XIII, XIV, and XV.

*No count made at this time.

Figure 1

Growth curves of 5 Strains of S. pullorum grown in 1 per cent tryptose-water

(These graphs are based on the data in table I)

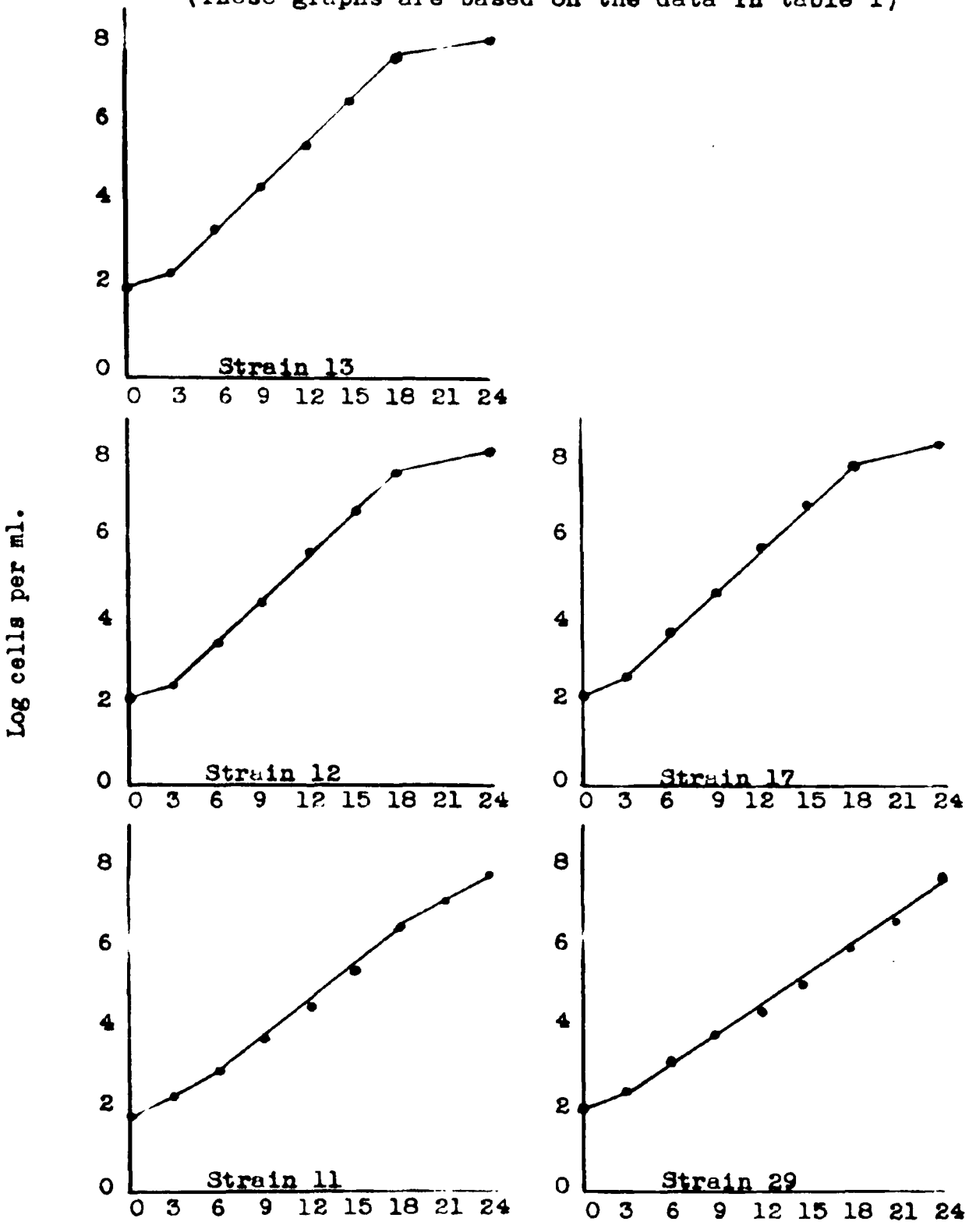


TABLE II

REPRODUCTIVE RATE AT 3 HOUR INTERVALS OF 5 STRAINS OF *S. PULLORUM* GROWN IN 1 PER CENT TRYPTOSE-WATER

Hours	Strains									
	11		12		13		17		29	
	n	g	n	g	n	g	n	g	n	g
0	-	-	-	-	-	-	-	-	-	-
3	1.62	111.1	1.37	131.4	1.06	169.8	1.67	107.8	1.48	121.6
6	1.88	95.7	3.54	50.8	3.62	49.7	3.79	47.5	2.31	77.9
9	3.03	59.4	3.42	52.6	3.23	55.7	3.23	55.7	2.22	81.1
12	2.34	76.1	3.40	52.9	3.49	51.6	3.38	53.2	2.06	87.4
15	2.87	62.7	3.54	50.8	3.73	48.3	3.41	52.9	2.36	76.3
18	3.56	50.6	3.07	58.6	3.23	55.7	3.34	53.9	2.98	60.4
24	4.32	83.3	1.67	215.6	1.71	210.5	1.88	191.5	5.87	61.3

These figures were computed from the data given in Table I.
n = number of generations occurring in time (t) in minutes.
g = generation time in minutes, or time for one complete cellular division to take place.
n and g were computed on the basis of the following two formulae:

$$n = 3.3 \log_{10} \frac{b}{B} \qquad g = \frac{t}{3.3 \log_{10} \frac{b}{B}}$$

where b = number of bacteria at the end of a given time (t), and B = number of bacteria at the beginning of a given time, taken as t = 0.

TABLE III

MEAN POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH

Hours	Strains				
	11	12	13	17	29
0	76	62	50	61	105
3	166	202	212	281	468
6	1,141	1,811	4,585	5,383	6,258
9	17,100	53,400	138,500	55,800	114,167
12	162,000	940,000	3,971,666	2,458,333	3,015,000
15	2,323,000	22,466,666	39,550,000	27,625,000	78,833,333
18	25,516,666	56,333,333	150,333,333	102,333,333	172,000,000
21	138,833,333	148,666,666	213,500,000	186,000,000	203,000,000
24	205,500,000	231,500,000	223,000,000	232,666,666	270,333,333

Figures are based on the average of the populations of 3 different growth determinations and represent organisms per ml. The populations of the 3 individual growth determinations are given in the Appendix, Tables XVII, XVIII, and XIX.

Figure 2
Growth curves of 5 strains of S. pullorum grown in nutrient broth

(These graphs are based on the data in table III)

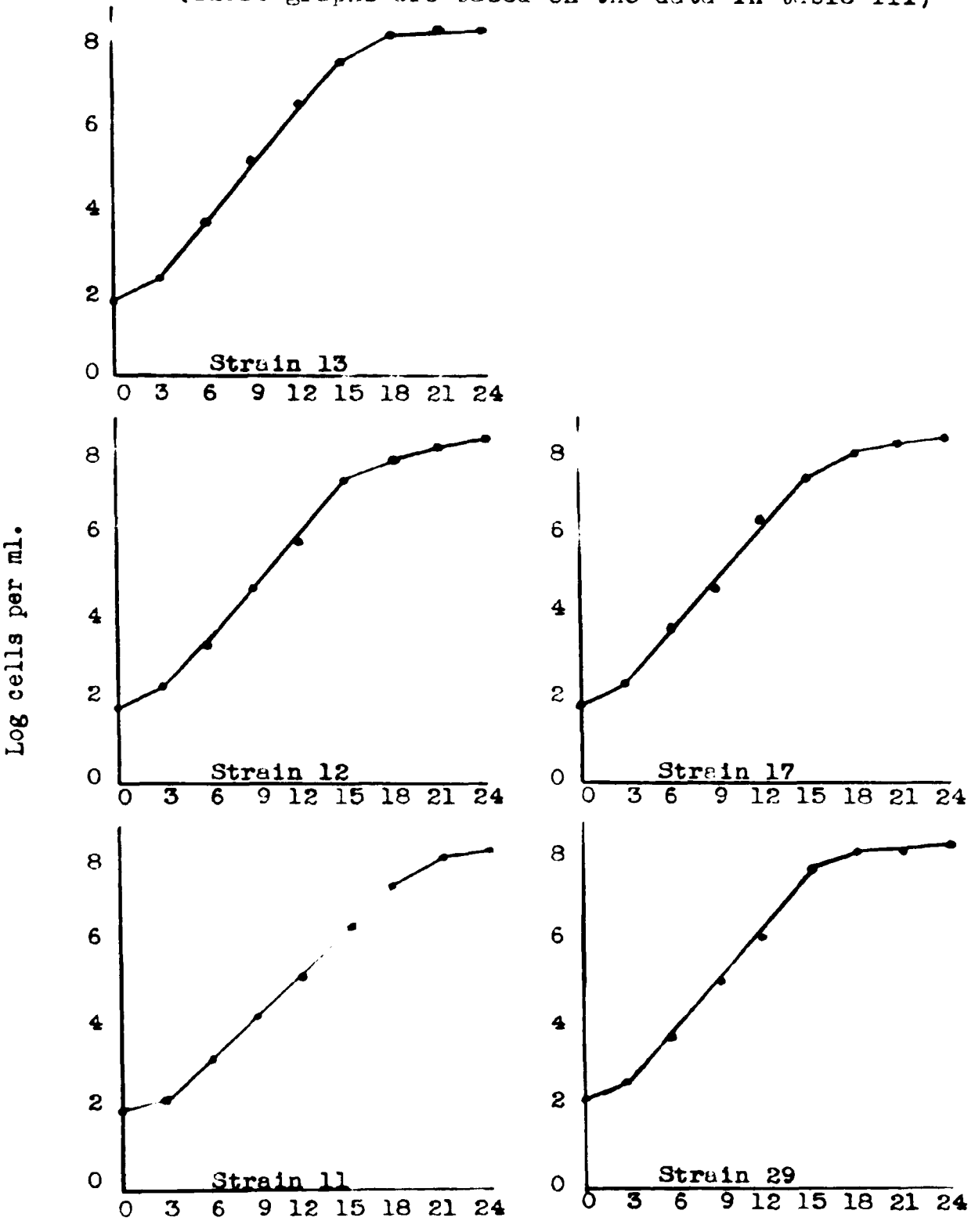


TABLE IV

REPRODUCTIVE RATE AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH

Hours	Strains									
	11		12		13		17		29	
	n	g	n	g	n	g	n	g	n	g
0	-	-	-	-	-	-	-	-	-	-
3	1.13	159.3	1.71	105.3	2.06	87.4	2.19	82.2	2.12	84.9
6	2.77	65.0	3.15	57.1	4.40	40.9	4.23	42.6	3.72	48.4
9	3.87	46.5	4.85	37.1	4.88	36.9	3.36	53.6	4.16	43.3
12	3.23	55.7	4.11	43.8	4.81	37.4	5.43	33.1	4.69	38.4
15	3.81	47.2	4.55	39.5	3.30	54.5	3.46	52.0	4.67	38.5
18	3.42	52.6	1.31	137.4	1.91	94.2	1.88	95.7	1.13	159.3
21	2.42	74.4	1.37	131.4	0.48	375.0	0.84	214.3	0.26	666.6
24	0.58	310.3	0.67	268.7	0.06	3000.0	0.38	473.7	0.36	500.0

These figures were computed from the data given in Table III.
n = number of generations occurring in time in minutes.
g = generation time in minutes, or time for one complete cellular division to take place.
"n" and "g" were computed on the basis of the same 2 formulae given below Table II.

TABLE V

MEAN POPULATION AT 6 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN A SYNTHETIC MEDIUM

Hours	Strains				
	11	12	13	17	29
0	42	130	126	106	126
6	301	488	512	446	538
12	2,063	2,768	3,266	2,092	6,075
18	8,353	19,700	17,200	6,858	47,725
24	44,033	178,800	119,692	44,050	467,083
30	255,916	2,957,416	983,333	221,908	5,065,000
36	2,089,750	53,666,666	8,720,833	1,004,166	75,166,666
42	16,153,250	286,291,666	44,933,333	9,180,000	439,000,000
48	114,283,333	969,166,666	347,333,333	47,745,833	1,544,333,333
54	341,500,000	930,000,000	905,666,666	349,666,666	932,500,000
60	612,500,000	809,000,000	887,250,000	1,232,500,000	977,250,000

Figures are based on the average of the populations of 3 different growth determinations and represent organisms per ml. The populations of the 3 individual growth determinations are given in the Appendix, Tables XXI, XXII, and XXIII.

Figure 3

Growth curves of 5 strains of *S. pullorum* grown in a synthetic medium

(These graphs are based on the data in table V)

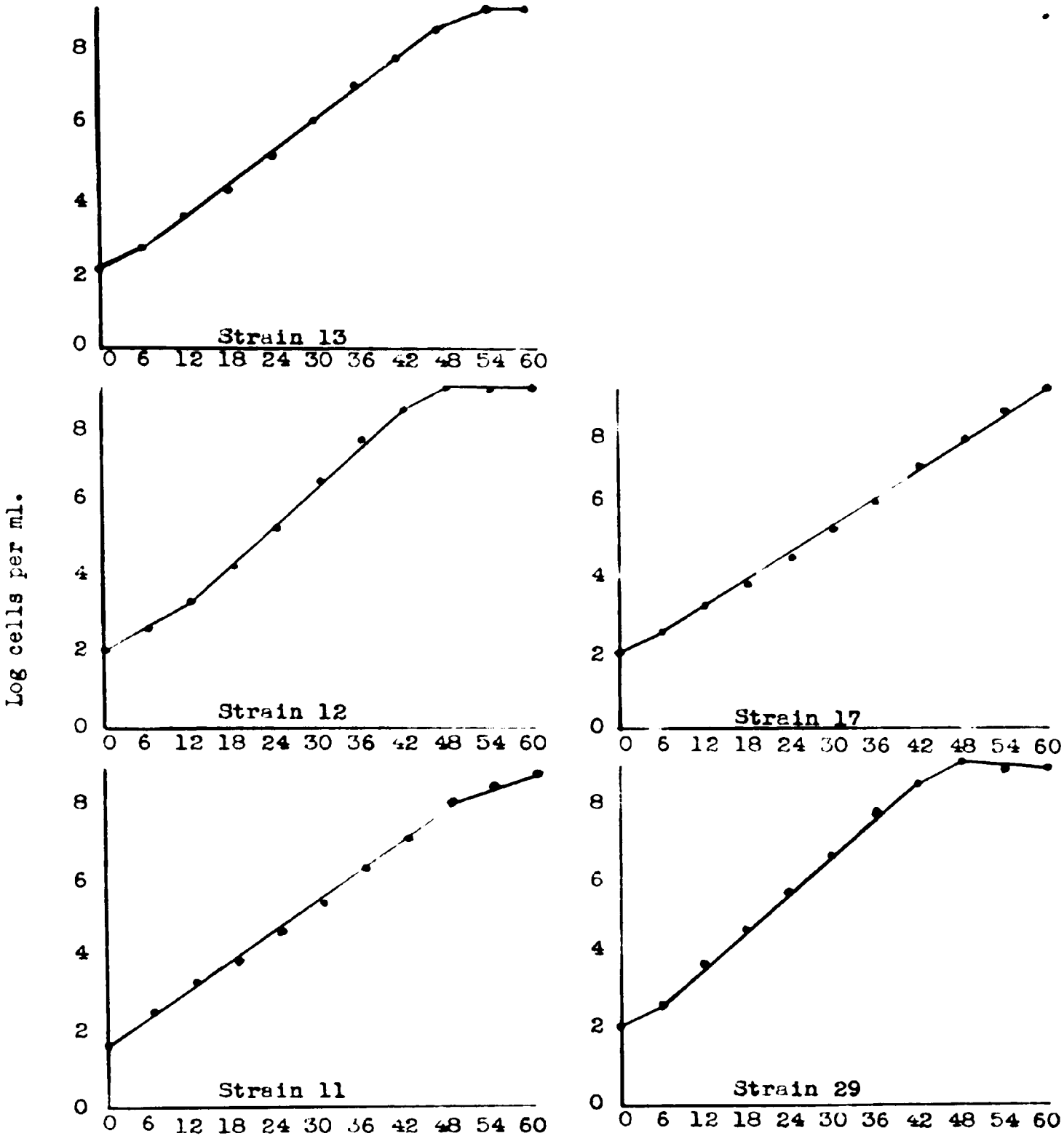


TABLE VI

REPRODUCTIVE RATE AT 6 HOUR INTERVALS OF 5 STRAINS OF *S. PULLORUM* GROWN IN A SYNTHETIC MEDIUM

Hours	Strains									
	11		12		13		17		29	
	n	g	n	g	n	g	n	g	n	g
0	-	-	-	-	-	-	-	-	-	-
6	2.83	127.2	1.91	188.5	2.02	178.2	2.06	174.8	2.09	172.2
12	2.77	130.0	2.49	144.6	2.66	135.3	2.22	162.2	3.48	103.4
18	1.99	180.9	2.81	128.1	2.39	150.6	1.71	210.5	2.96	121.6
24	2.39	150.6	3.16	113.9	2.79	129.0	2.66	135.3	3.27	110.1
30	2.52	142.9	4.02	89.6	3.02	119.2	2.31	155.8	3.41	105.6
36	3.02	119.2	4.15	86.7	3.13	115.0	2.16	166.7	3.86	93.3
42	2.93	122.9	2.39	150.6	2.36	152.5	3.16	113.9	2.52	142.9
48	2.81	128.1	1.75	205.7	2.93	122.9	2.36	152.5	1.80	200.0
54	1.57	229.3	*	*	1.37	262.8	2.85	126.3	*	*
60	0.84	428.6	*	*	*	*	1.80	200.0	*	*

These figures were computed from the data given in Table V.
n = number of generations occurring in time in minutes.
g = generation time in minutes, or time for one complete cellular division to take place.
"n" and "g" were computed on the basis of the same 2 formulae given below Table II.
*Organisms decreasing in number.

In 1 per cent tryptose-water a negative acceleration phase of approximately 3 hours followed the logarithmic phase. In nutrient broth there was a negative acceleration phase of approximately 3 hours for all strains except number 12 which was in this phase for about 6 hours. This phase varied in duration from 0 to 12 hours for these strains in the synthetic medium. Strain 17 had none; strains 11 and 29 had a 12 hour one, from the forty-eighth to sixtieth hour and from the thirty-sixth to the forty-eighth hour of incubation respectively; strains 12 and 13 had a 6 hour one, from the forty-second to the forty-eighth hour and from the forty-eighth to the fifty-fourth hour of incubation respectively.

The stationary phase was entered after 27 hours by strains 11 and 29, and after 21 hours by 12, 13, and 17 when they were grown in 1 per cent tryptose-water. When cultured in nutrient broth, strains 13, 17, and 29 entered it in 18 hours, and strains 11 and 12 reached it in 21 hours. In the synthetic medium, 11 and 17 attained the stationary phase in 60 hours, strains 12 and 29 arrived at this phase in 48 hours, and 13 reached it in 54 hours. The maximum number of viable organisms in the stationary phase was about the same for all strains when they were grown in either 1 per cent tryptose-water or nutrient broth for 36 hours, but it was a good deal higher when they were grown in the synthetic medium for 60 hours. See tables XVI, XX, XXI, XXII, and XXIII in the appendix. These results do not support the assumption of Bail (1929) who studied the problem of maximum population and decided that for each individual bacterial species there was a maximum population which could not be surpassed no matter what factors were controlled. Korinek (1939) found

that the maximum population was directly proportional to the concentration of food.

In summarizing the material on growth presented thus far it should be pointed out that there was no initial stationary phase evidenced when a sample was taken as late as 3 hours after inoculation. When grown in 1 per cent tryptose-water, all the strains, except 11, were in the lag phase for a period of 3 hours. Strain 11 was in this phase for about 6 hours. All the strains were in the lag phase for 3 hours when grown in nutrient broth. Two of the 5 strains had no lag phase when grown in the synthetic medium and the others had one of 6 to 12 hours, depending upon the strain. When cultured in nutrient broth, all these strains of S. pullorum had the shortest logarithmic phase and most of them were in it for about the same duration of time; in 1 per cent tryptose-water this phase was somewhat more variable in duration from strain to strain and all strains were in it longer than when cultured in nutrient broth; in the synthetic medium the reproductive rate was slow and thus extended this phase considerably over that in the other two media. The negative acceleration phase was about the same length for most of these strains whether they were growing in 1 per cent tryptose-water or in nutrient broth but varied from 0 to 12 hours when they were cultured in the synthetic medium. The stationary phase was reached in 18 to 21 hours by the nutrient broth cultures, in 21 to 27 hours by the 1 per cent tryptose-water cultures, and in 48 to 60 hours by the synthetic medium cultures. The maximum number of viable organisms in the stationary phase was higher in the synthetic medium than in either 1 per cent tryptose-water or nutrient broth. The maximum number of viable organisms

in the stationary phase was about the same in the latter two media.

When growth determinations were made in 1 per cent tryptose-water or nutrient broth using a different tube (tube method) at each 3 hour interval, a pattern almost identical with that given by use of a single source culture was found with nutrient broth. Three of the strains (13, 17, and 29) showed little, if any, negative acceleration phase when grown in 1 per cent tryptose-water and, as a result, reached the stationary phase 3 hours sooner by this method than when each pour-plate was made from the same culture for a 24 hour period. Also, strains 11 and 12 had a three-hour-shorter logarithmic period by the tube method. However, by this method the negative acceleration phase was 6 hours for strain 11; hence it reached the stationary phase in 27 hours, just as with the other method. Strain 12 had the usual 3 hour negative acceleration phase and attained the stationary phase in 18 hours. It must be kept in mind that this method was used only once in order to determine whether the two methods were at all comparable and, since the results were so much alike, the procedure was not repeated. These results are given in the appendix, tables XVI and XX.

Synthetic medium. A short discussion of the development of the synthetic medium is included because the survey of the amino acid and vitamin requirements of S. pullorum was of sufficient scope to be of value to anyone interested in the nutrition of this organism. On the basis of the statements of Lederberg (1947) and Gray and Tatum (1944), which have been cited in the review of literature, it was assumed in the beginning of this study that a synthetic medium such as they used would be suitable

for the strains of S. pullorum being studied. Furthermore, it was decided arbitrarily that the synthetic medium should be one in which all strains would grow sufficiently to change it macroscopically in 24 hours when an inoculum of about twenty-five thousand organisms was added to 2.5 ml. of it and in which subcultures would grow similarly when prepared in the same manner. Growth, in this discussion, is considered synonymous with visible growth.

One of the media used by Lederberg (1947) was composed of glucose, asparagine, cystine, leucine, methionine, salts, and trace elements. The concentrations of these substances have been given in the chapter on materials and methods. These concentrations were used throughout this investigation unless otherwise mentioned. None of the strains grew in this medium in 24 hours. In 48 hours strain 12 gave evidence of growth. When either cystine, or leucine and cystine were omitted from this medium, there was no growth in 48 hours. By plating out these media after 48 hours incubation, it was found that the organisms in the medium with all three amino acids were increasing in number, but slowly.

In the following discussion the above medium containing all three amino acids is designated by the term "base." A vitamin mixture of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and calcium pantothenate added to the base accelerated the growth of strains 12, 13, and 29 so that there was a macroscopic change in 24 hours. By the end of 48 hours growth had occurred in the tube containing strain 17 but not in the one containing strain 11. Strain 12 grew in a mixture of para-aminobenzoic acid, nicotinamide, i-inositol, and pimelic acid,

plus the base, but the growth did not become evident until it had been incubated for 48 hours. This is not an acceleration as it grew this well in the base alone. Strains 29 and 17 grew in 48 hours in a mixture of choline hydrochloride, nucleic acid, folic acid, and biotin, plus the base, but they grew only slightly - the latter strain doing this on the third subculture. Strains 12, 13, and 29 grew in 24 hours in a mixture of all the vitamins and other organic nutrients plus the base. Strain 17 grew in it in 48 hours; strain 11 did not grow in it in 48 hours. From this set of data it was concluded that more than just a supplement of the vitamins and other organic nutrients tested is needed to stimulate more rapid growth of strain 11, and that each vitamin of the mixture containing thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and calcium pantothenate should be tested by itself.

The following results are based on experimentation with the vitamins just mentioned, each tested separately with the base. No acceleration of growth occurred in tubes in which either riboflavin or pyridoxine hydrochloride was added to the base. Strains 13 and 17 grew in 48 hours when thiamine hydrochloride was added to the base. Strain 29 responded similarly with added calcium pantothenate.

In summary, these experiments showed that a vitamin mixture of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and calcium pantothenate accelerated the growth of strain 12 but that they did not accelerate growth of strain 12 when added individually to the base; a supplement of thiamine hydrochloride accelerated strains 13 and 17; a supplement of calcium pantothenate accelerated strain 29; neither the base itself nor any of the vitamin and other organic nutrient

mixtures tested accelerated strain 11; and, finally, none of these media were satisfactory for all 5 strains according to the criteria set down at the outset of this discussion.

Experiments to determine what amino acids were actually essential for these strains were tried by the method outlined in the chapter on materials and methods. The medium used was composed of glucose, salts, trace elements, and 17 amino acids. Essentially, the procedure was to omit one amino acid at a time from the entire mixture and observe whether the organisms would grow in the medium remaining. When a strain failed to grow 2 out of 3 times in 24 hours in the absence of an amino acid, that amino acid was then designated as essential; when a strain failed to grow at a maximum rate 2 out of 3 times in 24 hours in the absence of an amino acid, that amino acid was then designated as accessory. These designations were designed for this study only. They were merely used in order to have some means of classifying the data accumulated from three different experiments with all 17 amino acids. Leucine, histidine, valine, arginine, and proline were essential to at least one or more strains; histidine, valine, cystine, serine, tryptophane, phenylalanine, and glutamic acid were accessory to at least one or more strains. Strain 11 seemed to need the largest number of amino acids. Leucine, histidine, valine, arginine, and proline were essential and cystine, serine, and glutamic acid were accessory. Strain 29 needed the fewest amino acids. Leucine was essential and histidine was accessory. Arginine was essential to strains 12 and 13. Cystine and serine were accessory to the former strain; valine and cystine were accessory to the latter strain. Leucine only was

essential to strain 17; valine, cystine, and serine were accessory. Only the absence of leucine curtailed growth of all strains for over 24 hours. Without leucine no growth occurred even after 2 weeks incubation. It was concluded that leucine is an essential amino acid for these strains when this word is used in its narrowest sense of meaning. Johnson and Rettger (1943) concluded that leucine was one of the most important amino acids for most of the strains of S. pullorum with which they worked.

Since leucine seemed to be the key amino acid, an experiment was tried in which leucine, in 3, 6, and 9-fold greater concentrations than that ordinarily used, was added to glucose, salts, and trace elements. No growth occurred in any of these concentrations during a two week period of observation. Also, a 10-fold increase in concentration of leucine was tried with glucose, thiamine hydrochloride, calcium pantothenate, salts, trace elements, and ascorbic acid (at the rate of 0.62 mg./100 ml. of medium). No growth occurred in 48 hours.

Leucine, histidine, arginine, valine, serine, and cystine were tried in different combinations with glucose, salts, and trace elements. The combinations tried were: leucine; leucine and histidine; leucine, histidine, and cystine; leucine, histidine, cystine, and arginine; leucine, cystine, histidine, arginine, and valine; leucine, histidine, cystine, serine, and arginine; leucine, arginine, and cystine; leucine and arginine. It should be noted that proline was not included among this group of amino acids because it was specific for strain 11 only. Cystine and serine were tried in preference to proline, although not essential, because their absence consistently resulted in reduced

growth of the majority of strains. All strains grew well in 48 hours when arginine, cystine, histidine, leucine, and valine were combined, except 11, which required a larger inoculum to get a similar growth response. When the concentrations of arginine, cystine, histidine, and valine were doubled and leucine was quadrupled, growth occurred in 24 hours in all strains after the third subculture but growth was very slight for strains 11 and 13.

From the results of the experiments with the amino acids the following summarizing statements may be made. Leucine was essential for all strains and the usage of essential here means that without leucine they will not grow. None of the strains grew in a medium containing glucose, salts, and trace elements with leucine added in 3 different amounts. None of the strains grew in 48 hours in a medium in which leucine, in 10-fold greater concentration than that ordinarily used, was added to glucose, thiamine hydrochloride, calcium pantothenate, salts, trace elements, and ascorbic acid. Good growth occurred in 48 hours in a medium composed of arginine, cystine, histidine, leucine, valine, glucose, salts, and trace elements except for strain 11 which responded similarly only when a larger inoculum was used. Doubling the concentration of all these amino acids except leucine, which was increased 4-fold, gave a medium in which all strains grew in 24 hours after at least 3 subcultures.

Finally, either asparagine or one of the combinations (thiamine hydrochloride and calcium pantothenate; asparagine and thiamine hydrochloride; asparagine and calcium pantothenate; asparagine, thiamine

hydrochloride, and calcium pantothenate) was added separately to the medium containing glucose, salts, trace elements, arginine, cystine, histidine, leucine, and valine to determine whether it would enhance growth. The concentrations of the amino acids were those stated at the outset of the discussion on the development of the synthetic medium. All strains grew in 24 hours in a medium containing the combination of asparagine, thiamine hydrochloride, and calcium pantothenate; however, strain 11 did not grow consistently in 24 hours. This medium also gave best results when subcultures were made from it. Finding that this medium gave better results than any other combination tested was expected since it had already been determined that strains 13 and 17 grew better with the addition of thiamine hydrochloride and that strain 29 grew better with the addition of calcium pantothenate. None of the strains grew consistently in 24 hours in any of the other combinations of media.

The synthetic medium that was used for the growth studies and the work with neomycin was composed of arginine, cystine, histidine, leucine, valine, thiamine hydrochloride, calcium pantothenate, glucose, salts, asparagine, and trace elements. This medium was chosen over the one with these amino acids alone in higher concentrations because growth occurred upon initial inoculation in 24 hours. Subculturing was necessary to attain similar results with the amino acids alone in higher concentrations and even then strains 11 and 13 grew only slightly in 24 hours.

Sensitivity to neomycin. As is shown in tables VII and VIII (pages 41 and 43), about the same concentration of neomycin was sufficient to stop the growth of all the strains when they were grown and tested in either 1 per cent tryptose-water or nutrient broth. However, when the

bactericidal concentration of neomycin necessary in 1 per cent tryptose-water and nutrient broth was compared with that necessary in the synthetic medium, shown in table IX (page 43), it was revealed that from 41 to 321 times more neomycin was needed to kill the organisms when they were grown and tested in the synthetic medium. The latter fact was probably due to the interference of neomycin action by the inorganic salts of the synthetic medium. This point will be discussed further under the heading of the effect of the constituents of the medium and sodium chloride on the antibacterial action of neomycin. In only one case when they were grown and tested in either 1 per cent tryptose-water or nutrient broth was there growth after 72 hours. This was exactly diametrical to the results with the synthetic medium in which all except one strain grew after 72 hours in a tube with a higher concentration of neomycin. Although there was growth after 24 hours in tubes with a higher concentration of neomycin, this growth usually did not occur in a tube that contained greater than a 4-fold increase in concentration of the antibiotic. And, since only very small concentrations of neomycin were necessary to kill these strains of S. pullorum, this difference in concentrations of neomycin which stopped growth for 24 hours and that which inhibited growth for a week may be considered negligible. It was concluded from these observations that neomycin is bactericidal in concentrations only slightly higher than that in which it is bacteriostatic. This finding is in agreement with those reported by Waisbren and Spink (1950a), Waksman, Frankel, and Graessle (1949), Waksman, Lechevalier, and Harris (1949), and Waksman and Lechevalier (1949).

TABLE VII

SENSITIVITY OF S. PULLORUM TO NEOMYCIN AT VARIOUS pH VALUES

Strain	µg./ml. Neomycin								
	1.25	0.625	0.312	0.156	0.078	0.039	0.019	0.009	Control
					pH 5.0				
11	72*	72	72	72	72	72	72	72	48
12	48	48	48	48	48	48	48	48	48
13	72	72	72	72	72	72	72	72	72
17	48	48	48	48	48	48	48	48	48
29	24	24	24	24	24	24	24	24	24
					pH 6.0				
11	-**	24	24	24	24	24	24	24	24
12	-	-	48	24	24	24	24	24	24
13	-	-	48	24	24	24	24	24	24
17	-	48	24	24	24	24	24	24	24
29	-	24	24	24	24	24	24	24	24
					pH 7.0				
11	-	-	-	72	24	24	24	24	24
12	-	-	-	-	168	48	48	24	24
13	-	-	-	-	48	48	24	24	24
17	-	-	-	-	-	24	24	24	24
29	-	-	-	-	48	24	24	24	24
					pH 8.0				
11	-	-	-	-	-	-	96	24	24
12	-	-	-	-	-	-	-	96	24
13	-	-	-	-	-	-	48	48	24
17	-	-	-	-	-	-	120	48	24
29	-	-	-	-	-	-	-	48	24
					pH 9.0				
11	-	-	-	-	-	96	96	48	48
12	-	-	-	-	-	168	48	24	24
13	-	-	-	-	-	120	48	24	24
17	-	-	-	-	-	48	24	24	24
29	-	-	-	-	-	144	24	24	24

* Indicates the number of hours before growth occurred.
** Indicates no growth after incubation for 1 week.

Figure 4

Effect of pH on the antibacterial activity of neomycin

(These graphs are based on data in table VII)

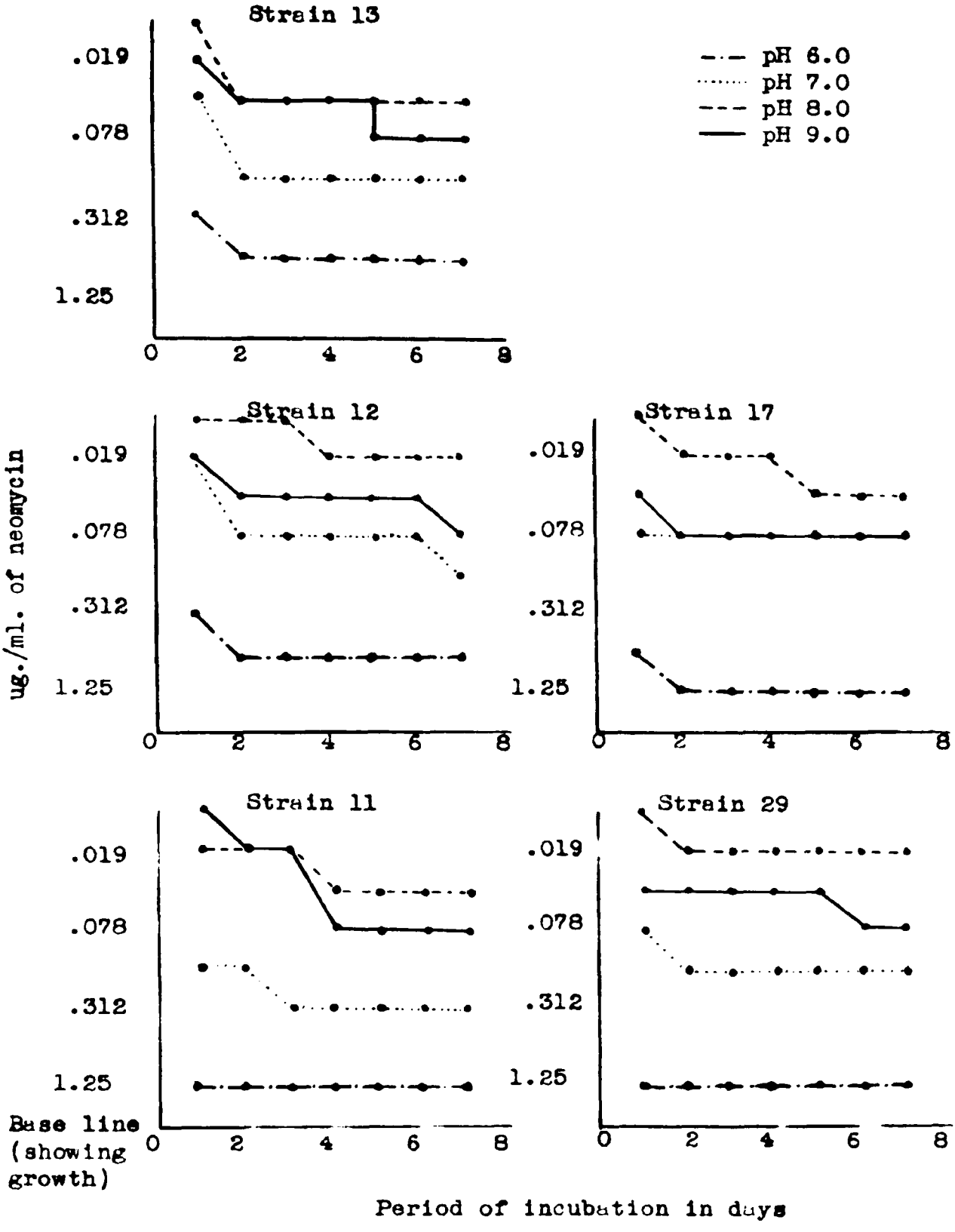


TABLE VIII

SENSITIVITY OF S. PULLORUM TO NEOMYCIN WHEN GROWN AND TESTED IN NUTRIENT BROTH

Strain	µg./ml. neomycin						
	1.25	0.625	0.312	0.156	0.078	0.039	Control
11	-*	-	-	-	72**	48	24
12	-	-	-	72	48	24	24
13	-	-	-	-	72	48	24
17	-	-	-	72	48	48	24
29	-	-	-	-	48	48	24

*Indicates no growth after incubation for 1 week.
**Indicates the number of hours before growth occurred.

TABLE IX

SENSITIVITY OF S. PULLORUM TO NEOMYCIN WHEN GROWN AND TESTED IN A SYNTHETIC MEDIUM

Strain	µg./ml. neomycin						
	50.0	25.0	12.5	6.25	3.12	1.56	Control
11	-*	-	168**	168	24	24	24
12	-	-	-	72	24	24	24
13	-	-	168	48	24	24	24
17	-	-	168	72	24	24	24
29	-	-	168	48	24	24	24

*Indicates no growth after incubation for 1 week.
**Indicates the number of hours before growth occurred.

To test whether there was any difference in the sensitivity of the organisms themselves when grown in different media, they were grown in the synthetic medium, then washed and centrifuged three times with physiological saline, and tested with different concentrations of neomycin in both the synthetic medium and 1 per cent tryptose-water. A greater concentration of neomycin was found necessary to kill an organism which had been grown and tested in the synthetic medium than when it had been grown in the synthetic medium and tested in 1 per cent tryptose-water. Moreover, about the same concentration of neomycin was necessary to kill all organisms when they were tested in 1 per cent tryptose-water whether they had been grown previously in the synthetic medium or in 1 per cent tryptose-water. Although experimentation along this line was limited to one strain of S. pullorum, the tentative conclusion may be drawn that the sensitivity of an organism to neomycin is governed by the medium in which it is tested rather than by that in which it is grown. Therefore, when the sensitivity of an organism to a particular antibiotic is reported, the medium in which the tests were carried out should be an integral part of the report.

The effect of pH on the antibacterial activity of neomycin. The data in table VII (page 41) and figure 4 (page 42) show that a pH of 8.0 definitely favored the action of neomycin over that of the other pH values at which the experiments were run. At pH 5.0 the activity of neomycin was destroyed. The organisms grew in all dilutions of neomycin at this pH. When the bactericidal titers of neomycin at pH values

6.0, 7.0, and 9.0 were compared with the bactericidal titer at pH 8.0 it is seen that 16 to 64 times more neomycin was needed at pH 6.0, 4 to 16 times more neomycin was needed at pH 7.0, and 2 to 4 times more neomycin was needed at pH 9.0 than at pH 8.0. The general conclusion may be drawn that neomycin activity is favored by an alkaline medium. Similar results have been reported by Waksman, Lechevalier, and Harris (1949) and Waksman and Lechevalier (1949). The most likely explanation for this phenomenon is that the basic property of neomycin, which probably is essential to its activity, is neutralized by acid or neomycin becomes more dissociated in an alkaline medium.

The effect of temperature on neomycin activity. As is shown in table X (page 46), neomycin at 4°C. and 20°C. was not quite as effective as it was at 37°C. At 4°C., 96.5 per cent to 99.9 per cent of the organisms were killed; at 20°C., 99.9 per cent of the organisms were killed; at 37°C., 99.9 per cent of the organisms were killed - less than 10 organisms survived. On the basis of these results, when temperature alone was considered as a factor which might influence the action of neomycin, it was concluded that the action of neomycin is not affected significantly by lowering the temperature from 37°C. to 4°C. These results seem to indicate that neomycin is somewhat unique with respect to the effect of temperature, as a closely related antibiotic, streptomycin, has been shown to be practically useless at 5°C. and to have a marked reduction in its activity at 20°C. (Garrod, 1948). Hobby, Meyer, and Chaffee (1942) investigated the effect of temperature on penicillin activity and reported results which are similar to those given above for streptomycin.

TABLE X

EFFECT OF TEMPERATURE ON NEOMYCIN ACTIVITY

Strain	Incubation Temperature	Organisms per ml. at 0 hours	Organisms per ml. after 18 hours incubation					
			4°C.		20°C.		37°C.	
			Control	0.5 µg./ml. neomycin	Control	0.5 µg./ml. neomycin	Control	0.5 µg./ml. neomycin
11	4°C.	6.9×10^4	6.5×10^4	2.3×10^3				
11	20°C.	6.9×10^4			7.4×10^5	2×10^1		
11	37°C.	6.2×10^4					1.6×10^8	<10
12	4°C.	2.9×10^6	3.1×10^6	1.1×10^3				
12	20°C.	2.9×10^6			4.5×10^7	5×10^1		
12	37°C.	4.8×10^6					2.3×10^8	<10
13	4°C.	3.1×10^6	3.4×10^6	1.3×10^3				
13	20°C.	3.1×10^6			5.4×10^7	5×10^1		
13	37°C.	3.3×10^6					2.2×10^8	<10
17	4°C.	1.7×10^6	1.7×10^6	1.4×10^3				
17	20°C.	1.7×10^6			4.8×10^7	5×10^1		
17	37°C.	3.1×10^6					2.7×10^8	<10
29	4°C.	8.8×10^4	7.1×10^4	1.0×10^3				
29	20°C.	8.8×10^4			5.2×10^5	<10		
29	37°C.	2.0×10^4					2.6×10^8	<10

Data for the part of the experiment at 37°C. came from another experiment.

It should be noted that the number of organisms in the control tube remained about the same after 18 hours incubation at 4°C. The fact that neomycin killed 96.5 per cent to 99.9 per cent of the organisms at this temperature then indicates that it was very effective when they were multiplying and metabolizing at a very slow rate. There was only a slight increase in the total population in the control tube after 18 hours incubation at 20°C. Nevertheless, 99.9 per cent of the organisms were killed by neomycin, which again indicates the antibacterial activity of neomycin was not restricted to organisms that were multiplying rapidly. The organisms in the control tube incubated at 37°C. continued to divide rapidly and were in the stationary phase at the end of the 18 hour incubation period. The fact that neomycin killed 99.9 per cent (less than 10 organisms survived) then indicates that it was very effective when organisms were multiplying rapidly. These results were further substantiated in another set of experiments which will be discussed next under the heading of the effect of growth phase on neomycin activity.

Effect of growth phase on neomycin activity. The antibacterial action of neomycin did not seem to be affected significantly by the particular phases of growth tested. This is revealed in table XI (page 48) which shows that, with either concentration of neomycin, almost as large a per cent of organisms was killed when they were in the stationary phase as when they were in the logarithmic phase. The slight difference in the per cent killed in the 2 phases might have have been due to some factor in the old culture which interfered slightly with the action of neomycin.

TABLE XI

EFFECTIVENESS OF NEOMYCIN AGAINST S. FULLORUM IN THE LOGARITHMIC AND STATIONARY PHASES OF GROWTH

Strain	Growth phase	µg./ml. neomycin	Organisms per ml. before adding neomycin	Organisms per ml. remaining after 18 hours incubation with neomycin	Per cent of Organisms killed
11	Log.	1.0	31,650	<10	99.9
11	Log.	3.2	24,750	<10	99.9
11	Sta.	1.0	240,000,000	2,910,000	98.7
11	Sta.	3.2	259,000,000	620,000	99.7
12	Log.	1.0	3,230,000	<10	99.9
12	Log.	3.2	3,150,000	<10	99.9
12	Sta.	1.0	276,000,000	16,075,000	94.2
12	Sta.	3.2	232,000,000	1,047,500	99.6
13	Log.	1.0	4,445,000	<10	99.9
13	Log.	3.2	3,470,000	<10	99.9
13	Sta.	1.0	267,000,000	30,700,000	88.5
13	Sta.	3.2	277,500,000	1,710,000	99.4
17*	Log.	1.0	1,845,000	<10	99.9
17	Log.	3.2	1,910,000	<10	99.9
17	Sta.	1.0	282,000,000	1,295,000	99.5
17	Sta.	3.2	273,000,000	139,500	99.9
29	Log.	1.0	25,900	<10	99.9
29	Log.	3.2	34,000	<10	99.9
29	Sta.	1.0	370,500,000	8,200,000	97.8
29	Sta.	3.2	247,500,000	1,507,000	99.4

Log. - logarithmic; Sta. - stationary.

Controls with each strain were run simultaneously with the above experiment. They followed the normal growth pattern.

*Data for strain 17 added from another experiment.

The significant thing about the results of this set of experiments and those results gathered with the experiments on the effect of temperature was that neomycin acted just as lethal when the organisms were not multiplying rapidly as when they were multiplying rapidly. On the other hand, Hobby, Meyer, and Chaffee (1942) reported that penicillin appeared to be effective only when active multiplication was taking place; Lenert and Hobby (1947) stated that streptomycin more constantly and completely inhibited the growth of 6 hour cultures than 16 hour ones. Chang, Kuan-how and Stafseth (1950) reported that the older the culture of S. pullorum the higher the bactericidal titer of streptomycin.

Effect of size of inoculum on the action of neomycin. The results of this set of experiments are given in figures 5, 6, 7, 8, and 9 (pages 50, 51, 52, 53, and 54). The first part of this discussion pertains to those results gathered when various quantities of organisms were incubated at 37°C. for 240 minutes with a quantity of neomycin large enough to give a final concentration of 0.495 µg./ml. It was concluded from previous experiments, see results of sensitivity tests in 1 per cent tryptose-water at pH 7.0, table VII, (page 41) that this quantity of neomycin would render a culture sterile in a week or less. Under the above stated conditions, the size of inoculum, within rather broad limits, did not seem to affect the action of neomycin significantly. That is, with all strains the inoculum could be varied from 1,000 to 1,000,000 organisms without influencing the action of neomycin. The time that it took this concentration of neomycin to reduce the number of organisms to below 10 per ml. was definitely influenced by the size of the inoculum. As the inoculum was increased in size so was the time lengthened for neomycin to reduce the number of organisms below 10 per ml.

Figure 5

Effect of size of inoculum on action of neomycin (Strain 11)

(These graphs are based on data in appendix, table XXIV)

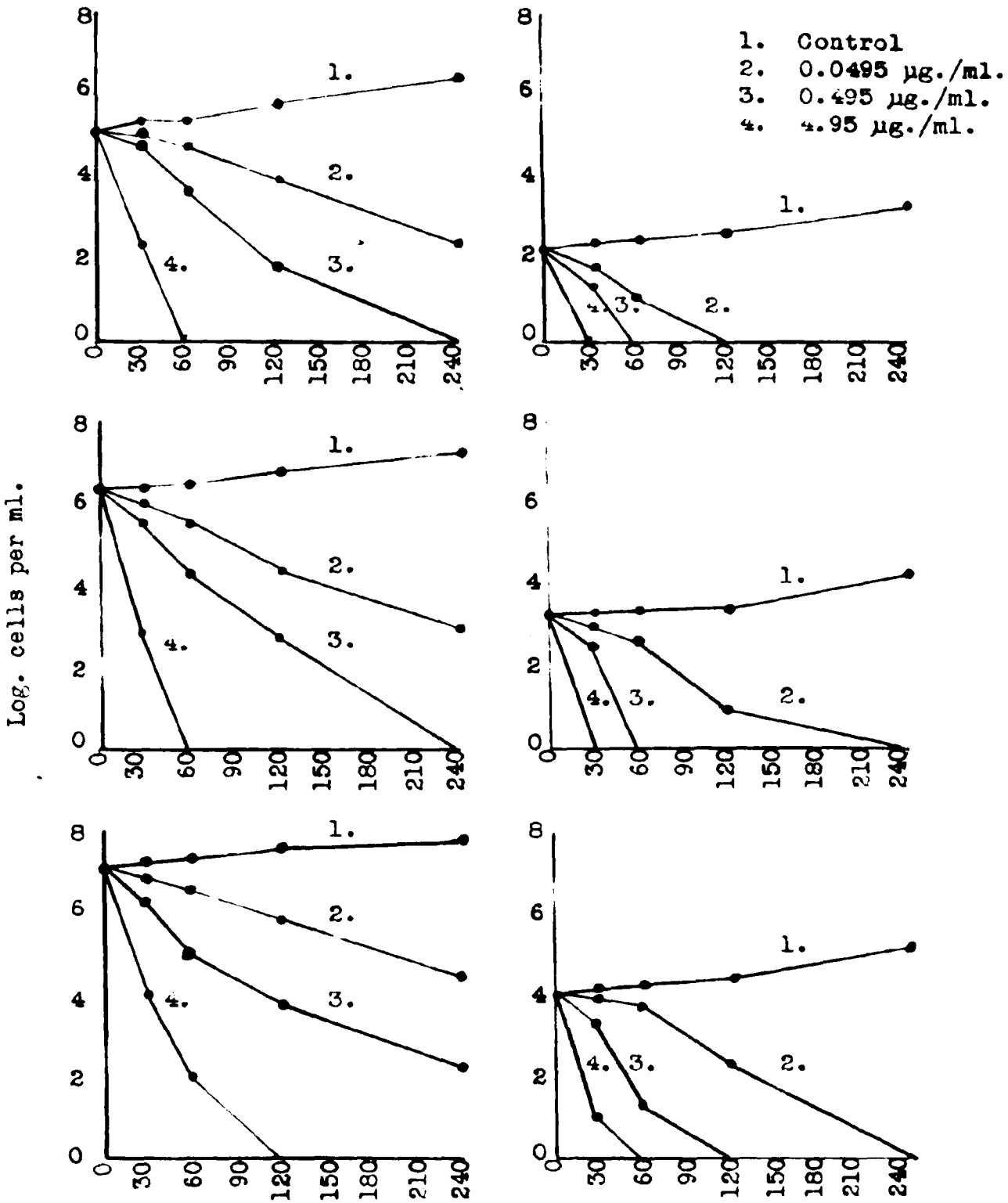


Figure 6

Effect of size of inoculum on action of neomycin (Strain 12)

(These graphs are based on data in appendix, table XXIV)

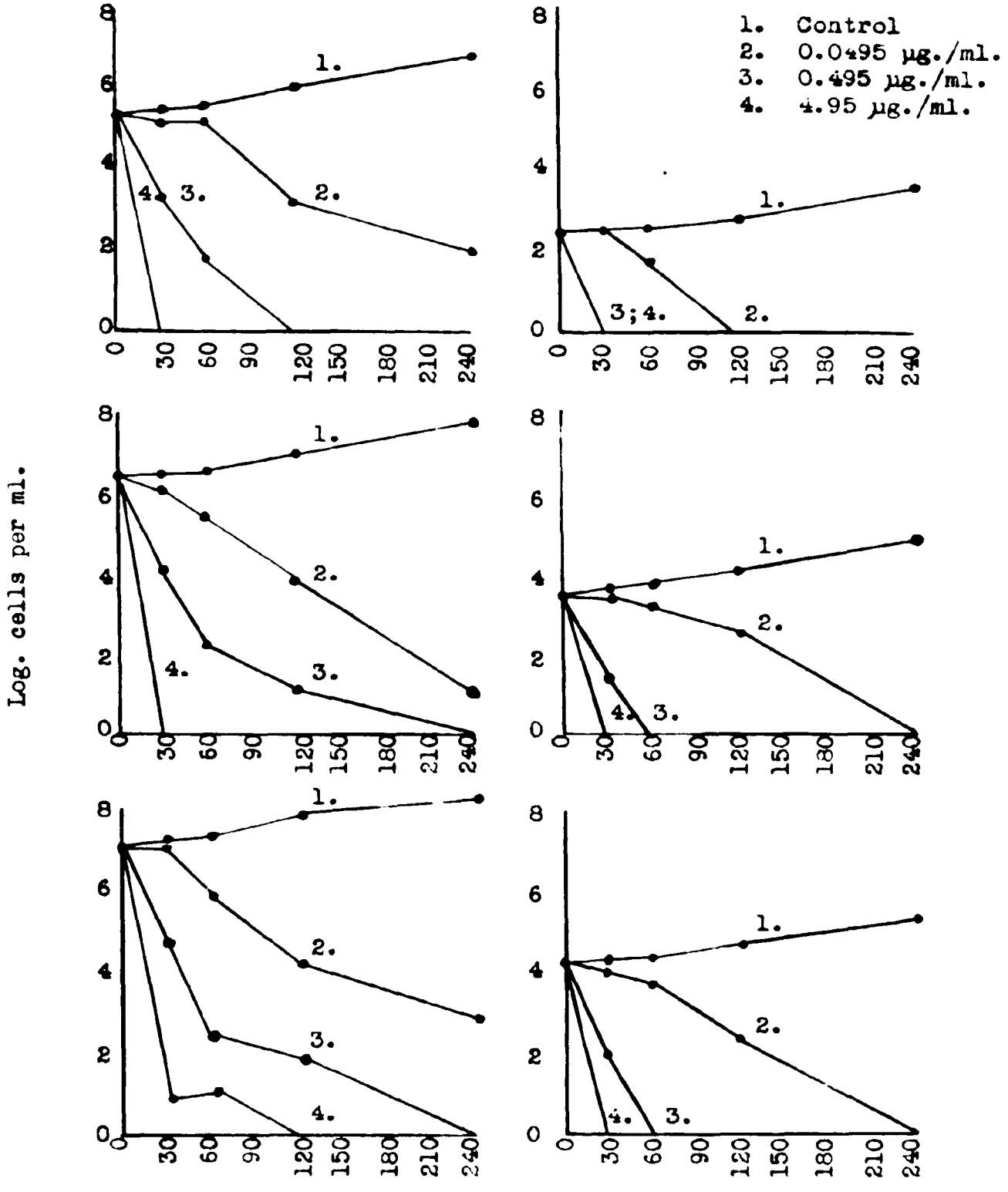


Figure 7

Effect of size of inoculum on action of neomycin (Strain 13)

(These graphs are based on data in appendix, table XXIV)

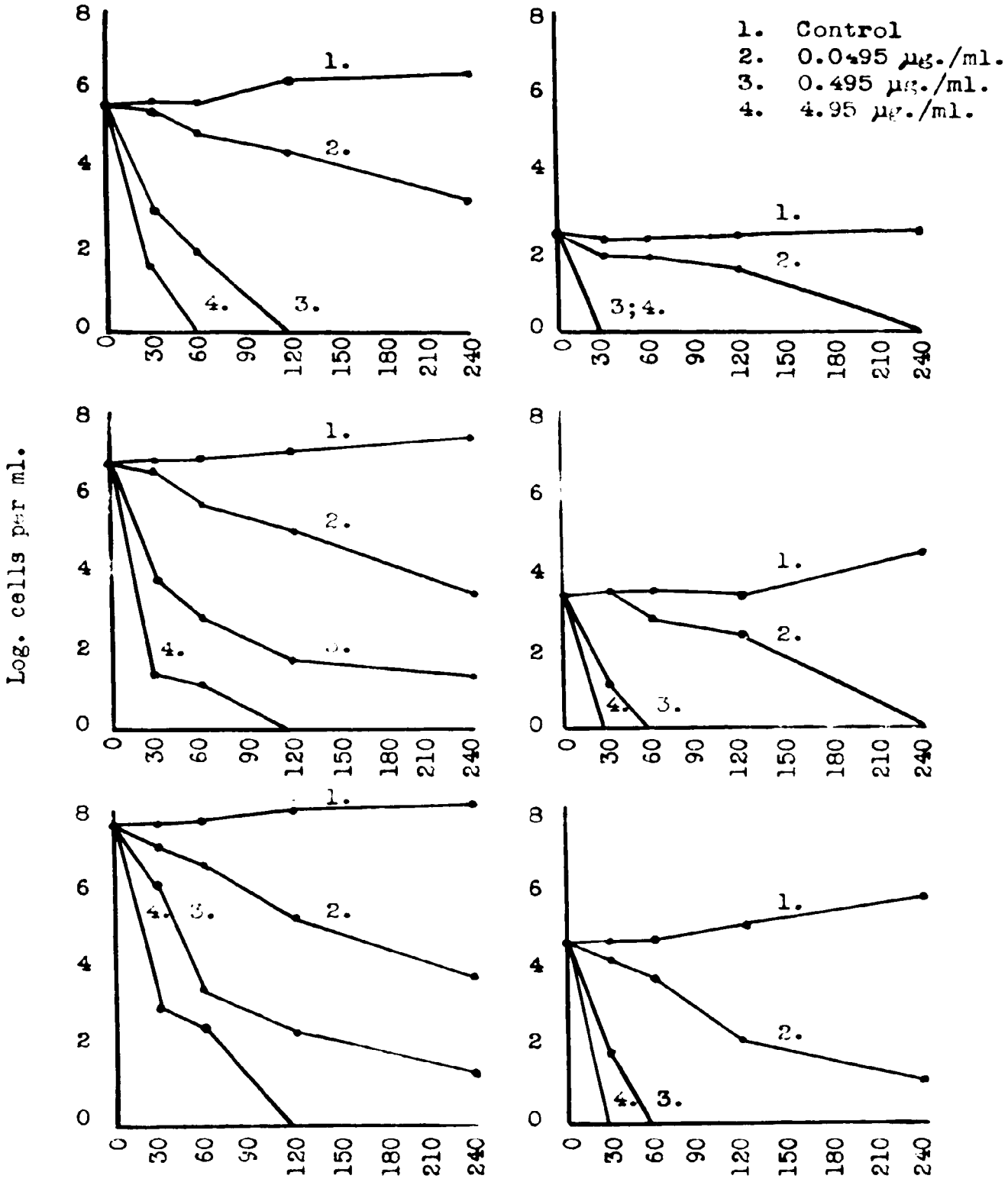


Figure 8

Effect of size of inoculum on action of neomycin (Strain 17)

(These graphs are based on data in appendix, table XXIV)

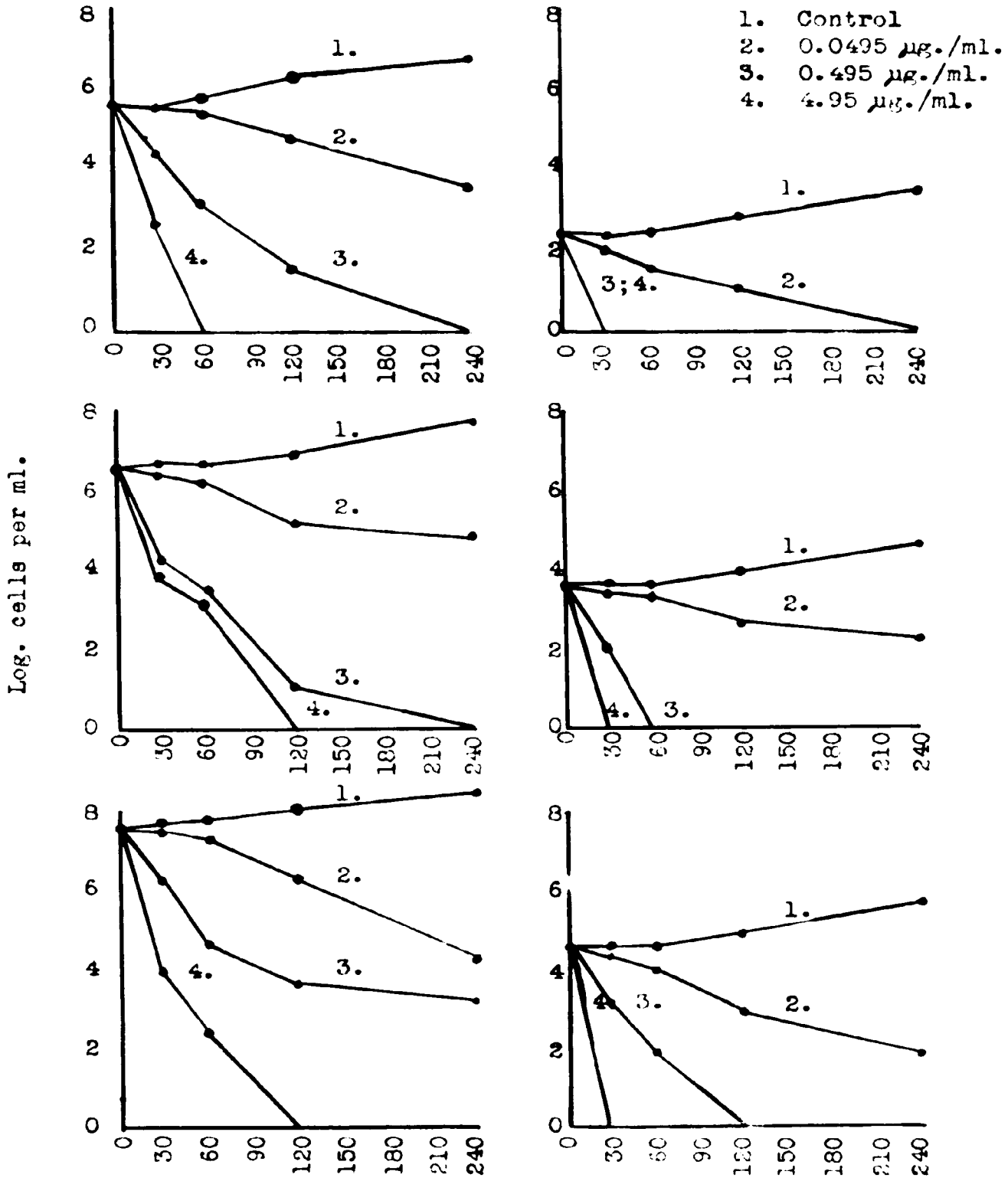
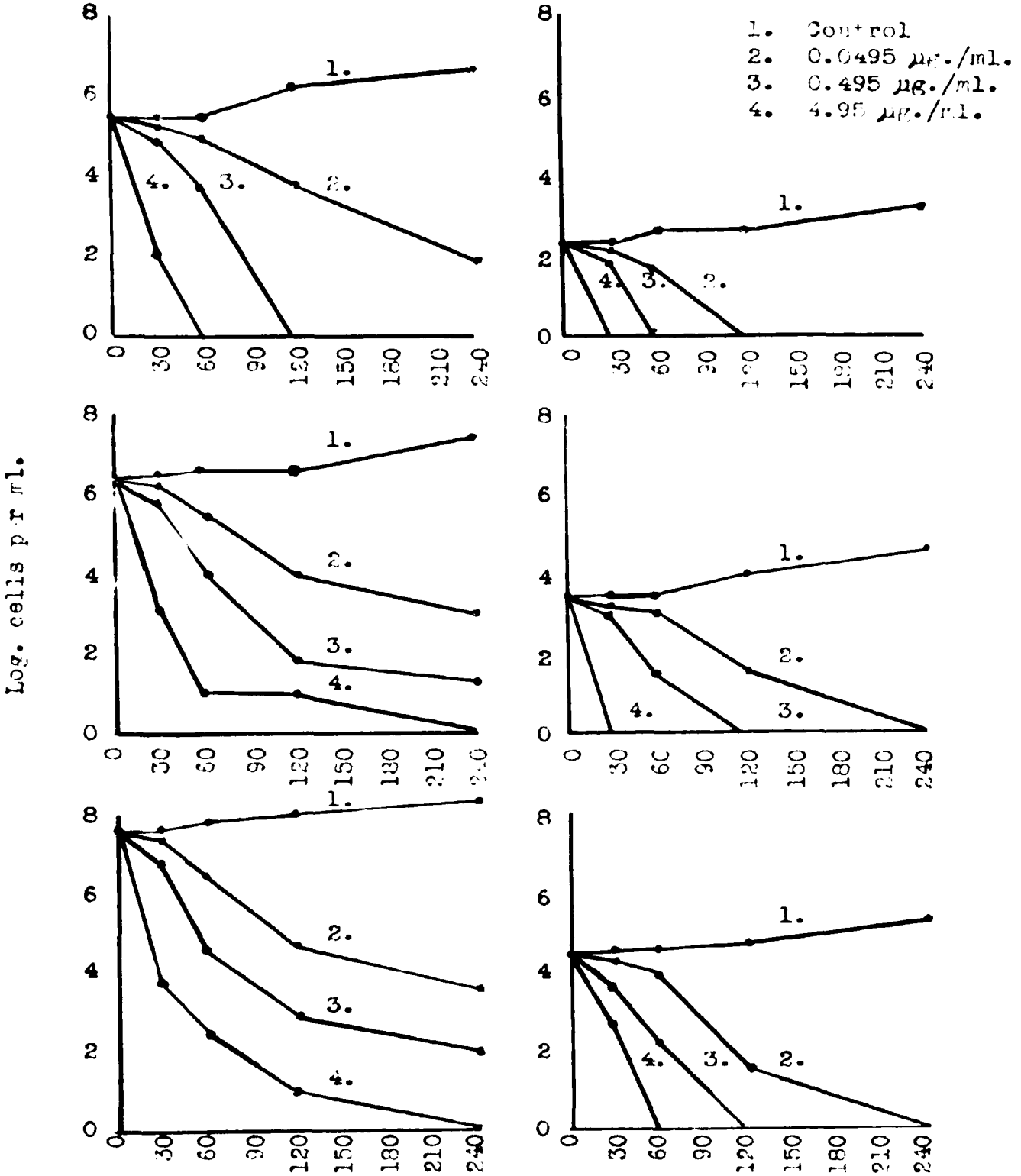


Figure 9

Effect of size of inoculum on action of neomycin (Strain 29)

(These graphs are based on data in appendix, table XXIV)



With a 10-fold increase in the concentration of neomycin, 4.95 µg./ml., all 6 of the different sized inocula tested were reduced to less than 10 organisms per ml. by the end of 2 to 4 hours; inocula of ten thousand organisms or less were reduced to less than 10 organisms per ml. by the end of 30 minutes. Table XXIV in the appendix shows that there was considerable reduction in the number of organisms before the original sample for a pour-plate could be removed from the tubes containing 4.95 µg./ml. of neomycin. That is, the action of neomycin was so rapid that the original (0 time) colony count of those pour-plates containing neomycin was considerably less than that of the control plate. Garrod (1948) reported that streptomycin acts more rapidly at higher concentrations but that the action of penicillin is not accelerated by an increased concentration of it above a minimum level. This same phenomenon was demonstrated earlier with penicillin by Hobby, Meyer, and Chaffee (1942).

A concentration of 0.0495 µg. of neomycin per ml. was effective in 4 hours for all strains only when the smallest inoculum, one thousand organisms, was used. It failed to reduce the growth of any of the strains to below 10 organisms per ml. when an inoculum of one million organisms or more was used. However, this concentration of neomycin did kill over 99 per cent of the organisms of each different sized inoculum in 4 hours. Chang, Kuan-how and Stafseth (1950) reported that the smaller the size of inoculum of S. pullorum, the higher the bactericidal titer of streptomycin. Lenert and Hobby (1947) found that with many strains the number of organisms in relation to the concentration of streptomycin so greatly influences its effect that size of inoculum

is probably a limiting factor in the usefulness of streptomycin. These same authors state that the number of units of penicillin necessary for the inhibition of a culture is altered only slightly by a large variation in the number of organisms present. Garrod (1948) reported that a small inoculum is destroyed rapidly by streptomycin, whereas a proportion of a large one always survives.

In summary, with all strains the inoculum could be varied from one thousand to one million organisms without influencing the action of 0.495 µg. of neomycin per ml. significantly when there was an incubation period of 4 hours. An increased concentration of neomycin, 4.95 µg./ml., was effective in 2 to 4 hours against all 6 of the different inocula tested. A decreased concentration of neomycin, 0.0495 µg./ml., reduced the number of organisms to below 10 per ml. only in the tube with the smallest inoculum but did kill over 99 per cent of the organisms of each different inoculum in 4 hours. In these experiments with the 5 strains of S. pullorum used, it was possible to change the size of the inoculum one hundred thousand-fold with 3 different concentrations of neomycin, 4.95, 0.495, 0.0495 µg./ml., without influencing significantly its action. Using E. coli and a beta streptococcus, Worth, Chandler, and Bliss (1950) studied the effect of the size of inoculum on the action of neomycin and concluded that the necessary increase in bactericidal concentration of neomycin with increase in inoculum is of the same order of magnitude as that for aureomycin, chloromycetin, and penicillin. The rate of action of neomycin was most rapid in the highest concentration used; it decreased noticeably with each 10-fold decrease in concentration. This condition held for all

inocula. Waisbren and Spink (1950a) reported that 20 µg. of neomycin per ml. killed all of the organisms of a 2 X 10^6 inoculum of E. coli in 2 hours. Waksman, Frankel, and Graessle (1949) have shown that 20 units of neomycin, assay 30 to 100 units per mg., incubated with a heavy suspension of S. pullorum were completely bactericidal in 3½ hours.

Effect of some of the constituents of two culture media and sodium chloride on the antibacterial activity of neomycin. The data pertaining to the effect of arginine, cystine, histidine, leucine, and valine on the antibacterial activity of neomycin is presented in table XII (page 61). Repetition of experiments to test the effect of these acids on neomycin activity did not consistently give the same results. That is, although the amount of neomycin necessary to kill strain 11, when leucine was added, was 2-fold greater than that in the control tube according to this particular data, it was found in another experiment that the same concentration of neomycin was lethal in both the tube to which leucine had been added and in the control tube. Also, in the presence of different amino acids, the bactericidal titer of neomycin varied in different experiments with other strains. It may be tentatively concluded, therefore, that no one particular amino acid or group of amino acids, in the concentrations tested, affects markedly the action of neomycin. The fact that the bactericidal concentration of neomycin varied as much as 2 to 4-fold when the same experiments were repeated, although all conditions were maintained constant as much as possible, is probably not unique for this antibiotic or this

set of experiments. West, Doll, and Edwards (1945) tested the sensitivity of Salmonella cultures to streptomycin and reported that its bactericidal titer varied when the same experiments were repeated. Chang, Kuan-how (1949) reported similar difficulties when he tested the sensitivity of S. pullorum to streptomycin.

Table XII (page 61) gives the results of an experiment in which asparagine, calcium pantothenate, and thiamine hydrochloride were added individually to 1 per cent tryptose-water, and the effect on the action of neomycin was noted. The bactericidal titer of neomycin often varied from 2 to 4-fold with the same strain in the same medium when the same experiment was repeated. As with amino acids, there seems to be no distinct influence on the action of neomycin by these substances.

When glucose was added to 1 per cent tryptose-water, a 2-fold increase in concentration of neomycin was needed to kill strains 11, 13, 17, and 29; and a 4-fold increase was needed to kill strain 12. These results are given in table XII. Similar results were obtained when this experiment was repeated. Also, all strains were tested in the synthetic medium with the concentration of glucose increased 4 and 8-fold. A 2 to 4-fold increase in concentration of neomycin was necessary to kill all strains in both media. These results then indicate that glucose interferes slightly with the activity of neomycin. Waksman, Lechevalier, and Harris (1949) reported that the presence of glucose in a test medium reduces the potency of neomycin by favoring either acid production or growth of the test organisms.

Referring to table XII again, it is seen that a 2 to 4-fold

increase in the concentration of neomycin was necessary to kill each strain when either peptone or beef extract was added to 1 per cent tryptose-water. Similar data were obtained when this experiment was repeated. On the other hand, the data from the sensitivity tests (see tables VII and VIII, pages 41 and 43) show that about the same concentration of neomycin was bactericidal in either 1 per cent tryptose-water or nutrient broth. On the basis of these results it was concluded that either peptone or beef extract in 1 per cent tryptose-water interfere slightly with the action of neomycin but that peptone and beef extract together in nutrient broth do not interfere.

A 10 to 20-fold increased concentration of neomycin was necessary to kill these strains when a quantity of sodium chloride sufficient to give a final concentration of 0.85 per cent was added to 1 per cent tryptose-water. This effect was noted consistently and is given in table XII. Thus, there seems to be no doubt that sodium chloride interferes with the action of neomycin. As was pointed out in the discussion of the sensitivity tests in the synthetic medium, a greater concentration of neomycin was necessary to kill the different strains in it than when they were tested in either 1 per cent tryptose-water or nutrient broth. This might be due to the interference of the salts of the synthetic medium for it had as part of its composition a mineral base of different salts.

In summary, arginine, cystine, histidine, leucine, valine, calcium pantothenate, asparagine, and thiamine hydrochloride, in the concentrations tested, did not seem to influence significantly the action of neomycin; glucose, peptone, and beef extract, in the concentrations

tested, seemed to interfere slightly with the action of neomycin; sodium chloride, in the concentration tested, definitely affected the action of neomycin.

TABLE XII

THE EFFECT OF SOME OF THE CONSTITUENTS OF 2 CULTURE MEDIA AND SODIUM CHLORIDE ON THE ANTIBACTERIAL ACTIVITY OF NEOMYCIN

Strain	µg./ml. neomycin						
	0.312	0.156	0.078	0.039	0.019	0.009	Control
				Arginine			
11	-*	-	48**	24	24	24	24
12	-	-	-	48	24	24	24
13	-	-	-	-	48	24	24
17	-	-	168	48	24	24	24
29	-	-	-	48	24	24	24
				Cystine			
11	-	-	48	24	24	24	24
12	-	-	-	168	48	24	24
13	-	-	-	-	48	24	24
17	-	-	-	-	24	24	24
29	-	-	-	48	24	24	24
				Histidine			
11	-	-	48	24	24	24	24
12	-	-	-	48	48	24	24
13	-	-	-	-	48	24	24
17	-	-	-	48	24	24	24
29	-	-	48	48	24	24	24
				Leucine			
11	-	72	48	24	24	24	24
12	-	-	72	48	48	24	24
13	-	-	-	-	48	24	24
17	-	-	-	48	24	24	24
29	-	-	168	48	24	24	24
				Valine			
11	-	-	48	24	24	24	24
12	-	-	-	48	24	24	24
13	-	-	-	-	48	24	24
17	-	-	-	48	24	24	24
29	-	-	72	48	24	24	24
				Control			
11	-	-	48	24	24	24	24
12	-	-	-	-	24	24	24
13	-	-	-	-	48	24	24
17	-	-	72	48	24	24	24
29	-	72	48	48	24	24	24

*Indicates no growth after incubation for 1 week.
**Indicates the number of hours before growth occurred.

TABLE XII (continued)

THE EFFECT OF SOME OF THE CONSTITUENTS OF 2 CULTURE MEDIA AND SODIUM CHLORIDE ON THE ANTIBACTERIAL ACTIVITY OF NEOMYCIN

Strain	µg./ml. neomycin						
	1.25	0.625	0.312	0.156	0.078	0.039	Control
				Peptone			
11	-	-	-	72	24	24	24
12	-	-	-	24	24	24	24
13	-	-	-	24	24	24	24
17	-	-	72	48	24	24	24
29	-	-	72	48	24	24	24
				Beef Extract			
11	-	-	72	72	24	24	24
12	-	168	72	48	24	24	24
13	-	-	-	24	24	24	24
17	-	-	-	24	24	24	24
29	-	-	-	24	24	24	24

Strain	µg./ml. neomycin						
	0.312	0.156	0.078	0.039	0.019	0.009	Control
				Control			
11	-	-	168	48	24	24	24
12	-	-	72	24	24	24	24
13	-	-	48	24	24	24	24
17	-	-	48	24	24	24	24
29	-	-	48	24	24	24	24

TABLE XII (continued)

THE EFFECT OF SOME OF THE CONSTITUENTS OF 2 CULTURE MEDIA AND SODIUM CHLORIDE ON THE ANTIBACTERIAL ACTIVITY OF NEOMYCIN

Strain	µg./ml. neomycin						
	0.312	0.156	0.078	0.039	0.019	0.009	Control
				Glucose			
11	-	72	24	24	24	24	24
12	-	72	24	24	24	24	24
13	-	24	24	24	24	24	24
17	-	168	24	24	24	24	24
29	-	24	24	24	24	24	24
				Asparagine			
11	-	48	24	24	24	24	24
12	-	-	168	48	24	24	24
13	-	-	168	48	48	24	24
17	-	-	-	168	48	48	48
29	-	-	24	24	24	24	24
				Calcium Pantothenate			
11	-	-	168	72	24	24	24
12	-	-	-	48	24	24	24
13	-	-	168	24	24	24	24
17	-	168	72	24	24	24	24
29	-	-	-	72	48	24	24
				Thiamine Hydrochloride			
11	-	-	-	48	24	24	24
12	-	-	168	168	48	48	24
13	-	-	48	48	24	24	24
17	-	-	-	-	48	24	24
29	-	72	72	48	24	24	24
				Control			
11	-	-	48	48	24	24	24
12	-	-	-	48	48	24	24
13	-	-	168	168	48	24	24
17	-	-	48	48	48	24	24
29	-	-	72	48	24	24	24

TABLE XII (continued)

THE EFFECT OF SOME OF THE CONSTITUENTS OF 2 CULTURE MEDIA AND SODIUM CHLORIDE ON THE ANTIBACTERIAL ACTIVITY OF NEOMYCIN

Strain	µg./ml. neomycin						
	12.5	6.25	3.12	1.56	0.78	0.39	Control
				Sodium Chloride			
11	-	-	-	24	24	24	24
12	-	-	-	-	24	24	24
13	-	-	-	-	24	24	24
17	-	-	-	-	48	24	24
29	-	-	-	24	24	24	24

Strain	µg./ml. neomycin						
	0.312	0.156	0.078	0.039	0.019	0.009	Control
				Control			
11	-	-	48	48	24	24	24
12	-	-	-	48	48	24	24
13	-	-	168	168	48	24	24
17	-	-	48	48	48	24	24
29	-	-	72	48	24	24	24

SUMMARY AND CONCLUSIONS

The five strains of S. pullorum studied grew most rapidly in nutrient broth; they grew more rapidly in 1 per cent tryptose-water than in the synthetic medium.

There was no evidence of an initial stationary phase for any of the strains when they were grown in the three different culture media.

The lag phase was of about the same duration for all strains, except 11, when they were grown in 1 per cent tryptose-water. It was the same for all strains when they were grown in nutrient broth. Two of the 5 strains had no lag phase when grown in the synthetic medium, and the others had one of 6 to 12 hours, varying with the strain.

The average generation time during the logarithmic phase was longer when these strains were grown in 1 per cent tryptose-water than when they were grown in nutrient broth. As a result, they were in this phase longer when cultured in 1 per cent tryptose-water than when cultured in nutrient broth. The average generation time during this phase was the longest when they were grown in the synthetic medium, and thus extended considerably this phase.

The negative acceleration phase was of about the same length for most of these strains whether they were growing in 1 per cent tryptose-water or in nutrient broth, but varied from 0 to 12 hours when they were cultured in the synthetic medium.

The maximum number of viable organisms in the stationary phase was about the same for all strains when they were grown in either 1 per cent tryptose-water or nutrient broth for 36 hours, but it was a good deal higher when they were grown in the synthetic medium for 60 hours.

Comparable growth curves for organisms grown in the same medium, either 1 per cent tryptose-water or nutrient broth, were established by two different procedures.

The synthetic medium developed for these strains of S. pullorum was composed of arginine, cystine, histidine, leucine, valine, thiamine hydrochloride, calcium pantothenate, glucose, salts, asparagine, and trace elements.

Leucine was found essential for all strains.

About the same concentration of neomycin was sufficient to stop the growth of these strains when they were grown and tested in either 1 per cent tryptose-water or nutrient broth. Forty-one to 321 times more neomycin was needed to kill the organisms when they were grown and tested in the synthetic medium than when they were grown and tested in either 1 per cent tryptose-water or nutrient broth.

Neomycin was bactericidal in concentrations only slightly higher than that in which it was bacteriostatic.

The sensitivity of an organism to neomycin was governed by the medium in which it was tested rather than by that in which it was grown.

The action of neomycin was favored by an alkaline medium.

The antibacterial activity of neomycin was not affected significantly by lowering the temperature from 37°C. to 4°C.

Neomycin was just as lethal to these organisms when they were in the stationary phase or dividing very slowly as when they were in the logarithmic phase or dividing at a maximum rate.

With the 5 strains of S. pullorum used, it was possible to change the size of the inoculum one hundred thousand-fold with 3 different concentrations of neomycin, 4.95, 0.495, and 0.0495 µg./ml., without influencing significantly its action.

The rate of action of neomycin was most rapid in the highest concentration used; it decreased noticeably with each 10-fold decrease in concentration. This condition was true for the different sized inocula.

Arginine, cystine, histidine, leucine, valine, asparagine, calcium pantothenate, and thiamine hydrochloride, in the concentrations tested, did not influence significantly the action of neomycin; glucose, peptone, and beef extract, in the concentrations tested, seemed to interfere slightly with the action of neomycin; sodium chloride, in the concentration tested, definitely affected the action of neomycin.

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APPENDIX

TABLE XIII

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN 1 PER CENT TRYPTOSE-WATER*

Experiment # 17

Hours	Strains				
	11	12	13	17	29
0	68	60	99	65	111
3	95	172	241	276	236
6	750	1,490	2,950	2,400	1,600
9	3,000	24,300	21,000	40,000	6,900
12	14,000	330,000	400,000	680,000	47,000
15	160,000	3,500,000	4,100,000	2,350,000	160,000
18	1,430,000	28,100,000	35,000,000	44,000,000	1,440,000
21	**	**	**	**	**
24	68,000,000	138,000,000	186,000,000	278,000,000	102,000,000

Figures represent organisms per milliliter.

*Data derived from an experiment by the single source culture method.

**No count made at this time interval.

TABLE XIV

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN 1 PER CENT TRYPTOSE-WATER*

Experiment # 20

Hours	Strains				
	11	12	13	17	29
0	28	162	76	226	32
3	29	419	179	571	102
6	264	4,180	1,750	8,140	501
9	1,755	32,900	16,250	32,500	5,000
12	7,640	313,000	158,000	174,000	14,100
15	171,000	3,360,000	1,140,000	1,460,000	101,000
18	1,170,000	27,200,000	19,800,000	13,100,000	1,030,000
21	4,810,000	**	**	**	6,400,000
24	36,000,000	132,500,000	143,500,000	159,000,000	64,000,000
27	106,500,000				215,500,000
30	219,000,000				223,000,000
33	269,000,000				270,000,000
36	304,500,000				281,500,000

Figures represent organisms per milliliter.

Data for strains 11 and 29 have been added from a separate experiment with them.

*Data derived from an experiment by the single source culture method.

**No count made at this time.

TABLE XV

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN 1 PER CENT TRYPTOSE-WATER*

Experiment # 21

Hours	Strains				
	11	12	13	17	29
0	98	92	171	81	130
3	482	310	690	369	429
6	1,267	4,095	8,300	6,140	1,710
9	14,135	49,550	86,300	86,100	6,050
12	74,700	800,000	850,000	1,120,000	13,800
15	385,000	10,200,000	13,800,000	17,500,000	99,000
18	5,000,000	91,000,000	126,000,000	163,000,000	660,000
21	19,300,000	**	**	**	1,460,000
24	71,000,000	208,000,000	268,000,000	373,000,000	22,100,000

Figures represent organisms per milliliter.

Data for strains 11 and 29 have been added from a separate experiment with them.

*Data derived from an experiment by the single source culture method.

**No count made at this time.

TABLE XVI

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF *S. PULLORUM* GROWN IN 1 PER CENT TRYPTOSE-WATER*

Experiment # 46

Hours	Strains				
	11	12	13	17	29
0	175	165	223	129	115
3	260	440	530	420	350
6	380	10,900	14,400	8,300	1,830
9	2,100	121,000	88,000	102,000	13,000
12	15,000	1,190,000	1,220,000	990,000	42,000
15	830,000	30,300,000	22,300,000	15,400,000	210,000
18	3,000,000	113,000,000	178,000,000	147,000,000	2,300,000
21	30,000,000	214,000,000	174,000,000	220,000,000	18,000,000
24	110,000,000	201,000,000	185,000,000	208,000,000	141,000,000
27	220,000,000	175,000,000	190,000,000	248,000,000	223,000,000
30	230,000,000	200,000,000	220,000,000	302,000,000	261,000,000
33	227,000,000	210,000,000	256,000,000	340,000,000	280,000,000
36	**	231,000,000	230,000,000	320,000,000	250,000,000

Figures represent organisms per milliliter.

* Data derived from an experiment by the tube method.

**Failed to inoculate the tube.

TABLE XVII

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH*

Experiment # 9

Hours	Strains				
	11	12	13	17	29
0	101	40	42	39	95
3	208	50	135	120	619
6	1,337	280	1,180	1,100	9,025
9	16,400	60,000	62,000	43,000	210,000
12	161,000	310,000	1,060,000	430,000	6,105,000
15	2,860,000	3,800,000	7,600,000	7,100,000	57,500,000
18	25,350,000	21,000,000	116,000,000	26,000,000	113,000,000
21	132,000,000	138,000,000	232,000,000	168,000,000	213,500,000
24	208,000,000	265,000,000	182,000,000	223,000,000	254,000,000

Figures represent organisms per milliliter.

Data for strains 11 and 29 added from another experiment.

*Data derived from an experiment by the single source culture method.

TABLE XVIII

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH*

Experiment # 13

Hours	Strains				
	11	12	13	17	29
0	91	97	27	37	110
3	258	465	157	207	474
6	1,437	2,542	3,800	4,000	5,090
9	17,900	77,200	136,000	86,000	99,500
12	165,000	1,940,000	5,100,000	2,640,000	1,470,000
15	2,060,000	56,800,000	**	**	91,000,000
18	24,250,000	109,000,000	164,000,000	119,000,000	207,000,000
21	142,000,000	186,000,000	**	**	190,500,000
24	212,500,000	226,500,000	281,000,000	216,000,000	280,000,000

Figures represent organisms per milliliter.

Data for strains 13 and 17 added from another experiment.

*Data derived from an experiment by the single source culture method.

**No count made at this time.

TABLE XIX

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH*

Experiment # 11

Hours	Strains				
	11	12	13	17	29
0	35	50	81	108	105
3	33	92	345	517	310
6	650	2,610	8,775	11,050	4,652
9	17,000	23,000	217,500	384,000	133,000
12	160,000	570,000	5,755,000	4,305,000	1,470,000
15	2,050,000	6,800,000	71,500,000	48,150,000	88,000,000
18	26,950,000	39,000,000	171,000,000	162,000,000	196,000,000
21	142,500,000	122,000,000	195,000,000	204,000,000	205,000,000
24	196,000,000	203,000,000	206,000,000	259,000,000	277,000,000

Figures represent organisms per milliliter.

Data for strain 29 added from another experiment.

*Data derived from an experiment by the single source culture method.

TABLE XX

POPULATION AT 3 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN NUTRIENT BROTH*

Experiment # 45

Hours	Strains				
	11	12	13	17	29
0	152	73	121	179	105
3	290	142	340	320	175
6	2,450	5,500	9,700	11,400	6,400
9	20,000	157,000	408,000	226,000	114,000
12	193,000	3,300,000	5,200,000	4,000,000	1,590,000
15	1,520,000	20,000,000	87,000,000	70,000,000	46,000,000
18	30,800,000	97,000,000	123,000,000	108,000,000	138,000,000
21	116,000,000	164,000,000	164,000,000	212,000,000	127,000,000
24	109,000,000	159,000,000	193,000,000	210,000,000	189,000,000
27	120,000,000	167,000,000	165,000,000	187,000,000	141,000,000
30	142,000,000	140,000,000	150,000,000	179,000,000	121,000,000
33	110,000,000	250,000,000	240,000,000	300,000,000	130,000,000
36	100,000,000	**	340,000,000	250,000,000	230,000,000

Figures represent organisms per milliliter.

*Data derived from an experiment by the tube method.

**Failed to inoculate the tube.

TABLE XXI

POPULATION AT 6 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN A SYNTHETIC MEDIUM*

Experiment # 78

Hours	Strains				
	11	12	13	17	29
0	65	168	167	148	189
6	492	650	520	720	812
12	3,610	3,520	3,210	2,980	9,475
18	17,000	22,000	13,300	8,200	58,475
24	61,000	176,000	106,500	50,000	645,000
30	270,250	2,642,250	680,000	332,125	9,600,000
36	1,708,000	36,000,000	5,812,500	1,137,500	140,000,000
42	15,649,750	130,875,000	27,600,000	9,040,000	692,000,000
48	103,350,000	937,500,000	93,000,000	29,737,500	1,735,000,000
54	320,500,000	**	777,000,000	487,000,000	**
60	**	**	**	1,635,000,000	**

Figures represent organisms per milliliter.

*Data derived from an experiment by the single source culture method.

**No count made at this time.

TABLE XXII

POPULATION AT 6 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN A SYNTHETIC MEDIUM*

Experiment # 78a

Hours	Strains				
	11	12	13	17	29
0	31	98	92	78	85
6	181	378	503	179	473
12	1,180	2,750	3,680	1,055	4,750
18	4,850	19,550	22,450	3,900	43,000
24	38,100	214,900	174,875	29,150	369,750
30	253,500	5,080,000	1,570,000	165,000	3,345,000
36	2,311,250	93,500,000	17,550,000	845,000	45,500,000
42	16,660,000	558,000,000	87,200,000	7,800,000	395,000,000
48	119,500,000	1,062,000,000	856,000,000	36,500,000	1,785,000,000
54	384,000,000	1,085,000,000	1,390,000,000	180,000,000	1,330,000,000
60	585,000,000	407,000,000	691,500,000	962,500,000	838,500,000

Figures represent organisms per milliliter.

*Data derived from an experiment by the single source culture method.

TABLE XXIII

POPULATION AT 6 HOUR INTERVALS OF 5 STRAINS OF S. PULLORUM GROWN IN A SYNTHETIC MEDIUM*

Experiment # 78b

Hours	Strains				
	11	12	13	17	29
0	30	123	118	93	103
6	230	437	512	440	329
12	1,400	2,035	2,910	2,240	4,000
18	3,210	17,550	15,850	8,475	41,700
24	33,000	145,500	77,700	53,000	386,500
30	244,000	1,150,000	700,000	168,600	2,250,000
36	2,250,000	31,500,000	2,800,000	1,030,000	40,000,000
42	16,150,000	170,000,000	20,000,000	10,700,000	230,000,000
48	120,000,000	908,000,000	93,000,000	77,000,000	1,113,000,000
54	320,000,000	775,000,000	550,000,000	382,000,000	535,000,000
60	640,000,000	1,211,000,000	1,083,000,000	1,100,000,000	1,116,000,000

Figures represent organisms per milliliter.

*Data derived from an experiment by the single source culture method.

TABLE XXIV

EFFECT OF SIZE OF INOCULUM ON ACTION OF NEOMYCIN

A. Strain 11

Time	µg./ml. neomycin	Tube					
		1	2	3	4	5	6
0*	0.0495	11,000,000	1,210,000	78,000	12,300	1,120	110
0*	0.0	13,800,000	1,010,000	110,000	10,800	1,450	70
0	0.495	22,000,000	2,510,000	254,000	15,800	1,690	250
0	4.95	10,400,000	910,000	133,000	4,300	1,000	40
0	0.0	22,000,000	2,340,000	196,000	25,800	2,020	220
30*	0.0495	8,300,000	1,060,000	132,000	10,700	1,270	60
30*	0.0	13,500,000	2,000,000	130,000	12,500	1,060	80
30	0.495	1,872,000	416,000	65,000	2,180	380	20
30	4.95	13,650	740	220	10	<10	<10
30	0.0	22,900,000	2,390,000	227,000	22,800	1,850	270
60*	0.0495	4,300,000	310,000	53,000	6,000	580	10
60*	0.0	19,400,000	1,970,000	184,000	13,700	910	130
60	0.495	107,250	19,500	4,260	20	<10	<10
60	4.95	110	<10	<10	<10	<10	<10
60	0.0	19,900,000	2,630,000	210,000	17,200	2,280	180
120*	0.0495	767,000	28,000	7,400	190	90	<10
120*	0.0	21,000,000	2,880,000	630,000	22,000	2,410	170
120	0.495	7,800	600	80	<10	<10	<10
120	4.95	<10	<10	<10	<10	<10	<10
120	0.0	54,000,000	7,100,000	640,000	37,000	4,100	390
240*	0.0495	38,600	1,000	250	<10	<10	<10
240*	0.0	45,000,000	9,900,000	2,300,000	106,000	11,800	720
240	0.495	180	<10	<10	<10	<10	<10
240	4.95	<10	<10	<10	<10	<10	<10
240	0.0	87,000,000	21,200,000	2,560,000	166,000	25,000	1,290

Figures under time represent minutes; figures under tube represent organisms per ml.

*Data taken from another experiment.

TABLE XXIV (continued)

EFFECT OF SIZE OF INOCULUM ON ACTION OF NEOMYCIN

B. Strain 12

Time	µg./ml. neomycin	Tube					
		1	2	3	4	5	6
0	0.0495	22,100,000	2,960,000	242,000	13,800	2,260	270
0	4.95	5,600,000	1,290,000	88,000	2,300	450	70
0	0.0	21,100,000	2,440,000	222,000	15,100	2,280	240
0*	0.495	19,266,666	2,455,000	195,833	16,083	1,132	120
0*	0.0	20,933,333	2,436,666	206,333	17,766	1,930	245
30	0.0495	18,450,000	1,430,000	148,000	13,650	2,390	300
30	4.95	60	<10	<10	<10	<10	<10
30	0.0	27,200,000	2,760,000	283,000	23,000	2,880	260
30*	0.495	85,000	11,491	2,150	95	17	<10
30*	0.0	27,366,666	3,143,333	238,333	18,633	2,490	240
60	0.0495	1,105,000	260,000	180,700	5,910	1,240	50
60	4.95	120	<10	<10	<10	<10	<10
60	0.0	26,700,000	2,680,000	237,000	27,800	2,570	290
60*	0.495	225	138	50	<10	<10	<10
60*	0.0	26,333,333	3,106,000	285,000	25,766	3,530	205
120	0.0495	20,800	6,500	1,300	230	280	<10
120	4.95	<10	<10	<10	<10	<10	<10
120	0.0	71,000,000	8,800,000	910,000	59,000	8,000	460
120*	0.495	82	12	<10	<10	<10	<10
120*	0.0	63,500,000	6,150,000	795,000	38,500	6,050	400
240	0.0495	740	10	70	<10	<10	<10
240	4.95	<10	<10	<10	<10	<10	<10
240	0.0	146,000,000	47,000,000	5,020,000	198,000	37,200	2,560
240*	0.495	<10	<10	<10	<10	<10	<10
240*	0.0	345,000,000	33,000,000	4,650,000	219,500	31,500	2,700

*Data taken from the average of 3 individual experiments.

TABLE XXIV (continued)

EFFECT OF SIZE OF INOCULUM ON ACTION OF NEOMYCIN

C. Strain 13

Time	µg./ml. neomycin	Tube					
		1	2	3	4	5	6
0	0.0495	48,100,000	2,960,000	332,000	25,100	1,720	230
0	0.495	36,300,000	2,120,000	223,000	17,800	980	110
0	4.95	31,100,000	1,160,000	105,000	8,000	660	40
0	0.0	38,000,000	4,100,000	325,000	30,100	2,410	270
30	0.0495	17,200,000	2,910,000	327,000	13,500	1,600	60
30	0.495	931,600	3,800	880	50	10	<10
30	4.95	730	20	30	<10	<10	<10
30	0.0	31,500,000	4,920,000	382,000	43,800	2,900	150
60	0.0495	3,240,000	366,000	74,000	3,700	550	60
60	0.495	2,500	540	80	<10	<10	<10
60	4.95	250	10	<10	<10	<10	<10
60	0.0	44,500,000	5,100,000	427,000	42,800	2,400	170
120	0.0495	149,500	77,000	24,100	110	210	30
120	0.495	210	50	<10	<10	<10	<10
120	4.95	<10	<10	<10	<10	<10	<10
120	0.0	87,000,000	7,600,000	1,500,000	90,000	2,100	220
240	0.0495	4,740	1,920	1,640	10	<10	<10
240	0.495	20	20	<10	<10	<10	<10
240	4.95	<10	<10	<10	<10	<10	<10
240	0.0	92,000,000	14,900,000	2,360,000	502,000	25,000	210

TABLE XXIV (continued)

EFFECT OF SIZE OF INOCULUM ON ACTION OF NEOMYCIN

D. Strain 17

Time	µg./ml. neomycin	Tube					
		1	2	3	4	5	6
0	0.0495	34,600,000	3,620,000	342,000	28,700	4,140	340
0	0.495	34,900,000	3,760,000	364,000	17,400	2,840	240
0	4.95	26,000,000	2,400,000	301,000	12,200	1,000	60
0	0.0	34,800,000	3,520,000	355,000	28,500	3,800	300
30	0.0495	32,600,000	3,520,000	338,000	21,400	2,720	100
30	0.495	1,755,000	13,000	22,750	1,350	90	<10
30	4.95	8,450	8,760	370	<10	<10	<10
30	0.0	44,500,000	3,620,000	323,000	32,600	3,480	280
60	0.0495	17,000,000	1,300,000	227,000	8,100	1,880	30
60	0.495	33,150	3,240	1,350	60	<10	50
60	4.95	200	1,530	<10	<10	<10	<10
60	0.0	45,200,000	4,180,000	516,000	32,000	3,880	350
120	0.0495	1,625,000	150,000	61,600	650	390	10
120	0.495	430	<10	30	<10	<10	<10
120	4.95	<10	<10	<10	<10	<10	<10
120	0.0	109,000,000	8,500,000	2,640,000	67,000	8,600	700
240	0.0495	16,900	7,540	3,940	60	160	<10
240	0.495	160	<10	<10	<10	<10	<10
240	4.95	<10	<10	<10	<10	<10	<10
240	0.0	261,000,000	46,800,000	5,490,000	359,000	39,500	2,360

TABLE XXIV (continued)

EFFECT OF SIZE OF INOCULUM ON ACTION OF NEOMYCIN

E. Strain 29

Time	µg./ml. neomycin	Tube					
		1	2	3	4	5	6
0*	0.0495	17,200,000	2,910,000	185,000	20,400	1,710	100
0*	0.0	18,300,000	2,840,000	201,000	22,900	2,150	250
0	0.495	33,800,000	3,010,000	362,000	28,100	2,830	300
0	4.95	15,300,000	1,140,000	322,000	1,100	1,200	40
0	0.0	32,700,000	2,770,000	275,000	25,100	3,020	220
30*	0.0495	18,600,000	2,380,000	215,000	18,900	1,920	120
30*	0.0	22,800,000	2,480,000	198,000	20,300	2,440	220
30	0.495	4,797,000	565,500	80,000	3,620	940	60
30	4.95	6,630	1,180	130	370	<10	<10
30	0.0	31,900,000	2,930,000	256,000	30,000	2,390	180
60*	0.0495	2,800,000	241,000	75,000	4,000	920	40
60*	0.0	25,400,000	3,620,000	351,000	28,600	3,130	240
60	0.495	35,750	10,400	3,570	160	30	<10
60	4.95	200	10	<10	<10	<10	<10
60	0.0	44,900,000	3,580,000	321,000	31,200	3,430	390
120*	0.0495	41,700	9,900	4,500	30	30	<10
120*	0.0	60,000,000	6,700,000	640,000	45,600	7,300	400
120	0.495	720	70	<10	<10	<10	<10
120	4.95	10	50	<10	<10	<10	<10
120	0.0	85,000,000	4,200,000	1,310,000	40,000	9,000	430
240*	0.0495	3,700	900	75	<10	<10	<10
240*	0.0	149,000,000	13,200,000	1,880,000	147,000	22,700	1,080
240	0.495	100	20	<10	<10	<10	<10
240	4.95	<10	<10	<10	<10	<10	<10
240	0.0	172,000,000	24,000,000	3,970,000	198,000	31,400	1,790

*Data taken from another experiment.

SOURCE OF AMINO ACIDS AND OTHER ORGANIC NUTRIENTS

Biotin was purchased from Hoffmann-La Roche Inc., Nutley, New Jersey.

Pimelic acid was purchased from Eastman Kodak Co., Rochester, New York.

Alpha alanine, aspartic acid, choline, cystine, folic acid, lysine, glycine, histidine, inositol, leucine, methionine, nicotinamide, nucleic acid, pyridoxine, pantothenate, para-aminobenzoic acid, phenylalanine, riboflavin, thiamine, tryptophane, tyrosine, and valine were purchased from General Biochemicals, Inc., Laboratory Park, Chagrin Falls, Ohio.

Arginine, glutamic acid, proline, serine, and threonine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.