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In vitro Susceptibility of Pneumococci to Sulfonamide. Relationship between Size of Inoculum and Bacteriostasis.

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The importance of detecting sulfapyridine-fast strains of bacteria and the need of a convenient and dependable method for measuring "fastness" are now widely recognized.[1, 2] Several methods of determining the degree to which bacteria are inhibited by sulfonamide drugs *in vitro* have been described.[2-6] While attempting to apply these methods to routine use, questions arose as to the optimum inoculum and the optimum concentration of sulfonamide to use. It has been shown that sulfanilamide will inhibit or delay the growth of hemolytic streptococci when small inocula are tested, but large inocula of susceptible strains are not inhibited even by high concentrations of the drug.[7-10] A similar quantitative relationship has been shown for pneumococci and sulfapyridine.[6, 11] Since a more complete analysis of this relationship appeared to have an important bearing on the choice of a method for testing the susceptibility of bacteria *in vitro*, we have undertaken to determine the growth-inhibiting power of a wide range of sulfapyridine concentrations for varying numbers of pneumococci.

Materials and Methods. The medium used was beef heart infusion broth, pH 7.6 to 7.8, containing 2% neopeptone (Difco) and 0.5% NaCl. Sulfapyridine in the desired concentration was dissolved in this medium which was then reautoclaved. Two percent rabbit serum was added and the broth was dispensed in 1.8 ml amounts in

1 MacLeod, C. M., and Mirick, G. S., *Am. J. Pub. Health*, 1941, **31**, 34.

2 Lowell, F. C., Strauss, E., and Finland, M., *Ann. Int. Med.*, 1940, **14**, 1001.

3 Bliss, E. A., and Long, P. H., *New England J. Med.*, 1937, **217**, 18.

4 Fleming, A., *Lancet*, 1938, **235**, 74.

5 MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

6 Spring, W. C., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **19**, 163.

7 Colebrook, L., Buttle, G. A. H., and O'Meara, R. A. Q., *Lancet*, 1936, **231**, 1323.

8 Nitti, F., Bovet, D., and Depierre, F., *Compt. rend. Soc. Biol.*, 1937, **124**, 16.

9 Osgood, E. E., and Brownlee, I. E., *J. Am. Med. Assn.*, 1938, **110**, 349.

10 Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds*, New York, 1939, p. 97.

11 McIntosh, J., and Whitby, L. E. H., *Lancet*, 1939, **236**, 431.

13 x 100 mm tubes. Seven-hour cultures of pneumococci in rabbit blood broth were decimally diluted to 10^{-7} and 0.2 ml of each dilution added to tubes of the test medium containing sulfapyridine and to the control medium without sulfapyridine. Blood agar plates were poured with 0.5 ml of the 10^{-7} dilution in order to estimate the number of pneumococci in the inocula. Tests were incubated at a temperature of 35° to 36°C, because it was found that the growth of some strains was variable at temperatures slightly over 37°C. Readings were made at 17, 24 and 40 hours. There was seldom any visible change in serum broth after 40 hours. This reading was therefore taken as the endpoint. Although a rapid test was desired, it was found that growth at 17 and 24 hours, particularly with small inocula, was too variable to be of much value. The presence or absence of visible turbidity was recorded, and subcultures were made only to exclude the possibility of contamination.

Results. Table I records the results of growth inhibition titrations with 5 strains of *Diplococcus pneumoniae.* The largest numbers of organisms inhibited by varying concentrations of sulfapyridine, under the conditions of the test, are expressed as logarithms. At least 4 separate titrations were performed with each strain and the results were averaged. The data in Table I are shown in graphical form in Fig. 1, where the logarithms of the numbers of organisms inhibited are plotted against the concentrations of sulfapyridine, and smooth curves drawn through the points thus obtained.

TABLE I.
Logarithms of the Largest Numbers of Pneumococci Inhibited by Various Concentrations of Sulfapyridine.

Mg sulfapyridine per 100 ml broth	Strain				
	A47-III	Bog-XXIII	Bra-VII	41385-III	41382-XIV
0.1	<1.0	—	—	—	—
0.25	<1.1	—	—	—	—
0.5	2.9	<1.1	—	—	—
1	3.9	<1.3	—	—	—
2	5.1	1.9	—	—	—
3	5.1	3.1	<1.3	—	—
4	5.1	3.8	<1.8	<1.5	—
6	5.1	5.1	2.2	1.9	—
8	5.3	5.0	3.6	3.0	—
10	5.1	4.9	4.1	3.9	—
12	—	—	4.8	3.9	—
16	5.3	5.3	5.1	4.0	—
20	—	5.1	5.3	4.1	1.0
24	—	—	—	4.9	2.6
32	—	5.3	5.2	5.0	3.0
48	—	—	5.2	5.0	4.0
64	—	5.3	—	5.0	4.9

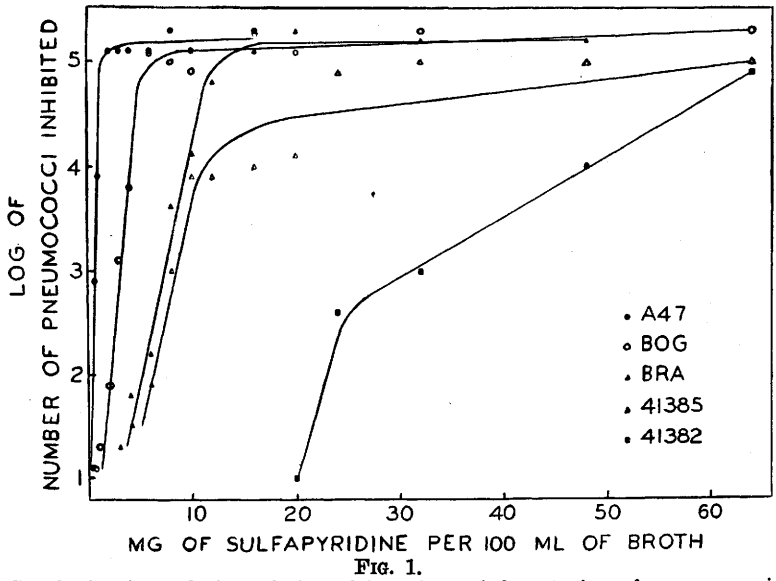

FIG. 1.

Graph showing relation of size of inoculum of five strains of pneumococci to inhibition of growth by sulfapyridine.

Differences in the susceptibility of the 5 strains are clearly illustrated.

Strains A47, Bog, and Bra were recently isolated in the course of routine bacteriological examinations. Strains 41385* and 41382* were stock strains known to be resistant to sulfapyridine. With 4 of the strains the concentration of the drug required to inhibit growth increased rapidly with the size of the inoculum up to about 100,000 organisms. The curve of this relationship appears to be an ascending straight line. Inocula above 100,000 were not inhibited regardless of the concentration of the drug and the relative susceptibility of the strain. The greater the resistance of the strain to sulfapyridine, the more gradual the slope of the curve. This was particularly noticeable with the most resistant strain, 41382. Three other resistant strains* were studied but sufficient data were not obtained to plot curves because the smallest inocula of these strains were only slightly inhibited by a concentration of 64 mg per 100 ml. Curves obtained for strains A47 and Bra with sulfathiazole were similar to those described with sulfapyridine except that less sulfathiazole than sulfapyridine was required to inhibit growth.

The results show that an attempt to titrate the sulfonamide susceptibility of a strain by testing varying inocula in a constant con-

* Furnished by the kindness of Dr. John K. Miller, New York State Department of Health, Albany.

centration of drug would have only a limited value because with any one concentration of sulfonamide an end point would be obtained for only a few of the strains which might be encountered. Using a single inoculum, such as 1000 organisms, and testing it in varying concentrations of sulfonamide, however, would theoretically give an end point with any strain. If 2 points on the logarithmic portion of the curve were determined, thereby indicating the slope of the curve and its position with reference to the abscissa, it should be possible, by interpolation, to determine the approximate concentration of drug required to inhibit the growth of any number of bacteria on this portion of the curve. Results could then be expressed in terms of the same number of bacteria for all strains tested, thereby avoiding the error due to variation in numbers of organisms present in different cultures of various strains. In our experience, the number of colonies developing from 0.2 ml of 10^{-6} dilutions of 7-hour blood broth cultures varied between 60 and 360.

In order to determine the degree of variation that might be expected in the results of repeated tests on the same strain the following experiment was performed. Twenty-five 7-hour cultures of strain Bog were decimally diluted. Two dilutions of each culture, 10^{-4} and 10^{-6}, were tested in beef heart infusion broth containing 2% rabbit serum and 1, 2, 3, 4, 5, 6, 8, and 10 mg of sulfapyridine per 100 ml. The tests were set up by 2 persons on 2 different days using different lots of sulfapyridine solutions on each day. The same lot of infusion broth was used throughout the experiment. The concentration of sulfapyridine required to inhibit visible growth for 40 hours was noted in each instance. The number of pneumococci present in the 2 inocula of each culture was estimated from colony counts. Two of the 25 tests were discarded because colonies failed to develop on poured plates. The concentration of sulfapyridine required to inhibit 1000 pneumococci was calculated algebraically from the observed data for each of the 23 remaining tests. The mean concentration of sulfapyridine required was 3.89 mg per 100 ml, with a standard deviation of 0.48. The lowest value obtained was 2.6 and the highest 4.8 mg per 100 ml. It was found that the concentration of sulfapyridine required to inhibit 1000 pneumococci could readily be estimated graphically within 0.2 mg of the figure determined algebraically.

The results of these repeated tests indicate that sulfonamide susceptibility can be determined by this method with a reasonable degree of accuracy. The expected variation is such, however, that tests should be performed at least in duplicate. The extent to which

results are reproducible depends upon the use of the same medium in comparative tests. One of our strains was tested in beef heart infusion broth with and without serum. Six mg of sulfapyridine per 100 ml were required to inhibit the growth of 24,000 pneumococci in broth containing 2% serum while the same inoculum failed to grow in a concentration of 1 mg per 100 ml without serum. Similar results were obtained with brain heart infusion broth (Difco) with and without serum. Unless serum was used, however, growth from small inocula in control tubes could not be depended upon. The increased amount of sulfapyridine required in broth containing serum was probably not due to the presence of sulfonamide inhibitor in the serum[5] but rather to the stimulation of growth by the serum.

Summary. Inhibition tests using varying numbers of pneumococci in varying concentrations of sulfapyridine and sulfathiazole indicated that except for highly resistant strains, there is a straight line relationship between the amount of drug required to inhibit growth and the number of pneumococci inoculated up to about 100,000. Larger inocula were not inhibited regardless of the amount of drug present. A method is described whereby this quantitative relationship may be applied to testing the sulfonamide susceptibility of pneumococci and expressing the results in terms of the concentration of sulfonamide required to inhibit the growth of a given number of bacteria.

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Sulfonamide Chemotherapy of Mouse Pneumonitis, Meningopneumonitis and Lymphogranuloma Venereum.

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Within the last few years two new virus-like agents which form elementary bodies have been described. The first of these, the agent of meningo-pneumonitis, was isolated by Francis and Magill[1] from ferrets which had received nasal washings from humans with symptoms of grippe. The authors believed the agent to have been carried in their stock of ferrets. The second, the agent of mouse pneumonitis, was isolated by Nigg[2] from an apparently normal stock

[1] Francis T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

[2] Nigg, C., *Science*, 1942, **95**, 49.