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Failure of Certain Growth Factors to Inhibit Sulfonamide Bacteriostasis in Simple Media.

LOWELL A. RANTZ. (Introduced by A. L. Bloomfield.)

From the Department of Medicine, Stanford University School of Medicine, San Francisco, California.

The mechanism of the action of the sulfonamide compounds in the inhibition of bacterial growth is, at the present time, imperfectly understood. Several substances, including peptones, purulent exudates,[1] methionine,[2] cozymase,[3] and para-amino benzoic acid,[4] have been shown to interfere with the bacteriostatic activity of these chemicals. It is now believed that para-amino benzoic acid is an essential growth factor which is required by most bacteria and synthesized by them.[5] It has been isolated from certain microörganisms. Because it resembles the sulfonamides chemically, and is an exceedingly potent inhibitor of their bacteriostatic activity, it is possible that the primary action of these chemicals on bacterial growth lies in their ability to interfere with the function of para-amino benzoic acid in the metabolism of these organisms.

Many other chemicals are also known to be essential to bacterial metabolism and several have been studied for possible activity as inhibitors of sulfonamide bacteriostasis. The most recent report is that of Strauss, Dingle and Finland,[6] who studied nicotinamide, thiamin, biotin, and yeast nucleic acids, and showed that these substances were without activity as sulfonamide inhibitors when tested in simple media.

It is the purpose of this report to describe somewhat similar observations which were begun before the appearance of their report, the growth factors studied being thiamin chloride, nicotinic acid, cocarboxylase, pyridoxine, riboflavin, calcium pantothenate and para-amino benzoic acid.*

Methods. Two organisms, *E. coli* and *Staphylococcus aureus* were used in these experiments. Casein hydrolysate known to be free of

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

2 Bliss, E. A., and Long, P. H., *Bul. J. H. Hosp.*, 1941, **69**, 14.

3 West, R., and Coburn, A. F., *J. Exp. Med.*, 1940, **72**, 91.

4 Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

5 Fildes, P., *Lancet*, 1940, **1**, 955.

6 Strauss, Elias, Dingle, J., and Finland, M., *J. Immunol.*, 1941, **42**, 313, 331.

* Obtained through the courtesy of Merck and Company, Rahway, New Jersey.

sulfonamide inhibitors was used as a nitrogenous base for the simple media used in the tests. Staphylococci grew well in a modification of the media described by Knight.[11] The observations on *E. coli* were conducted in a medium which contained .5% of ammonium sulphate, .5% of sodium chloride, .2% of dextrose, .3% of potassium diphosphate, and .2% of hydrolyzed casein in distilled water. Both media were adjusted to pH 7.6. The growth factors were dissolved in distilled water adjusted to pH 7, sterilized by Seitz filtration and added in serial dilutions to the base medium. A heavy growth of organisms within 24 hours was always obtained. Sulfathiazole was added to the medium in a concentration of 2.5 μg per milliliter, which produced marked bacteriostasis, but permitted slight visible growth within 48 to 72 hours. In each experiment a constant number of organisms were added to every culture tube, usually about 50 per milliliter.

The rate of bacterial growth under control and test conditions was determined by measuring the time of appearance of visible growth and the density of the cloud of organisms at intervals by means of a photoelectric nephelometer. The values obtained in this manner have been shown to be proportional to the number of viable organisms present during the period of rapid bacterial growth.

Results. Nicotinic Acid. Nicotinic acid has been shown to be an essential growth factor for many bacterial species, including *Staphylococcus aureus, C. diphtheriae, Shigella paradysenteriae,*[7] and *Brucella abortus.*[8]

The basal media described above failed to support the growth of *S. aureus* in the presence of an excess of thiamin chloride in the absence of nicotinic acid. The addition of 1 microgram per cubic milliliter was sufficient to cause the development of maximal bacterial growth, no further increase being induced by concentrations of the chemical to 100 μg per milliliter; 1000 μg per milliliter strikingly interfered with the rate of multiplication, but failed to prevent the eventual development of maximal growth. No effect of sulfonamide bacteriostasis was demonstrated in media containing from 1 to 100 μg per milliliter of nicotinic acid. The base media supported luxuriant growth of *E. coli* in the absence of additional growth factors. Nicotinic acid in concentrations of 100 μg per milliliter failed to enhance the rate of growth of control preparations and had no effect whatsoever on sulfonamide bacteriostasis.

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[7] Koser, S. A., and Saunders, F., *Bact. Rev.*, 1938, **2**, 99.

[8] Koser, S. A., Breslove, B. B., and Dorfman, A., *J. Infect. Dis.*, 1941, **69**, 114.

[11] Knight, B. C. J. G., *Brit. J. Exp. Path.*, 1941, **16**, 315.

Thiamin Chloride. Thiamin has been shown to be essential to the growth of *S. aureus,* certain molds,[7] and *Brucella abortus,*[8] among other bacteria. The base medium used in these experiments in the presence of excess nicotinic acid supported minimal growth of *S. aureus* and was, therefore, not entirely free of thiamin. The addition of .00001 μg per milliliter of this chemical as the chloride was necessary to obtain optimal growth. A further increase in concentration to 100 μg per milliliter failed to further increase, and concentration greater than 1000 μg decreased, the rate of growth. Within the range of optimal growth, increasing amounts of thiamin chloride in the medium had no effect on sulfonamide bacteriostasis.

Neither the growth rate of *E. coli* or sulfonamide bacteriostasis of this organism was affected by the presence, in the base medium, of 100 μg per milliliter of thiamin chloride.

Cocarboxylase. Diphosphothiamin (Cocarboxylase) is an essential intermediary substance formed during the metabolism of thiamin. It was substituted for thiamin chloride in the same concentrations in a series of experiments similar to those just described. The results with both *S. aureus* and *E. coli* were approximately the same in every respect as those obtained with thiamin chloride.

Riboflavin. Riboflavin has been shown to be essential to the development of certain lactic acid bacteria[7] and streptococci.[9] Its addition to the basal media containing excess thiamin chloride and nicotinic acid in concentrations up to 200 μg per milliliter failed to enhance the rate of bacterial growth of *S. aureus* or to interfere with sulfonamide bacteriostasis. Similar results were obtained with *E. coli* growing in the base medium to which no additional growth factors other than riboflavin had been added.

Pyridoxine. Pyridoxine (Vitamin B6) appears to be a growth factor for certain streptococci[9] and other organisms. In experiments similar to those conducted with riboflavin, its addition to the base media in concentrations up to 1000 μg per milliliter failed to enhance the rate of bacterial growth or interfere with sulfonamide bacteriostasis.

Pantothenic Acid. Pantothenic acid has been shown to be essential to the growth of certain lactic acid bacilli, yeasts, and hemolytic streptococci,[9] as well as other microörganisms. The addition of this substance as the calcium salt to the simple medium containing excess thiamin and nicotinic acid used in these experiments slightly but definitely increased the rate of multiplication of *S. aureus* over that obtained in its absence. A concentration of 200 μg per milliliter

[9] Woolley, D. W., *J. Bact.*, 1941, **42**, 155.

was necessary to obtain this effect. Attempts were made to demonstrate a similar augmentation of the rate of growth of *E. coli* but were unsuccessful.

Sulfonamide bacteriostasis of *S. aureus* and *E. coli* was not affected by concentrations of calcium pantothenate up to 200 μg per milliliter.

Para-amino Benzoic Acid. Para-amino benzoic acid is known to be a powerful inhibitor of sulfonamide bacteriostasis, a constituent of the cells of certain yeasts,[4] and an essential growth factor for at least one of the Clostridia.[10]

In these experiments the addition of this chemical to the basal media in concentrations up to 100 μg per milliliter failed to enhance the growth of either *S. aureus* or *E. coli.* As was to be expected, para-amino benzoic acid markedly inhibited sulfonamide bacteriostasis, .001 μg per milliliter being adequate for the inhibition of 2.5 μg per milliliter of sulfathiazole.

Summary and Conclusions. Nicotinic acid, thiamin chloride, cocarboxylase, riboflavin, pyridoxine and para-amino benzoic acid have been shown to have no effect on the photometrically determined growth rate of *S. aureus* and *E. coli* in simple media. Calcium pantothenate definitely accelerated the rate of multiplication of *S. aureus* but not of *E. coli* when present in a concentration of 200 μg per milliliter. None of these substances except para-amino benzoic acid antagonized sulfathiazole bacteriostasis although present in amounts 40 times as great as the sulfonamide. Concentrations of sulfonamide 2,500 times that of the accompanying para-amino benzoic acid were completely ineffective in inhibiting bacterial growth.

These observations, therefore, support the view that the sulfonamides directly affect that part of the microbial metabolic system which specifically utilizes para-amino benzoic acid, since relatively large amounts of 6 other important growth factors completely failed to inhibit their bacteriostatic activity.

[10] Rubbo, S. D., and Gillespie, J. M., *Nature*, London, 1940, **146**, 838.