THE BACTERICIDAL POWER OF SULFANILAMIDE UNDER ANAEROBIC CONDITIONS[1]

GEORGE M. KALMANSON

Department of Bacteriology and Immunology, Washington University, St. Louis, Mo.

Received for publication June 7, 1940

As a result of the studies of Locke, Main, and Mellon (1938), Fox, German, and Janeway (1939), and others, and the chemical experiments of Shaffer (1939), all of whom have advocated the importance of the presence of oxygen and the formation of hydrogen peroxide as essential to the action of sulfanilamide, interest has developed as to whether sulfanilamide can exert its action on organisms *in vitro* under biologically anaerobic conditions.

Bliss and Long (1939) have reported that they were able to demonstrate bacteriostasis by sulfanilamide under anaerobic conditions, but did observe less effect at low oxygen tension. Broh-Kahn (1939) has likewise studied this question and substantiated the results of Bliss and Long. Using *Escherichia coli* as the test organism, this worker has elicited the following facts. When the organism is grown in nutrient extract broth it develops much better aerobically than anaerobically, and there is marked bacteriostasis with sulfanilamide under aerobic but not under anaerobic conditions. This was explained by stating that the drug affects (of the two types of nutrition) only the aerobic mechanism of nutrition while the anaerobic mechanism is unaffected. In evidence of this fact the degree of growth aerobically in the presence of the drug was claimed to be the same as anaerobically without the drug. Further evidence for this point of view was adduced by the observation that in the presence of glucose no effect of the drug was noted

[1] This work was supported from a grant made to Dr. P. A. Shaffer by the Rockefeller Foundation.

either aerobically or anaerobically. In this case the glucose was assumed to supply a pathway of nutrition unaffected by the drug. However, when the organism was grown in a synthetic medium quite different results were obtained. *E. coli* grows well aerobically on the salt mixture of Quastel, Stephenson, and Whetham (1925) when one per cent lactate is added. In order to obtain anaerobic growth, however, it is necessary to add one per cent nitrate. It was found that sulfanilamide had no effect on this organism growing aerobically with or without nitrate, but that anaerobically there was marked bacteriostasis. The author explained this by assuming that sulfanilamide prevents the oxidation of lactate by nitrate and so exerts its effect. It was claimed by this worker that very rigid anaerobic conditions were attained, and that there were few molecules of oxygen present in the medium. Fox (1940) has criticized the findings of Broh-Kahn, claiming that reducing conditions were not established in the lactate-nitrate medium under anaerobic conditions as could he demonstrated by the fact that there was no reduction of methylene blue.

A bacteriostatic effect is by no means as clear cut as a bactericidal one, and it was therefore thought of interest, in the light of the findings of White and Parker (1938) that sulfanilamide is bactericidal at 40°C., to study the action at this temperature under anaerobic conditions.

TECHNIQUE

The technique of White and Parker was followed mainly, with the exception that tryptose broth with one-tenth per cent glucose was used as the medium. 0.4 ml. of a 1:1,000 dilution of a 5-hour culture of the organism was seeded into 9 ml. of tryptose broth and thoroughly mixed. 4.0 ml. of the mixture was placed into each of two 50 ml. Erlenmeyer flasks (this produced a very thin layer of liquid, thus allowing more rapid equilibration with the atmosphere). To one flask was added 0.2 ml. of 0.44 per cent solution of sulfanilamide in distilled water, and to the control 0.2 ml. of sterile distilled water. The flasks were then incubated at 40°C. for 48 hours, at which time subcultures were

made and the viable bacterial population determined. Similar results were obtained when the cultures were incubated as tall narrow columns in tubes without shaking.

The anaerobiosis was produced in a jar in which phosphorus was ignited under sealed conditions (Varney, 1926). At all times, unburned phosphorus was present in order to react with any residual oxygen present. This method of producing anaerobiosis has been found satisfactory for growing the strictest anaerobes. The sulfanilamide solution was made up in water, double-distilled from glass, and prepared fresh every few days. The media were heated twenty minutes in flowing steam at 100°C. before inoculation in order to destroy any peroxides present. To further aid in the establishment of reducing conditions, 30 mgm. per cent of crystalline ascorbic acid was added to both the sulfanilamide and control flasks. A streptococcus, strain C203, was adapted to grow at 40°C. and was used for all the experiments involving streptococci.

The technique with *E. coli* was the same. Two strains of *E. coli*, "P.C." and "K3B", both of which grew well on the Quastel medium, were used in these experiments.

RESULTS WITH STREPTOCOCCUS

It was found that at 40°C., 20 mgm. per cent sulfanilamide was able to sterilize small inocula within 48 hours, but not in 24 hours, either aerobically or anaerobically, with or without the presence of ascorbic acid. A typical experiment is given in table 1.

Under these conditions sulfanilamide was not found to have any demonstrable action at 37°C. Suspecting that a possible explanation of this marked difference might be that at 37° the organism grew so rapidly that it overwhelmed the drug, we attempted to slow down this growth by diluting the medium with saline. No action of the drug could be demonstrated in this way. As the medium was diluted with saline till growth was less and less, there was exactly the same amount of growth in the flask containing drug as in the control, down to the point of cessation of growth. Similarly, the addition of 1:10,000

$FeCl_3$ to the medium in the hope of "potentiating" the drug had no effect. The reason for the striking temperature difference is at present obscure.

EXPERIMENTS WITH E. COLI

The results with *E. coli* are in some respects the same, and in some different from those with the streptococcus. Contrary to the results of Broh-Kahn, no action of the drug on *E. coli* grown in nutrient extract broth could be discerned. Since the possibility existed that this might be due to the presence of a

TABLE 1

Action of sulfanilamide on streptococcus C203 at 40°C. under aerobic and anaerobic conditions

	AEROBIC				ANAEROBIC			
	Ascorbic		Control		Ascorbic		Control	
	Sulf.	Cont.	Sulf.	Cont.	Sulf.	Cont.	Sulf.	Cont.
Gross appearance.....	Clear	Turbid	Clear	Turbid	Clear	Turbid	Clear	Turbid
Subcult. to broth.....	−	+	−	+	−	+	−	+
Subcult. to blood plate.	−	+	−	+	−	+	−	+
Bact. count per ml.*....	−	5.1×10^7	−	3.1×10^7	−	8.2×10^7	−	4.7×10^7

* Initial concentration of bacteria was 450 per ml.

different kind of peptone, we repeated the experiment using the tryptose-phosphate-glucose broth previously used for our streptococcus, nevertheless, no action was demonstrable.

With the Quastel synthetic medium, however, results entirely similar to those obtained with the streptococcus were demonstrated. The drug was able to sterilize, or at least very markedly inhibit the growth of small inocula (less than 5,000 per ml.) of *E. coli* under either anaerobic or aerobic conditions. Again, our results were somewhat different from those of Broh-Kahn in that we were able to get bactericidal action in the presence of glucose as well as the lactate-nitrate medium. The results with

this organism differed from those with the streptococcus in that there was marked action at 37°C., although there was no sterilization such as usually occurred at 40°C.

DISCUSSION

The question can of course be raised as to whether we have accomplished complete *chemical anaerobiosis*. The answer is probably, no. However, we have been able to obtain complete *biological* anaerobiosis in the sense usually employed in bacteriology, since this method of producing anaerobic conditions, i.e., by burning phosphorus, is quite sufficient for bacteriological purposes. The piece of phosphorus usually burns out in a matter of a few minutes thus establishing anaerobic conditions fairly rapidly. The addition of ascorbic acid was resorted to since it has been shown to be possible to grow anaerobes aerobically by the addition of this agent. The protective effect of glucose noted by Broh-Kahn and explained as offering an alternative pathway of nutrition has not been observed in our experiments. The observation of Lockwood (1938) that peptone interferes with the action of sulfanilamide has not been observed in our experiments with streptococci, since the tryptose broth contains 2 per cent peptone. The failure of the drug to act against *E. coli* remains unexplained, though in this case peptone may be concerned.

SUMMARY

1. At 40°C., sulfanilamide in a concentration of twenty milligrams per cent, in tryptose-phosphate-glucose broth, is able to sterilize small inocula of streptococcus C203 in 48 hours either aerobically or anaerobically with or without the presence of ascorbic acid.

2. No action under these conditions was noted at 37°C., and attempts to aid the drug by slowing down the growth of the organism by diluting the medium with saline were negative. Likewise, attempts to potentiate the drug by ferric chloride were negative.

3. Sulfanilamide had no effect on two strains of *Escherichia*

coli when grown in nutrient or tryptose broth aerobically or anaerobically.

4. In synthetic medium containing either glucose or lactate-nitrate at 37° or 40°C. marked inhibition or sterilization of small inocula of two strains of *E. coli* was noted both aerobically or anaerobically in the presence of ascorbic acid.

BIBLIOGRAPHY

BLISS, E., AND LONG, P. 1939 Observations upon the mode of action of sulfanilamide and sulfapyridine. Proc. Third Intern. Congr. Microbiol., New York, 585–586.

BROH-KAHN, R. H. 1939 The bacteriostatic action of sulfanilamide under anaerobic conditions. Science, **90**, 543–544.

FOX, C. L. 1940 Is sulfanilamide bacteriostatic under anaerobic conditions? Science, **91**, 477–478.

FOX, C. L., GERMAN, B., AND JANEWAY, C. A. 1939 Effect of sulfanilamide on electrode-potential of hemolytic streptococcal cultures. Proc. Soc. Exptl. Biol. Med., **40**, 184–189.

LOCKE, A., MAIN, E. R., AND MELLON, R. R. 1938 Anti-catalase and the mechanism of sulfanilamide action. Science, **88**, 620–621.

LOCKWOOD, J. S. 1938 Studies on the mechanism of the action of sulfanilamide. III. The effect of sulfanilamide in serum and blood on hemolytic streptococci in vitro. J. Immunol., **35**, 155–193.

QUASTEL, J. H., STEPHENSON, M., AND WHETHAM, M. 1925 Some reactions of resting bacteria in relation to anaerobic growth. Biochem. J., **19**, 304–317.

SHAFFER, P. A. 1939 The mode of action of sulphanilamid. Science, **89**, 547–550.

VARNEY, P. L. 1926 A simple method for cultivating anaerobes by means of phosphorus. J. Lab. Clin. Med., **11**, 1–4.

WHITE, H. J., AND PARKER, J. M. 1938 The bactericidal effect of sulfanilamide upon beta hemolytic streptococci in vitro. J. Bact., **36**, 481–498.