

THE INACTIVATION OF STREPTOMYCIN BY CYANATE*

By ROBERT J. FITZGERALD

(From the Department of Physiology and Pharmacology, Duke University
School of Medicine, Durham, North Carolina)

(Received for publication, June 30, 1948)

The reversal of the bacteriostatic action of streptomycin for certain mycobacteria as well as *Escherichia coli* by urea has been the subject of a preliminary note by Fitzgerald and Bernheim (1). At that time the possibility was considered that urea was being directly assimilated by the organisms. Subsequent work to elucidate the nature of this phenomenon has revealed that the agent which causes the reversal of streptomycin is most probably not urea but some product formed when urea is autoclaved in the medium. The purpose of this paper is to present the experimental evidence for this conclusion as well as information on the possible nature of the active reverser and its mode of action.

Methods

The experiments were done with *Mycobacterium tuberculosis* 607 grown in the Tween medium of Dubos (2). The medium was dispensed in a final volume of 10 cc. in test-tubes 1 inch in diameter and suitable for use in the Evelyn photoelectric colorimeter. The inoculum consisted of 0.5 cc. of a 1:20 dilution of a 48 to 72 hour culture which was adjusted to a standard density prior to dilution. All tests were run at 37°. Ammonia determinations were done by a modified method of Van Slyke and Cullen (3) and urea determinations were done by the method of Ormsby (4). Stock 1 per cent solutions of urea were made in 0.05 M phosphate buffer at pH 7.0 and appropriate dilutions made in the medium prior to sterilization by autoclaving at 122° for 10 minutes. Originally this procedure was followed routinely, since only about 10 to 20 per cent of the urea was decomposed by this treatment.

The streptomycin was added aseptically from suitable dilutions of a stock solution of streptomycin sulfate (Winthrop) in sterile distilled water which contained 100,000 γ of streptomycin per ml. Growth was followed turbidimetrically by means of an Evelyn colorimeter with a No. 660 filter.

EXPERIMENTAL

The effect of various concentrations of urea autoclaved in Dubos medium on the bacteriostatic action of 100 γ per cent of streptomycin is shown in

* Aided by a grant from the Duke University Research Council.

Fig. 1. Under the test conditions the growth of *Mycobacterium tuberculosis* 607 is inhibited by 10.0 γ per cent of the drug, while in the presence of 12.0 mg. per cent of urea appreciable growth occurs with 100.0 γ per cent of streptomycin. Subcultures from the urea-streptomycin tubes onto veal infusion-glycerin agar containing various concentrations of streptomycin revealed that the sensitivity of these organisms to the drug was the same as the controls. In other words the organisms growing in the urea-streptomycin tubes had not become streptomycin-fast in spite of the fact that

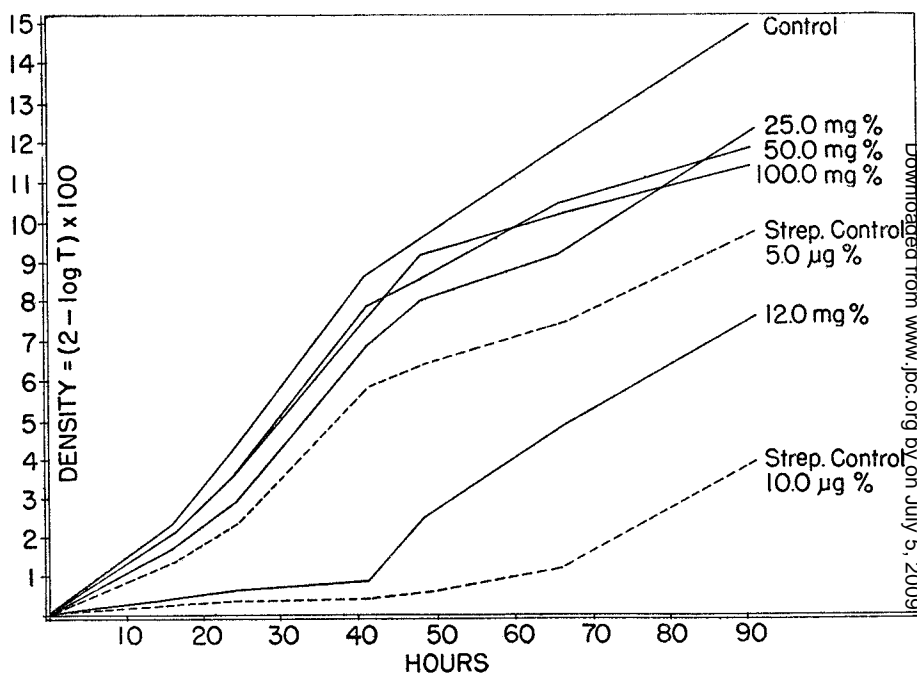


FIG. 1. The effect of urea concentration on the bacteriostasis of *Mycobacterium tuberculosis* 607 by 100.0 γ per cent of streptomycin.

under normal conditions *M. tuberculosis* 607 can rapidly develop resistance to this drug. The urea concentration of the medium decreases with growth of the organisms and, since similar results could be obtained with *Escherichia coli* 6522 which presumably contained no urease, the possibility was considered that urea was being assimilated directly by the organisms. However, it was found that any loss of urea could be accounted for by accumulation of ammonia in the medium. Subsequent studies showed that *M. tuberculosis* 607, *M. tuberculosis* BCG 8240, and the H37RV strain of *M. tuberculosis*, as well as *E. coli* 6522, all possessed definite urease activity.

Furthermore it was found that no reversal of streptomycin occurred when the urea was sterilized by filtration through Seitz pads or sintered glass filters and added aseptically to the medium. This would indicate that the active reverser is produced as a result of autoclaving the urea. In order to determine the conditions necessary for the formation of the reverser, urea was autoclaved separately in distilled water and also together with various components of the medium prior to addition to the test medium. The results of such an experiment are shown in Table I from which it may be seen that the most effective reversal of streptomycin occurs when the urea is autoclaved in the complete medium.

TABLE I

Reversal of Streptomycin by Urea Previously Autoclaved With Different Constituents of Dubos Medium

Supplement*	Streptomycin <i>γ per cent</i>	Density, $(2 - \log T) \times 100$		
		26 hrs.	42 hrs.	70 hrs.
Urea and phosphates	100	0.0	0.0	2.4
	50	0.0	2.4	6.9
“ citrates, and MgSO ₄ ·7H ₂ O	100	0.9	1.4	6.7
	50	2.4	6.7	8.3
Urea and complete medium	100	3.1	6.4	8.0
	50	3.4	7.3	8.6
“ “ distilled water	100	0.0	0.0	4.4
	50	0.0	0.0	0.0
Streptomycin control	100	0.0	0.0	0.0
	50	0.0	0.0	0.0
	5	0.0	0.9	7.4
Control	0	2.5	8.8	13.7

* Unless otherwise indicated, each tube contained the equivalent of 100 mg. per cent of urea (before autoclaving). The various supplements as well as the complete medium were autoclaved for 10 minutes at 122°.

To establish somewhat more precisely the conditions for the formation of the reverser samples of the urea-containing medium were boiled for 10 minutes or autoclaved for various periods of time. Neither boiling for 10 minutes nor autoclaving for 1 minute gave rise to any reverser. Best results were obtained when the urea-containing medium was autoclaved for from 5 to 15 minutes. Growth in media with urea autoclaved for longer periods was not optimal, presumably due to the pH changes resulting from accumulation of ammonia.

Removal of the residual urea after it had been autoclaved in the medium was accomplished by addition of purified urease (Squibb). It could be shown after such treatment that the reverser was still present.

A number of compounds that could be considered related to or derived from urea were tested as possible reversers. These included ammonium carbonate, ammonium carbamate, biuret, guanidine hydrochloride, thiourea, sodium cyanide, sodium thiocyanate, and potassium cyanate. All the compounds were made up in 0.05 M phosphate buffer at pH 7.0 and sterilized by Seitz filtration. Additions were made to the test mixtures from appropriate dilutions in Dubos medium. None of these compounds could function as a reverser, with the exception of potassium cyanate.

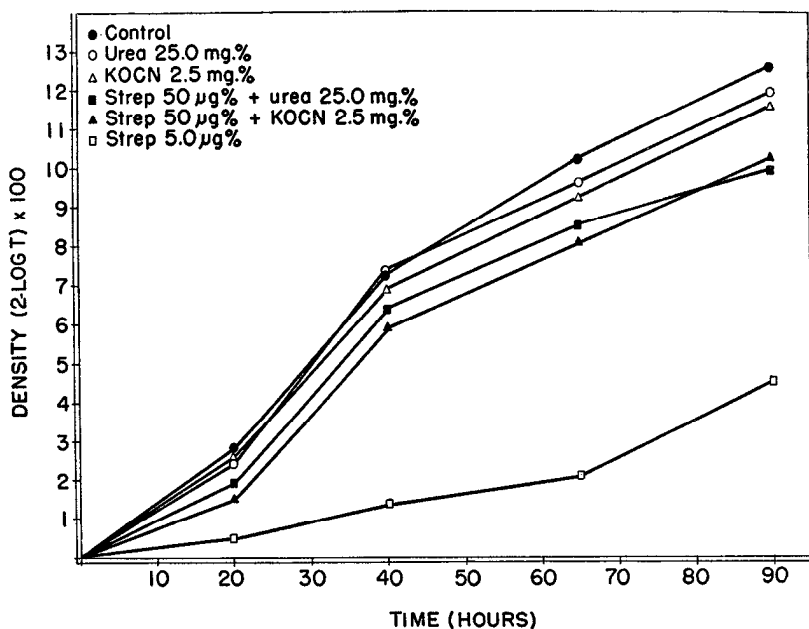


FIG. 2. Comparison of urea autoclaved in the medium and potassium cyanate added aseptically as reversers of streptomycin.

Fig. 2 shows a comparison of potassium cyanate added aseptically and urea autoclaved in the medium as streptomycin reversers. The cyanate has the greater activity, being approximately 10 times as effective as the autoclaved urea in counteracting the bacteriostatic effect of streptomycin on *Mycobacterium tuberculosis* 607. Similar results were obtained with *Escherichia coli* 6522 in the medium of Kohn and Harris (5), and a laboratory strain of *Staphylococcus aureus* in Difco nutrient broth. When the cyanate was autoclaved in the medium most of its ability to reverse streptomycin was lost.

Cyanate is probably not utilized by these organisms because concentrations above 10 mg. per cent inhibit growth. It seemed probable therefore

that cyanate was reacting with the streptomycin molecule. If this were so, cyanate added to washed suspensions of mycobacteria should counteract the inhibitory effect of streptomycin on the formation of the adaptive enzyme for benzoic acid (6). Experiments were done with *Mycobacterium tuberculosis* 607 and *Mycobacterium lacticola*. 1.0 mg. of potassium cyanate completely prevented the inhibition of enzyme formation by 0.2 mg. of streptomycin. Since cyanate alone inhibits the oxygen uptake under these conditions it probably is not metabolized. This experiment indicates therefore that cyanate reacts with the streptomycin molecule. Because of the ability of cyanate to react with amino groups, it is probable that it combines with these groups in streptomycin.

DISCUSSION

The inhibition of the bacteriostatic effect of streptomycin by urea autoclaved in Dubos medium is most probably due to formation of some new compound, since urea itself does not possess this property. Salt effects on streptomycin are ruled out because comparable concentrations of ammonium carbonate or ammonium carbamate are inactive. Of the compounds tested potassium cyanate is the only one which meets the requirements of the hypothetical compound in that it reverses streptomycin and small amounts of cyanates may be formed by autoclaving urea solutions.

In view of the fact that cyanate does not seem to be a normal metabolite of *Mycobacterium tuberculosis* 607 and may actually inhibit its growth and metabolic activity it is probable that it reacts with the streptomycin molecule. Although the nature of this reaction has not been elucidated, the reactivity of cyanates with free amino groups and the presence of these groups in the streptamine portion of the molecule suggest that this is the point of attack. Donovick *et al.* (7) have already demonstrated that streptomycin may be inactivated by certain carbonyl reagents such as semicarbazide, thiosemicarbazide, and hydroxylamine which presumably act upon the carbohydrate portion of the molecule. It would appear from the results herein presented that the free amino groups of streptomycin are also essential for antibiotic activity.

SUMMARY

1. When urea is autoclaved in Dubos medium a product is formed which blocks the bacteriostatic action of streptomycin.
2. Of the substances tested cyanate is the only one which satisfies the characteristics of this reverser.
3. Cyanate probably inactivates streptomycin directly, possibly by combining with its free amino groups.

BIBLIOGRAPHY

1. Fitzgerald, R. J., and Bernheim, F., *J. Biol. Chem.*, **172**, 845 (1948).
2. Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., *Am. Rev. Tuberc.*, **54**, 204 (1946).
3. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical physiological chemistry*, Philadelphia, 12th edition (1947).
4. Ormsby, A. A., *J. Biol. Chem.*, **146**, 595 (1942).
5. Kohn, H. I., and Harris, J. S., *J. Pharmacol. and Exp. Therap.*, **73**, 343 (1941).
6. Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, **55**, 765 (1948).
7. Donovick, R., Rake, G., and Fried, J., *J. Biol. Chem.*, **164**, 173 (1946).