ACTION OF FORMALDEHYDE ON MICROORGANISMS

III. Bactericidal Action of Sublethal Concentrations of Formaldehyde on *Aerobacter aerogenes*

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**ABSTRACT**

Neely, W. Brock (The Dow Chemical Co., Midland, Mich.). Action of formaldehyde on microorganisms. III. Bactericidal action of sublethal concentrations of formaldehyde on *Aerobacter aerogenes*. J. Bacteriol. 86:445-448. 1963.—Bacteriostatic concentrations of formaldehyde were found to be bactericidal during the initial growth period. This bactericidal activity was due to the primary induction of unbalanced growth by formaldehyde. The subsequent formation of 1,3-thiazane-4-carboxylic acid prevented the synthesis of methionine. This dual activity of formaldehyde resulted in an inhibition of both nuclear and cytoplasmic syntheses, and created the normal bacteriostatic condition that has been observed before with sublethal concentrations of formaldehyde.

In our previous investigation dealing with the action of formaldehyde on microorganisms (Neely 1963a), it was shown that formaldehyde had an initial bactericidal action when used in bacteriostatic concentrations. This action seemed worthy of further investigation. The subsequent identification of 1,3-thiazane-4-carboxylic acid in *Aerobacter aerogenes* treated with formaldehyde (Neely, 1963b) provided a lead to the understanding of this particular action. The formation of the above thiazane derivative by microorganisms effectively removes homocysteine from any further biological reaction. It may be assumed that homocysteine is the precursor for the biosynthesis of methionine, for there is good evidence that this assumption is correct (Harold, 1962). Then it logically follows that the synthesis of this essential amino acid will be prevented in the presence of formaldehyde. With this background, I decided to investigate both the formation of 1,3-thiazane-4-carboxylic acid and the action of methionine in *A. aerogenes* treated with formaldehyde.

**MATERIALS AND METHODS**

*Culture.* The organism, *A. aerogenes* ATCC 8308, was cultured and maintained as described by Neely (1963a). Mutant 24 of *A. aerogenes* A3(0) was generously supplied by F. M. Harold, Department of Experimental Chemistry, National Jewish Hospital, Denver, Colo. This particular mutant grew only if methionine or homocysteine were added to the medium. In the present case, the mutant was maintained on the minimal medium of Warren, Ellis, and Campbell (1960) supplemented with methionine (30 μg/ml). Unless otherwise stated, the use of the term *A. aerogenes* will mean the ATCC 8308 culture.

*Analytical techniques.* The procedures for measuring growth, viability, and CO₂ evolution were similar to those described previously (Neely, 1963a). The chromatographic identification of 1,3-thiazane-4-carboxylic acid has been reported (Neely, 1963b). To follow the formation of 1,3-thiazane-4-carboxylic acid, the following techniques were adopted. A portion (30 ml) of the chemically defined medium of Warren et al. (1960) was inoculated with a 24-hr culture of *A. aerogenes*. The resulting culture was adjusted with water so that when the appropriate formaldehyde was added the total volume would be 40 ml. The solution was then placed in a water bath (30°C) and aerated.

When turbidity measurements indicated that logarithmic growth had started, the formaldehyde was added. Samples (5 ml) were taken at periodic intervals and centrifuged immediately. The cells were washed twice with 2-ml portions of physiological saline, and the supernatant and washings were made up to 10 ml with water. The cells were then extracted twice with 2-ml portions of hot.
water, and the hot-water extracts were made up to 5 ml. Samples of the various fractions were counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.) as well as chromatographed on paper (Whatman no. 1), by use of the top layer of a n-butanol-acetic acid-water (4:1:5) solvent. For greater accuracy and sensitivity in measuring the C\textsuperscript{14} activity in the chromatogram, a 1-in. paper strip was sectioned into 1-in. squares and counted by means of liquid scintillation. The results are reported as counts per min per sample of bacterial extract chromatographed.

Chemicals. The C\textsuperscript{14}-labeled formaldehyde (specific activity of 10 mc/m mole) and the C\textsuperscript{14}-labeled methionine (specific activity of 5 mc/m mole) were supplied by the New England Nuclear Corp., Boston, Mass.

The methyl ester of methionine was prepared from the directions of Dekker, Taylor, and Fruton (1949). The product had a mp of 150 to 151 C as compared with the literature value of 151 C. In addition, the infrared spectra had a strong absorption at 5.8 \mu, supporting the presence of an ester linkage.

RESULTS

The formation and disappearance of 1,3-thiazane-4-carboxylic acid by \textit{A. aerogenes} treated with 50 \mu g/ml of labeled formaldehyde are shown in Fig. 1. Superimposed on this graph is the C\textsuperscript{14}O\textsubscript{2} evolution pattern from labeled formaldehyde and the decrease in total C\textsuperscript{14}-activity during growth. Finally, the appearance and disappearance of a new constituent produced by the organism is illustrated. This latter material on chromatography in the butanol solvent moved only slightly from the origin after 18 hr of chromatography.

Experiments were designed to see what effect the addition of methionine would have on the formaldehyde-treated organism. The first experiments with methionine were unsuccessful in that no effect was observed. With labeled methionine, it was discovered that \textit{A. aerogenes} was unable to incorporate preformed methionine from the medium. The initial C\textsuperscript{14}-activity was similar to the activity found in the supernatant after 24 hr of growth. Futhermore, the final activity was found by paper chromatography to reside completely in the methionine.

The experiments utilizing the methyl ester of methionine were more successful, as the results in Fig. 2 and 3 indicate. Figure 2 is a dose-response curve demonstrating the dramatic effect that increased concentration of methione ester has on the ability of the microorganism to metabolize formaldehyde. The previous results (Neely, 1963a) indicated that the shape of the C\textsuperscript{14}O\textsubscript{2} evolution curve was parallel to the viable count. Figure 2 indicates that, at the highest concentration of methionine, the viable count decreased and failed to recover during the time of the experiment. The viable-count and turbidity data in Fig. 3 verified this interpretation.

In an effort to determine that \textit{A. aerogenes} was utilizing the methyl ester of methionine as a
source of methionine, experiments were performed with the mutant strain A3(0). It was discovered that the ester was equally good as the free amino acid in supporting growth of this particular auxotroph. To rule out the possibility that the results in Fig. 2 and 3 were due in some way to the ester group, the experiments using the methyl ester of methionine were repeated with the methyl ester of glycine. (A sample of this material was supplied by H. C. White of the E. C. Britton Research Laboratory, The Dow Chemical Co., Midland, Mich.) This addition gave results which were comparable with the control experiments in Fig. 2 and 3.

**DISCUSSION**

Barner and Cohen (1954) and Cohen and Barner (1956) have shown that a thymine-requiring strain of *Escherichia coli* 15T loses the power to multiply or "dies" when it is permitted to metabolize and grow in the absence of exogenous thymine. The effect was described by these authors as death by unbalanced growth, a situation where nuclear synthesis was inhibited while cytoplasmic syntheses and growth continued.

Cohen and Barner (1956) also examined the action of sulfanilamide on microorganisms. The normal action of sulfanilamide is one of interfering with the folic acid cycle. This in turn causes the inhibition of the one-carbon transfer mechanism, a mechanism which is essential for the biosynthesis of a large number of metabolites. It is evident that the multiple deficiencies produced by this agent prevent both cytoplasmic and nuclear syntheses and bring about a bacteriostatic situation.

The experiments of Cohen and Barner (1956) also illustrated that sulfanilamide can become a bactericidal agent when the deficiency of metabolites is made specific for the nuclear constituent. This situation occurs when all the metabolites formed by the folic acid cycle are added back to the medium, with the exception of thymine. This set of circumstances causes the organism to die by unbalanced growth in a manner analogous to the thymine-requiring mutant of *E. coli*.

If the action of formaldehyde on *A. aerogenes* is examined in relation to the above discussion, all of these observations fall into a similar pattern. One of the actions of formaldehyde, which is probably connected with nuclear synthesis, is to inhibit cell division (Neely 1963a). Assuming that this is the sole action of formaldehyde during the early phase, then a situation occurs which causes death of the bacteria by unbalanced growth. However, another competing reaction of formaldehyde is the formation of 1,3-thiazane-4-carboxylic acid, the formation of which reaches a maximum shortly after the loss in viability occurs. This reaction prevents the synthesis of methionine, an essential metabolite connected with cytoplasmic synthesis. Consequently, during the remaining action of formaldehyde, both nuclear and cytoplasmic syntheses are inhibited with the resultant achievement of a bacteriostatic condition.

If this mechanism is correct, then the addition of preformed methionine to the culture should accentuate the bactericidal action of formaldehyde. In the present case it was discovered that *A. aerogenes* would not utilize preformed methionine (see Results). However, the addition of the methyl ester of methionine to the medium did cause an increased drop in viability as compared with formaldehyde alone. The experiments with the methionine-requiring mutant of *A. aerogenes* A3(0) demonstrated that the methyl ester of this amino acid was satisfactory as a source of methionine. This would confirm that the observed action of the methionine ester on formaldehyde-treated *A. aerogenes* was nutritional and not due to some other phenomenon. The inability of the methyl ester of glycine to increase the bactericidal action of formaldehyde eliminated the activity of the methionine ester as being due to the ester group.
The presence of the new C\textsuperscript{14}-labeled component, which moved only slightly on chromatography in the butanol solvent, is being examined and the results will be reported at a later date.

Literature Cited


