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INFLUENCE OF BIOLOGICAL METHYLATION ON THE BIOSYNTHESIS OF MITOMYCIN A

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ABSTRACT

KIRSCH, E. J. (Lederle Laboratories, Pearl River, N.Y.), AND J. D. KORSHALLA. Influence of biological methylation on the biosynthesis of mitomycin A. J. Bacteriol. 87:247-255. 1964.—Methionine-methyl-C^{14} was shown to contribute radioactive carbon to the mitomycin antibiotic complex synthesized by *Streptomyces verticillatus* in a simple synthetic medium containing glucose and inorganic salts. The position of radioactivity in mitomycin A was determined by selective hydrolysis of the 7 and 9a methoxyl functions. Essentially all of the radioactivity incorporated was distributed evenly between these two substituent groups. Mitomycin A, synthesized by washed resting cells of *S. verticillatus* at the expense of internal metabolites, also incorporated methyl label. When the methionine antagonist, D,L-ethionine, was added to resting cells at a concentration causing 65% inhibition of antibiotic synthesis, incorporation of radioactive methyl groups was reduced to the same extent. Synthetic medium supplemented with D,L-ethionine supported about 90% maximal growth of the culture, but antibiotic biosynthesis was markedly inhibited. The addition of the inhibitor during the period of rapid antibiotic synthesis resulted in cessation of further increases in antibiotic titer. L-Methionine was shown to be capable of reversing ethionine inhibition; the extent of reversal was dependent on the concentration, as well as on the time of addition of amino acid. The data suggest the critical nature of a methyl transfer system in the biogenesis of biologically active mitomycins.

Hata et al. (1956) described the production, isolation, and properties of mitomycins A and B, two component members of the mitomycin antibiotic complex. The third component, mitomycin C, was described by Wakaki et al. (1958); the fourth mitomycin, named porfiromycin, was introduced by DeBoer et al. (1961). More recently, Lefemine et al. (1962) announced the discovery of mitiromycin, the fifth compound in this group. The structures of mitomycins A, B, C, and porfiromycin were clearly established by Webb et al. (1962); the structure and stereochemistry of mitomycin A was confirmed in X-ray diffraction studies reported by Tulinsky (1962). The mitomycins are related chemically by a common structural nucleus which has been assigned the trivial name, mitosane, by Webb and his co-workers. These compounds are chemically differentiated from one another by methoxyl, methyl, hydroxyl, or amino group substitutions of the mitosane nucleus; thus, mitomycin A is a 7,9a-dimethoxymitosane.

The presence of substituent *O*-methyl and *N*-methyl groups in these molecules suggested that methyl transfer reactions were involved in the biosynthetic origin of the completed antibiotics. It has been shown repeatedly that the methionine antagonist, ethionine, interferes with the transfer of methyl groups. Hendlin et al. (1962) described the increased formation of 6-demethylchlortetracycline by *Streptomyces viridifaciens* as a consequence of the addition of D,L-ethionine to the fermentation. Zygmunt (1962) reported that selective inhibition of the tetracyclines produced by *S. rimosus* likewise resulted from the inclusion of D,L-ethionine in the fermentation. In addition, it has been suggested that ethionine can substitute for methionine, and can participate in a transethylation reaction analogous to transmethylation. Parks (1958) observed that *Torulopsis utilis* grown in the presence of ethionine produced *S*-adenosylethionine, which, in an in vitro enzyme reaction, was shown to donate an ethyl group to homocysteine, yielding ethionine. The formation of the 2′-ethyl homologue of griseofulvin by *Penicillium griseofulvum* (Jackson et al., 1962), and the biosynthesis of *N*-methylethyl oxytetracycline by *S. rimosus* (Dulaney et al., 1962) presumably resulted from the participation of ethionine acting

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as an ethyl group donor in transethylation reactions analogous to methionine in transmethylation.

In this communication, the results of our studies on the relationship of L-methionine to the origin of substituent methyl groups in mitomycin A, and the effect of D,L-ethionine in inhibiting methylation and subsequent antibiotic biosynthesis, are reported.

MATERIALS AND METHODS

Mitomycin-producing organism. A single colony of *S. verticillatus*, Lederle isolate no. AB929, was obtained by surface-plating portions of a diluted spore suspension and incubating the plates at 28 C for 5 days. A master culture was started from this colony. Subcultures were made on yeast agar slants having the following composition: yeast extract, 0.4%; malt extract, 1.0%; glucose, 0.4%; and refined agar, 2.0%. These cultures were used in all of the work to be reported.

Production of mitomycins for growth experiments. Cultures were started on a complex medium (IM-1) containing Trypticase (BBL), 1.0%; glycerol, 1.0%; soya peptone, 0.5%; glucose, 0.5%; $(NH_4)_2HPO_4$, 0.2%; K_2HPO_4, 0.15%; $MgSO_4 \cdot 7H_2O$, 0.025%; NaCl, 0.2%; and trace element solution, 0.1% (v/v). The trace element solution consisted of $AlK(SO_4)_2$, 0.015%; KI, 0.003%; KBr, 0.003%; $MnCl_2 \cdot 4H_2O$, 0.04%; $ZnSO_4$, 0.006%; $CaSO_4 \cdot H_2O$, 0.006%; $CoCl_2$, 0.007%; $(NH_4)_6Mo_7O_{24}$, 0.003%; $K_2Cr_2O_7$, 0.001%; and $CuSO_4 \cdot 5H_2O$, 0.003%. After inoculation of 50 ml of medium IM-1 in 250-ml Erlenmeyer flasks, the cultures were incubated for 72 hr at 28 C on a Gump rotary shaker rotating at 240 rev/min. The cells were collected by centrifugation at 600 to 900 $\times$ *g* in an International centrifuge (model 2). The supernatant fluid was decanted. The cells were dispersed by homogenization in a Waring Blendor, washed five times in sterile distilled water, and resuspended to their original volume in sterile distilled water.

The suspension was inoculated at the 4% level into a synthetic fermentation medium (SPM) having the following composition: $(NH_4)_2SO_4$, 0.1%; glucose, 0.5%; $FeSO_4 \cdot 7H_2O$, 0.01%; $MgSO_4 \cdot 7H_2O$, 0.02%; KH_2PO_4, 0.02%; $CaCO_3$, 0.05%; and trace element solution, 0.2% (v/v). Glucose was sterilized separately and added to the medium aseptically. The cultures, in 50 ml of SPM medium contained in 250-ml Erlenmeyer flasks, were incubated at 28 C with shaking on a Gump rotary shaker revolving at 240 rev/min. Antibiotic production reached a maximal level at about 120 hr.

Toluene (2.0%, v/v) was added to each flask 30 min prior to harvesting the cultures to prevent the destruction of the mitomycins by the culture mycelia. This phenomenon was described in a publication by Gourevitch, Pursiano, and Lein (1961). The cells were separated from the culture fluid by filtration through tared Whatman no. 2 filter-paper discs. Growth was determined by drying the cells collected on filter paper to a constant weight at 70 C. Samples of the culture filtrate were diluted in sterile distilled water in preparation for biological assay. Additional samples were shaken with 0.5-volume amounts of ethyl acetate for paper chromatographic examination.

Resting-cell experiments. Complex medium IM-1 was inoculated from a slant of *S. verticillatus* as described for the growth experiments. After 3 days of incubation, the cells were collected by centrifugation, washed five times in sterile distilled water, and resuspended in 0.25 volume of sterile distilled water. To 15 ml of sterile 0.1 M phosphate buffer (pH 6.0) containing the supplement being investigated were added 5 ml of cell suspension. The cultures were incubated at 28 C on a Gump rotary shaker at 240 rev/min, and were sampled both at 0 time and after 48 hr.

Biological assay. The total antibiotic activities of diluted culture filtrates were assayed by the deep-well agar plate diffusion method, with *Bacillus subtilis* (Lederle no. 115) as the test organism. Mitomycin A was employed as a reference standard; activity is expressed in terms of micrograms of mitomycin A equivalents per milliliter.

Paper chromatography. Measured amounts of ethyl acetate extracts were applied to 0.5- or 1-in. strips of Whatman no. 1 filter paper. The mitomycin components were resolved by descending development in our system 86, consisting of water-benzene-methanol (2:1:1). The antibiotics were located by bioautography on trays seeded with *Streptococcus pyogenes* NY-5.

Radioactive tracer experiments. The following radioactive supplements were employed in these studies: L-methionine-methyl-C^{14} (specific ac-

tivity, 10.9 μc/μmole) and L-methionine-methyl-C^{14} (specific activity, 4.96 μc/μmole). Direct measurements of radioactivity in fermentation samples or extracts were made by plating appropriately diluted samples on stainless-steel planchets. The samples were dried at 100 C in a drying oven, and were counted with a Compu/matic II scaler (Tracerlab, Inc., Waltham, Mass.) with a thin-window Geiger-Müller tube housed in a shielded sample changer. Platings were made so that self-absorption error was minimal and counts were below the level requiring coincidence correction. All counts were corrected for background activity.

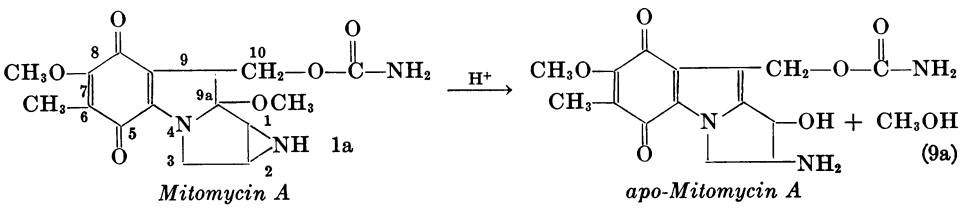
Paper chromatograms bearing radioactive compounds were scanned with the drive mechanism of a Brown recorder (model 30-NGP10) to advance the strip at a speed of 8 in. per hr under a Geiger-Müller detector, model 1063 (Nuclear-Chicago Corp., Des Plaines, Ill.). The signal was amplified and integrated by a Baird Atomic ratemeter (model 432A; Atomic Accessories Inc., Valley Stream, N.Y.), and was recorded with a Brown and a Varian (model G-10) recorder. After scanning, the strips were bioautographed on plates seeded with *S. pyogenes* NY-5 to facilitate location of biologically active mitomycins. Radioactivity was estimated by relating the area under respective peaks to similar measurements made for radioisotopic reference standards of known concentrations.

Scheme for partial degradation of mitomycin A. The following sequence of reactions was described in a report by Webb et al. (1962), and was adapted in the present studies for the purpose of determining the position of radioactive carbon incorporated into mitomycin A:

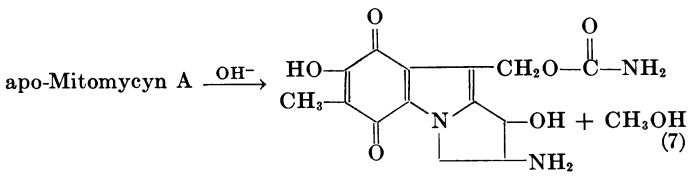
Isolation and degradation of radioactive mitomycin A. To a *S. verticillatus* culture were added 20 μc of L-methionine-methyl-C^{14} (specific activity 4.96 μc/μmole) 40 hr after growth had been initiated in medium SPM. The culture was incubated for an additional 80 hr to permit antibiotic synthesis and incorporation of radioactive carbon. The culture broth was separated from the cells by filtration, extracted three times with 0.5-volume amounts of ethyl acetate, and concentrated under vacuum with minimal heating.

The concentrate was applied by streaking on several sheets of Whatman no. 1 chromatography paper, and the mitomycins were resolved in solvent system no. 86. Mitomycin A was located by bioautography and by scanning for radioactivity. The antibiotic was eluted from paper with 10% methanol.

Cold carrier mitomycin A (2.86 μmoles) was added to the concentrated eluate containing approximately 0.1 μmole of radioactive mitomycin A to facilitate further purification of the compound. A small acid-washed Celite diatomaceous earth column (Johns-Manville, New York, N.Y.) was prepared, and the antibiotic preparation was purified by partition chromatography in a developing system containing benzene-methanol-0.01 M phosphate buffer at pH 6.8 (20:1:1), as described by Lefemine et al. (1962). Mitomycin-containing fractions were located by visible color and biological activity. Each fraction was also examined qualitatively by paper chromatography, and the peak cuts were pooled and concentrated. Spectrophotometric analysis of a sample diluted in methanol and measured at 318 mμ revealed the recovery of 1.6



Mitomycin A *apo-Mitomycin A*



7-Demethyl apo-mitomycin A

μmoles of purified mitomycin A. Radioactive measurement indicated that the specific activity of the compound was 3.9 mμc/μmole.

Selective removal of the 9a methyl group of radioactive mitomycin A was accomplished by hydrolysis with 0.1 N HCl for 3 hr at 25 C. A small amount of methanol was added to act as a carrier for the radioactive methanol released. The yield of apo-mitomycin A was essentially quantitative, as determined by spectrophotometric analysis in methanol at 285 mμ. Qualitative identification of the yellow chromophore of apo-mitomycin A was obtained by absorption spectra in a Beckman DB spectrophotometer. Samples were also plated on stainless-steel planchets, dried with heat, and counted. Any methanol contained in these samples was volatilized during drying, thus permitting an isotopic evaluation of the apo-mitomycin A without interference.

After acid hydrolysis, the remaining compound was subjected to alkaline hydrolysis. The sample was adjusted to pH 7.0 with 0.1 N NaOH, and sufficient 3 N NaOH was added to raise the hydroxide concentration to 0.1 N. The reaction was continued for 3 hr at 25 C. A new purple chromophore was formed with absorption characteristics identical to that of the 7-demethylated analogue of apo-mitomycin described by Webb et al. (1962). The 7-methyl group was split off selectively under these conditions. The conversion of apo-mitomycin A to 7-demethyl apo-mitomycin A was quantitated by spectrophotometric analysis at 254 mμ in 0.1 N NaOH. Radioactivity remaining in the chromophore was determined in the usual way, after driving off methanol with heat.

To determine directly the extent of labeling in the methyl groups split off by hydrolysis, a hydrolyzed sample of mitomycin A was shaken with activated carbon to remove the chromophore, and the reaction liquor was then cleared by centrifugation. The methanol present was oxidized with alkaline potassium permanganate at 100 C, and the CO_2 evolved was trapped as sodium carbonate. Radioactivity in plated samples was measured as previously described. The recovery in this step was not quantitative; however, it appeared to be of sufficiently high order to be useful in determining whether the carbon released as methanol was, in fact, radioactive.

RESULTS

Incorporation of methyl-C^{14} into mitomycins. The data presented in Table 1 describe the incorporation of radioactive carbon derived from methionine-*S*-methyl-C^{14} into mitomycin A. Although all of the components of the mitomycin complex appeared to be labeled to some extent, the selection of mitomycin A for analysis was predicated on the fact that it was in greatest abundance and was most clearly separable from the other components by paper chromatography.

When exogenous methionine-*S*-methyl-C^{14} was added to a proliferating cell system in synthetic growth medium SPM at the start of the fermentation, 1.1% of the radioactivity added was found in the mitomycin A synthesized. Although this level of methyl incorporation does not appear to be high, it is quite probable that the small amount of radioactive methionine initially added was diluted by methionine synthesized de novo by the culture. It was observed that the culture dry weight increased 30- to 35-fold during the first 24

TABLE 1. *Incorporation of radioactive methyl carbon into mitomycin A by resting cells and proliferating cells of Streptomyces verticillatus*

Cell type*	Supplements	Radioactivity added	Radioactivity found in mitomycin A	Per cent incorporation
		mμc	mμc	
Washed resting cells	Methionine, methyl-C^{14}	4,000	9.2	0.23
Washed resting cells	Methionine, methyl-C^{14} plus D,L-ethionine (400 μg/ml)	4,000	3.3	0.063
Proliferating cells	Methionine, methyl-C^{14}	3,000	33.2	1.1

* Resting cells were prepared by growing the culture in complex IM-1 medium for 72 hr, washing four times, and resuspending in 0.1 M phosphate buffer. Supplements were added at time 0, and the cultures were incubated for 48 hr prior to analysis. Proliferating cells refer to similarly washed inocula which were added to synthetic medium SPM containing radioactive methionine, and were incubated for 120 hr.

hr of incubation. Antibiotic was not actively synthesized during this period of time; only trace amounts were detected in the medium. It seems possible, therefore, that the percentage of methyl incorporation is, in fact, higher than that which is reflected in the value presented for the uptake of methyl-group radioactivity. This does not, of course, rule out the possibility that other factors, such as low permeability of the cells to exogenous methionine, may influence the seemingly small amount of methyl carbon incorporated.

Resting cells of the mitomycin-producing culture were found to excrete a small but measurable amount of antibiotic activity in aerated phosphate buffer within 24 hr. The concentration of antibiotic released was directly related to the size of the population employed. This observation suggested at least two possibilities: that the antibiotic excreted represented complete preformed material which had been synthesized under growing conditions, was carried internally by the cells, and was released during incubation in the phosphate buffer; or, that newly formed antibiotic was synthesized by nonproliferating cells at the expense of internal metabolites. The data in Table 1 support the latter contention, although the first may be operative as well. It appears that some de novo biosynthesis does occur, because 0.23% of the radioactivity added to resting cells as methionine-*S*-methyl-C^{14} was found in the mitomycin A released. However, the data presented can only be interpreted to be suggestive of de novo synthesis, because it is possible that the radioactivity observed in the antibiotic resulted from an exchange of methyl groups. It is particularly interesting to note that both the incorporation of methyl carbon and the yield of antibiotic was reduced approximately 65% when the transmethylation inhibitor, D,L-ethionine, was added to a resting-cell system containing radioactive methionine.

Distribution of methyl-C^{14} in mitomycin A. Gross inspection of the structural formula of mitomycin A suggests that a transmethylation reaction sequence involving methionine could account for the presence of methyl groups at positions 6 and 7 on the quinone ring, and position 9a on the pyrrole ring. To determine the extent of C^{14} incorporation at these positions, radioactive mitomycin A was isolated from a culture which had been exposed to methionine-methyl-C^{14}. The crude radioactive antibiotic was diluted with cold mitomycin A, extensively purified by chromatography, and subjected to sequential hydrolytic treatment which permitted removal of 9a and 7 methyl carbons, in that order. At the termination of each hydrolytic step, the chromophore remaining was identified and measured spectrophotometrically. In addition, the radioactivity residing in the remaining ring structure was measured.

The data indicate that acidic hydrolytic removal of 9a carbon effects nearly a 50% decrease in specific radioactivity of the apo-mitomycin A chromophore remaining (Table 2). Alkaline treatment of apo-mitomycin A, which selectively removes the 7 methyl group, resulted in the loss of almost all of the remaining radioactivity. There appeared to be no significant amount of radioactivity left in the 7-demethyl-apo-mitomycin A, suggesting that 6-methyl carbon does not arise from methionine.

In addition to measuring the loss of radioactivity, an attempt was made to recover the methyl carbon released. Under the conditions of hydrolysis, the methyl groups were released as methanol. The chromophore was removed by adsorption on activated carbon, and the methanol was oxidized by alkaline permanganate to CO_2, which was trapped as sodium carbonate. The overall recovery in control systems was not quantitative, but ranged from 40 to 60%. It was observed that 50 to 60% of the radioactivity in mitomycin A could be accounted for as methanol. This indicates quite clearly that methionine does contribute methyl groups to substituent ring positions 7 and 9a.

TABLE 2. *Partial degradation of mitomycin A showing distribution of radioactive carbon in positions 7 and 9a*

Treatment	Compound formed	Specific radioactivity	Per cent of radioactivity remaining
		mμc/μM	
None	Mitomycin A	3.9	100.0
Acid hydrolysis	Apo-mitomycin A	2.2	56.0
Acid + alkaline hydrolysis	7-Methyl apo-mitomycin A	0.12	3.0
Acid + alkaline hydrolysis	Methylalcohol	—	52.5

Inhibition of mitomycin biosynthesis. The participation of methionine in the biogenesis of the mitomycins suggested the possibility that external control of methyl transfer reactions could be exerted by supplementation of the growth medium with compounds known to influence these reactions. A widely used antagonist of biological transmethylation, the methionine analogue ethionine, was tested for its effect on the synthesis of the mitomycins; it was found to be essentially innocuous to growth of the organism, while highly effective in inhibiting the formation of the antibiotics.

The extent to which antibiotic biosynthesis was prevented was affected by several factors. The effects of the concentration of inhibitor, and the time of addition to the growing culture, are shown in Table 3. The data show quite clearly that the addition of ethionine to the culture at a time preceding the onset of rapid antibiotic formation results in a marked decrease in total yield, provided that a sufficiently high concentration of inhibitor is used. At lower levels of ethionine, there is an obvious interplay of several factors which appear to make the inhibitory effect seem more erratic and interpretation less precise. In all subsequent inhibitor studies, ethionine was employed at a level of 100 μg/ml, or higher.

TABLE 3. D,L-*Ethionine inhibition of mitomycin biosynthesis in synthetic medium*

D,L-Ethionine concn	Time of addition	Total activity after 120 hr of fermentation*	Per cent inhibition
μg/ml	*hr*	*μg/ml*	
Not added	—	10.5	—
100	0	0.4	96.2
100	24	0.3	97.1
100	48	0.2	98.1
100	72	2.8	73.4
50	0	7.6	27.6
50	24	7.1	31.4
50	48	0.3	97.2
50	72	5.7	45.8
25	0	9.9	6.0
25	24	10.0	5.0
25	48	6.0	43.0
25	72	5.2	50.5

* Total activity expressed as μg/ml of mitomycin A equivalent activity determined by assay with *Bacillus subtilis* Lederle no. 115 at pH 6.0.

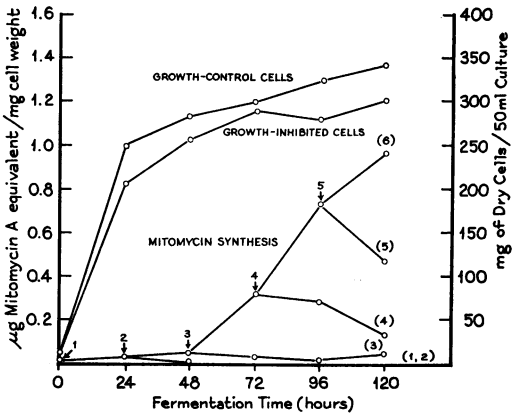


FIG. 1. *Kinetics of ethionine inhibition of mitomycin biosynthesis in synthetic medium SPM. Numbered arrows refer to the introduction of 100 μg/ml of* D,L*-ethionine to the fermentation. Corresponding numbers in parenthesis designate the plot of antibiotic synthesis resulting from such additions. Curve 6 is the plot of antibiotic synthesis in the uninhibited control fermentation.*

In an attempt to develop a more perceptive view of the effect of ethionine on the culture during antibiotic biosynthesis, a study was made to determine the relationship of time of inhibitor addition to the course of growth and antibiotic synthesis. The data obtained in this experiment are shown in Fig. 1, where growth and antibiotic production of the unsupplemented and the ethionine-supplemented cultures are plotted as a function of time. The culture increased rapidly in cell mass during the first 24 hr of incubation, with the ethionine-inhibited culture lagging slightly in growth rate. The cells continued to increase in weight at a lower rate in the succeeding period of incubation. In general, ethionine added at time 0 prevented complete expression of growth by a factor of about 10%. Antibiotic production, however, was almost completely inhibited throughout the entire period of incubation. In the uninhibited culture, rapid antibiotic synthesis followed the cessation of rapid growth, and continued through 120 hr. The addition of ethionine at a time preceding the onset of rapid antibiotic synthesis prevented further synthesis and accumulation of antibiotic. When the inhibitor was added during the phase of rapid antibiotic synthesis, there was an abrupt termination of antibiotic accumulation, followed by a gradual disappearance of activity in the culture

filtrate. The decrease in accumulated antibiotic after addition of the inhibitor may represent chemical instability or enzymatic destruction of the compounds. No attempt has been made to determine a causal relationship between the presence of ethionine and the cessation of antibiotic accumulation. Whether the inhibitor is terminating antibiotic synthesis, stimulating antibiotic destruction, or inducing the formation of a less active antibiotic is not clearly understood.

Reversal of ethionine inhibition. Additional evidence for the participation of methionine in reactions leading to the methylation of mitosane, resulting in the ultimate biosynthesis of the mitomycins, was obtained by determining the ability of methionine to overcome ethionine inhibition. When 100 μg/ml of D,L-ethionine was added during the first 48 hr of fermentation, and when antibiotic yield was measured after 120 hr of incubation, the reduction of activity was in all cases nearly complete. L-Methionine added at the level of 50 μg/ml at an early stage in the fermentation was not able to reverse ethionine inhibition. A small amount of reversal (10 to 20%) was noted when this level of methionine was added during the period of active antibiotic synthesis. The most striking reversal effects were noted when the inhibitor was added at the onset of antibiotic synthesis, and when the reversor was added any time after the early growth period, either prior to, coincident with, or after, inhibitor addition (Table 4). These data might suggest that, when methionine is added to the culture at an early stage, it is used preferentially for synthesis of protoplasm so that its effective reversal concentration with respect to the concentration of ethionine is reduced.

Although the data are not tabulated in this report, it was noted that the concentration of L-methionine used in reversing ethionine inhibition influenced the extent to which reversal was achieved. For example, when both inhibitor and reversor were added at 24 hr, 50 μg/ml of methionine permitted the synthesis of about 30% of the uninhibited antibiotic yield, whereas 100 μg/ml of methionine affected 80% reversal. The relationships between the time of addition and the concentration of inhibitor and reversor seem to be quite complex and unpredictable, even though the effects produced are quite striking.

TABLE 4. *L-Methionine reversal of D,L-ethionine inhibition of mitomycin biosynthesis in synthetic medium*

Time of ethionine addition*	Time of methionine addition†	Total activity after 120 hr. of fermentation‡	Per cent inhibition
hr	*hr*	*μg/ml*	
0	0	0	100.0
0	24	0.4	95.8
0	48	2.0	79.0
0	72	1.0	87.5
24	24	3.6	73.0
24	48	3.6	73.0
24	72	4.0	80.0
48	0	0.7	93.1
48	24	9.6	0
48	48	9.5	0
48	72	5.6	30.0

* D,L-Ethionine added at a final concentration of 100 μg/ml.

† L-Methionine added at a final concentration of 50 μg/ml.

‡ Total activity expressed as μg/ml of mitomycin A equivalent activity determined by assay with *Bacillus subtilis* Lederle no. 115 at pH 6.0.

DISCUSSION

It seems apparent from the foregoing studies that the methyl-group transfer-reaction sequence plays a critical role in the biosynthesis of biologically-active mitomycins. When methyl C^{14} methionine is added to a culture of *S. verticillatus* growing under conditions which permit the synthesis of the mitomycins, radioactivity is incorporated into the antibiotics. This observation in itself does not preclude the possibility that radioactive carbon may have entered the molecule at positions other than substituent methyl groups. However, it seems clear from the results of degradation studies of mitomycin A, in which the 7 and 9a methoxyl groups are selectively hydrolyzed, that essentially all of the radioactivity incorporated resides in these two methyl functions. Furthermore, each methyl carbon accounts for approximately 50% of the radioactivity taken up in the antibiotic molecule.

Additional support for the participation of methyl transfer in the biogenesis of the mitomycins is found in the striking selective inhibition of apparent biosynthesis by D,L-ethionine and in the reversal of this effect with L-methionine.

The kinetics of inhibition indicate that there is rapid cessation of antibiotic synthesis even when the inhibitor is added late in the period of rapid antibiotic synthesis, suggesting the possibility that the inhibitor interferes directly with some terminal biosynthetic process. Unfortunately, there is no clear-cut evidence for the nature of the metabolic block caused by ethionine in this system. It has been suggested by other investigators, and it is tempting in this situation to postulate, that ethionine interferes with the methyl transport system in the reactions leading to the formation of active methyl donor, *S*-adenosyl methionine. It is conceded, however, that ethionine may have some other involvement in methionine metabolism which results indirectly, but ultimately, either in the inability of the organism to complete mitomycin biosynthesis, or in the disappearance of antibiotic activity from the culture filtrate.

If the assumption is made that ethionine does, in fact, prevent completion of antibiotic synthesis by inhibiting the donation of methyl groups to some receptor structure, questions arise as to the nature of this receptor compound and to the role methyl groups play in inducing, promoting, or maintaining the antimicrobial activity of the completed molecule. By way of speculation, examination of the chemical structures describing the known mitomycins reveals that positional or functional alterations at positions 1a, 7, and 9a confer unique chemical, physical, and biological properties of sufficient magnitude to characterize each compound. The data obtained to date suggest that one or more substituent methyl groups located in positions 7 or 9a are requisite in maintaining the biological integrity of these antibiotic compounds. If this were not so, it would be difficult to understand the marked decrease in antibacterial activity mediated by the inhibitor ethionine, which in some fashion appears to prevent *O*-methylation in mitomycin A.

Since methionine methyl groups are, in fact, donated to the 7 and 9a methoxyl functions of mitomycin A, one might conclude that the receptor structure for this methyl transfer is hydroxylated at these positions. It is interesting to note that among the active mitomycins characterized to date, a free hydroxyl group is found only in mitomycin B, and this at position 9a. None of the known mitomycins have free hydroxyl groups at position 7. The inference is that the presence of hydroxyl groups at position 7, and under certain conditions at 9a, may have either a direct or indirect influence in negating the biological activity of these compounds. This could form the basis for a working hypothesis which would explain the influence of ethionine in inducing the accumulation of inactive mitomycins, if one can assume that hydroxylated mitomycins of the type described require protective methylation as a means of stabilization of their chemical structure, and thus, of their biological activity.

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