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Microcin 15m from *Escherichia coli*: Mechanism of Antibiotic Action

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It has been previously established that a high proportion of enterobacteria isolated from the intestinal content of humans, mostly *Escherichia coli* strains, secrete into the culture media antibiotic substances of low molecular weight, which have been called microcins. It was also found that the synthesis of these antibiotics is determined by plasmids. In this paper, a method for the purification of microcin 15m is described, and experimental data are given on the mechanism of its action. The data indicate that this action is based mainly on the inhibition of the first enzyme of the methionine biosynthetic pathway, homoserine-*O*-transsuccinylase, a similar way to the allosteric inhibition caused by this amino acid.

A number of enterobacteria, mostly *Escherichia coli* strains, have been isolated which secrete into the culture media antibiotic substances of low molecular weight, called microcins (1). It has been found that the synthesis of most microcins is determined by plasmids (3). In the culture supernatant of one of these strains (*E. coli* LP15), two different antibiotic activities have been identified. One of them, microcin 15 (hereafter referred to as microcin 15m since the finding of other microcin activity), is antagonized in its antibiotic action by L-methionine and has a molecular weight below 500.

In this paper, we describe a procedure for purifying microcin 15m and provide experimental data on the mechanism of its antibiotic action. Our results indicate that this action is due mainly to the inhibition of the first enzyme of the methionine biosynthetic pathway, homoserine-*O*-transsuccinylase, in a similar way to the allosteric inhibition caused by this amino acid (5, 7).

MATERIALS AND METHODS

Bacterial strains and assay of antibiotic activity. *E. coli* LP15, a producer of microcin 15m, and *E. coli* 405(15^r), resistant to microcin 15m, were isolated in our laboratory (1). An *E. coli* LP15 *met* mutant was obtained by using nitrosoguanidine (9). *E. coli* K-12, *E. coli* B, *E. coli* 405 (McLeod strain) and *E. coli* W were used as indicators of antimicrobial activity.

The antimicrobial spectrum of microcin 15m was studied on 64 bacterial strains belonging to 13 different genera; 60 strains were of clinical origin, and 4 (*E. coli* B and K-12, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923) were used as susceptibility test control strains. All tests were performed on minimal glucose agar medium (see below). Strains able to grow on unsupplemented agar were seeded by flooding with a suspension containing 10^5 colony-forming units. As microcin sources, dense spots of an overnight culture of *E. coli* K-12 RYC3108, which harbors the plasmid encoding for microcin 15m production, and a 500× concentrate of the supernatant of a liquid culture of the same strain were used (100 μl of this preparation was dropped into a 6-mm sterile blank susceptibility disk just before each test). Blank disks with 100 μl of a 0.2 M L-methionine solution were placed near the bacterial spots or disks containing microcin 15m. A methionine-reversible inhibition zone of at least 15-mm diameter around disks or spots after 18 h of incubation at 37°C was considered a positive result. In all cases, there was a coincidence between spot and disk tests. Appropriate controls of the immune strain *E. coli* RYC3108 were included in each experiment. Strains which are unable to grow in minimal medium (*Staphylococcus*, *Streptococcus*, and some *Proteus*) were similarly seeded on agar plates as before, and then a Whatman 2 sterile paper strip (0.5 by 5 cm) with 0.2 ml of brain heart infusion was placed on each plate. These strips were crossed by others with 0.2 ml of the 500× microcin 15m preparation, with and without 0.2 ml of 0.2 M L-methionine. The lack of an inhibition zone after 18 h of incubation at 37°C beside the microcin-containing strips in the area corresponding to the very limited growth of the tested strain was considered a negative result.

Media and growth conditions. *E. coli* LP15 was grown in minimal medium 63 (13) with 0.2% glucose at 37°C, either in liquid with stirring at 200 rpm or in solid (1.5% agar) media. For preparative cultures, incubation was allowed to reach an optical density of approximately 0.9 at 660 nm.

Chemicals. L-[*methyl*-^{3}H]methionine, L-[^{35}S]methionine, L-[*U*-^{14}C]leucine, and L-[*U*-^{14}C]phenylalanine were obtained from The Radiochemical Centre, Amer-

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sham, England. The tRNA mixture was from Sigma Chemical Co. Succinyl coenzyme A was from Serva or was prepared according to Nagai and Flavin (10). Dowex resins were from Serva.

Microcin 15m purification. The supernatants from cultures of *E. coli* LP15 were treated as described elsewhere (1). The 500× concentrate was thereafter thoroughly mixed with activated charcoal (5%, wt/vol) for 15 min. After filtration, the colorless, active supernatant was heated at 100°C for 10 min. This preparation was acidified to pH 2.5 and added to a column of Dowex 50W × 4 (H) equilibrated with HCl at the same pH. The column was first washed with HCl at pH 2.5, and the microcin activity was thereafter eluted with distilled water. The active fractions were pooled, lyophilized, dissolved in water, and added to a column of Dowex 1 × 8 (HCO_3) equilibrated with 1 mM ammonium hydrogen carbonate. A gradient of ammonium hydrogen carbonate (1 to 500 mM) was passed through the column. The active fractions, which eluted at approximately 300 mM ammonium hydrogen carbonate, were pooled and lyophilized. Finally, to eliminate the bulk of salts, the most active fractions were passed through a Dowex 50W × 4 (H) column as before. Antibiotic activities were estimated in arbitrary units. One arbitrary unit corresponded to about 0.5 μg, which was the minimal amount of microcin 15m that produced a visible inhibition halo on a lawn of *E. coli* 405 grown in solid minimal medium with glucose.

Incorporation of labeled amino acids, uracil, and thymidine into macromolecules. To estimate the effect of the microcin on protein synthesis, the in vivo incorporation of L-[*U*-^{14}C]leucine was measured by using susceptible *E. coli* strains (14). Three flasks with 5 ml of minimal medium were inoculated with 0.1 ml of an overnight culture of *E. coli* 405 and incubated at 37°C until an optical density of 0.4 at 660 nm was reached. To each flask, 0.5 μmol of labeled leucine with a specific activity of 5 μCi/μmol was added; one of them received 1,000 U of microcin 15m, and the other received the same amount of microcin plus 30 mM methionine. Portions of 1 ml were removed from the flasks at different times and mixed with 2 ml of 7.5% trichloroacetic acid for 15 min at 100°C and then chilled in ice. Each sample was filtered through a Whatman GF/C filter and washed with cold trichloroacetic acid. The radioactivity retained on the filters was estimated in a Nuclear-Chicago Mark II liquid scintillation counter.

The same procedure was used to estimate the incorporation of labeled uracil and thymidine into RNA and DNA except that the treatment with boiling water was omitted (14). The [2-^{14}C]uracil used had a specific activity of 2 μCi/μmol, and the [6-^{3}H]thymidine had a specific activity of 20 μCi/μmol. In both cases, 1 μmol of each metabolite was added.

Enzymatic activities. For the aminoacyl-tRNA synthetase assay, cells were grown to an optical density of 0.8 and centrifuged at 18,000 × *g* for 10 min. The pellet was washed twice in a buffer containing: 10 mM Tris-hydrochloride, pH 7.5; 10 mM mercaptoethanol; 5 mM magnesium acetate; and 10 mM KCl. The cells were homogenized with alumina (1:3) and resuspended in the same buffer. Alumina, unbroken cells, and membrane fragments were removed by centrifugation at 39,000 × *g* for 30 min in an SS34 rotor. The supernatant was centrifuged again at 105,000 × *g* in a 50.1 rotor for 2 h to eliminate the ribosomes. The resulting supernatant (S105) contained the aminoacyl-tRNA synthetases, which remained active for about 10 days at 4°C. The assay mixture contained, in a final volume of 250 μl: 40 mM imidazole, pH 7.5; 4 mM mercaptoethanol; 25 mM $MgCl_2$; 4 mM ATP; 50 μl of a mixture of 20-mg/ml *E. coli* tRNAs; 8 μM L-[*methyl*-^{3}H]methionine with a specific activity of 20 μCi/μmol; 20 μl of the S105 preparation; and, when appropriate, variable amounts of microcin preparations. Incubation was carried out at 37°C and stopped by addition of 2 ml of 7.5% trichloroacetic acid to the reaction mixture; the tubes were then chilled at 0°C, filtered through Whatman GF/C paper, and washed with cold 5% trichloroacetic acid. After drying, the filters were counted as above.

To estimate the homoserine-*O*-transsuccinylase activity, the cells were cultured as described, harvested, washed, and suspended in 50 mM cold phosphate buffer, pH 7.6. Crude extracts were obtained by sonication and subsequent centrifugation at 27,000 × *g*. Nucleic acids were eliminated by addition of 1% protamine sulfate up to a 0.2% final concentration. A further purification was achieved by precipitation with ammonium sulfate between 35 and 55% saturation; the precipitate was dissolved in potassium phosphate (50 mM, pH 7.6) and passed through a Sephadex G-25 column (10 by 1.2 cm) to remove amino acids and salts. This column was washed with 50 mM phosphate buffer, pH 7.6. The extract could be stored at −20°C with no appreciable loss of activity after 48 h. The enzymatic activity was determined by the conversion of L-[*U*-^{14}C]homoserine to [^{14}C]*O*-succinylhomoserine according to Nagai and Flavin (10) with some modifications. The reaction mixture contained, in a volume of 200 μl: 20 μmol of potassium phosphate buffer, pH 7.6; 0.2 nmol of succinyl-CoA; 15 nmol of L-[*U*-^{14}C]homoserine with a specific activity of 4 μCi/μmol; and approximately 200 μg of protein. The reaction was started by the addition of succinyl-CoA to the preincubated tubes (34°C) and was halted by adding 0.1 ml of 1 M KOH. The tubes were then set in a boiling water bath for 2 min to bring about the conversion of *O*-succinylhomoserine to *N*-succinylhomoserine (10); after boiling, the samples were vigorously mixed with Dowex 50W × 4 (H), and the unbound *N*-succinylhomoserine was counted in scintillation liquid plus Triton X-100. Proteins concentrations were determined according to Lowry et al. (8).

Radioactive labeling of cells and microcin extraction. To label microcin 15m, three 2-liter flasks, containing 1 liter of medium 63 each, were inoculated with 0.1 ml of an overnight culture of *E. coli* LP15 *met*. One received 0.1 mCi of L-[*methyl*-^{3}H]methionine with a specific activity of 0.77 μCi/μmol; the second received 66 μCi of L-[^{35}S]methionine with a specific activity of 0.49 μCi/μmol; and the third contained 10 μCi of L-[*U*-^{14}C]leucine with a specific activity of 77 μCi/mmol plus cold methionine to a final concentration of 0.13 mM. The flasks were incubated at 37°C until the end of exponential growth, and the cells were subsequently removed by centrifugation. Supernatants were lyophilized, and each was dissolved in 50 ml of water; 200 ml of cold methanol was added to each concentrate to remove salts, and the supernatants obtained upon centrifugation were concentrated under vacuum at

TABLE 1. Susceptibility of bacterial strains to microcin 15m[a]

Organism	No. of strains tested	No. of susceptible strains
Escherichia coli	14	10
Salmonella typhimurium	9	9
S. enteritidis	1	1
Citrobacter freundii	2	2
Klebsiella pneumoniae	7	5
Enterobacter cloacae	5	0
E. liquefaciens	2	0
Serratia marcescens	1	0
Erwinia sp.	1	0
Proteus mirabilis	4	1
P. morganii	1	0
Pseudomonas aeruginosa	9	0
P. maltophilia	1	0
Acinetobacter calcoaceticus	1	0
Staphylococcus aureus	3	0
Streptococcus faecalis	1	0
S. agalactiae	1	0
Bacillus subtilis	1	0

[a] See Materials and Methods for details.

30°C, down to a 2-ml volume. The concentrates were brought to pH 2.5 with HCl and applied to a column of Dowex 50W × 4 (10 by 0.5 cm) equilibrated at pH 2.5, which was washed successively with 100 ml of HCl (pH 2.5) and 100 ml of water. Fractions with microcin activity eluted after the addition of water. The active fractions were chromatographed by applying 100 μl on Whatman 3MM paper, using *n*-butanol–acetic acid–water (12:3:5) as the elution system, in an ascending sheet. Afterwards, the chromatograms were cut at 1-cm intervals to count radioactivity and its distribution in a liquid scintillation counter.

RESULTS

***E. coli* LP15 growth and microcin production.** Microcin 15m activity was found primarily in the supernatant of minimal medium cultures of the producing strain. The antibiotic activity increased with time during growth, and the highest activity, about 2,000 arbitrary units of the 500× concentrates per ml (1), was always found at the end of the exponential phase of growth. Activity declined later on, which might have been due to inactivation of the microcin or its reutilization by the producing strain.

Purification and some properties of microcin 15m. The starting material was a 500× concentrate (1), obtained from the 60-liter culture supernatant of *E. coli* LP15. From 2 to 4 mg of active material was normally obtained.

As previously established (1), microcin 15m is a compound of low molecular weight (below 500). Furthermore, its antibiotic activity is quickly destroyed by oxidants such as hydrogen peroxide (90% of activity lost after 6 h at 20°C in the presence of 0.6 mM H_2O_2). Current studies on its molecular structure indicate a molecular weight of 240, including a methionine moiety that can be separated under mild acid conditions; apparently, it does not correspond to any of a series of methionine analogs described in the literature (namely, selenomethionine, ethionine, norleucine, methionine-sulfoximine, methionine-sulfone, methionine-sulfoxide, and α-methyl-methionine) according to chromatographic techniques, infrared spectra, and microbiological criteria (J. M. Fernández-Sousa, personal communication).

Antimicrobial specificity of microcin 15m. Studies summarized in Table 1 on the spectrum of activity of microcin 15m are only qualitative, since no sufficiently pure preparations of the active product are yet available. Nevertheless, specificity of the detected activities is assured by their reversibility in the presence of L-methionine. Moreover, since L-methionine is a growth factor for many bacterial genera, only organisms growing in minimal glucose media can be properly tested. The triple-strip test that we used can, however, offer some preliminary results. Microcin 15m seemed to have a rather restricted spectrum of activity, which included strains of *Escherichia, Citrobacter, Salmonella*, and *Klebsiella* and apparently excluded *Pseudomonas* and gram-positive organisms (Table 1).

Antagonism of microcin 15m activity by L-methionine. The antibiotic effect of microcin 15m on susceptible cells was bacteriostatic, according to viable counting estimations (data not shown) of cultures treated with purified preparations of microcin. The inhibitory action did not appear when susceptible cells were growing in a medium containing methionine (1).

Effect of microcin 15m on the in vivo incorporation of [^{14}C]leucine, [2-^{14}C]uracil and [6-^{3}H]thymidine by proteins, RNA, and DNA. The capability of L-methionine to antagonize the antibiotic effect of microcin 15m suggested that its structure could be related to that of the amino acid and, consequently, its mechanism of action could be located in some step of protein synthesis involving L-methionine metabolism. To test this hypothesis, the effect of microcin 15m on the incorporation of labeled leucine by susceptible cells was investigated. Microcin 15m markedly inhibited the incorporation of the labeled amino acid (Fig. 1). L-methionine (see Materials and Methods) prevented the inhibition produced by the microcin (data not shown). In a similar experiment using cold leucine, equal portions were removed after various periods of incubation to count viable cells. The data obtained (not shown) confirmed the bacteriostatic character of the effect of microcin 15m. The effect of microcin 15m on RNA and DNA synthesis was not significant compared with the marked effect

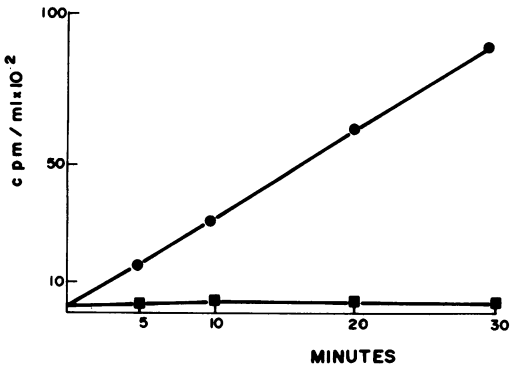

FIG. 1. Effect of microcin 15m on the incorporation of L-[*U*-^{14}C]leucine in protein synthesis of *E. coli* 405. Incorporation without (○) and with (■) microcin, as described in Materials and Methods.

observed on protein synthesis (Fig. 2). In fact, RNA synthesis was partially inhibited by the antibiotic, possibly due to a "stringent response" (6) of the cells as a result of methionine starvation caused by microcin 15m.

Effect of microcin 15m on the activity of homoserine-*O*-transsuccinylase. In a series of experiments homoserine-*O*-transsucinylase activity in the presence or absence of L-methionine, *S*-adenosylmethionine (SAM), and microcin 15m was assayed (Fig. 3). Figure 3A shows the inhibitory effects of L-methionine alone and together with SAM on the enzyme, which are in good agreement with previous data (5, 7). According to our results, a mixture of 5 mM methionine and 0.1 mM SAM inhibited 85% of the enzyme activity. Figure 3B illustrates the effect of the addition of microcin 15m alone and together with 0.1 mM SAM. The patterns of the two inhibition curves were rather similar, although the concentration of microcin 15m required to obtain an equivalent inhibition of the enzyme (in the presence of 0.1 mM SAM) was about an order of magnitude lower than that of methionine under similar conditions.

The concerted inhibition shown by either L-methionine or microcin 15m with SAM was not observed when, in the absence of SAM, the amino acid and the microcin were added together (data not shown). This result points to a similar mechanism of action for both compounds, a hypothesis that is strengthened by the study of a microcin 15m-resistant *E. coli* 405 mutant obtained from the parental, susceptible strain. The homoserine-*O*-transsuccinylase assay of the extracts prepared from this mutant showed that this enzyme had lost its susceptibility to methionine, SAM, and microcin 15m when added separately. It was necessary to use a high concentration of SAM plus methionine or microcin 15m to detect a measurable inhibition. Thus,

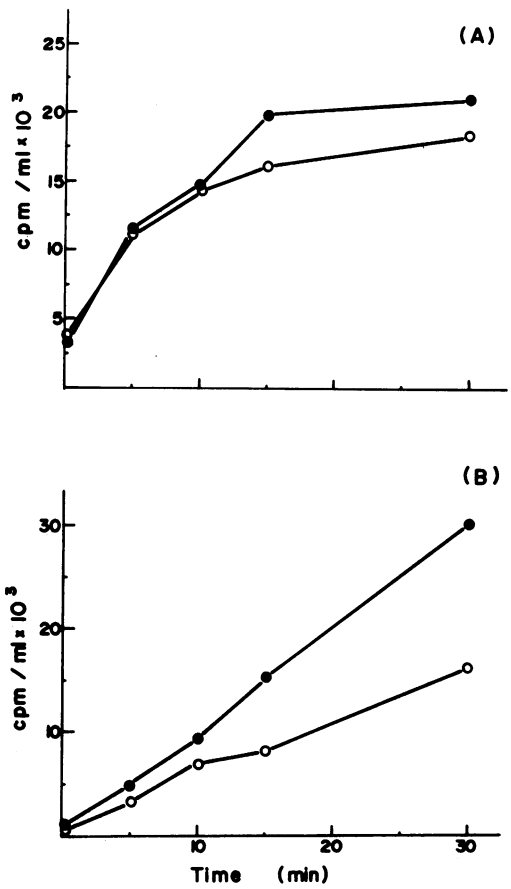

FIG. 2. Effect of microcin 15m on the incorporation of [6-^{3}H]thymidine and [2-^{14}C]uracil in DNA and RNA. (A) Incorporation of [6-^{3}H]thymidine without (●) and with (○) microcin 15m. (B) Incorporation of [2-^{14}C]uracil without (●) and with (○) microcin 15m, as described in Materials and Methods.

when 0.5 mM SAM was added with 3 mM microcin, 80% of the maximal activity was observed, a figure that should correlate with the weak inhibition observed in vivo when resistant mutants are grown in the presence of a relatively high concentration of microcin 15m.

Biosynthesis of microcin 15m. In a preliminary experiment, we observed that resting cells of *E. coli* LP15 produced a measurable amount of microcin 15m activity in the suspending medium after 3 h of incubation, whereas an auxotrophic derivative requiring methionine (*E. coli* LP15 *met*) gave no detectable activity under similar conditions. This observation suggested that L-methionine is a precursor for the biosynthesis of the microcin.

To test this hypothesis, an experiment was carried out to investigate whether specifically labeled parts of L-methionine given to *E. coli* LP15 *met* appeared to be associated with micro-

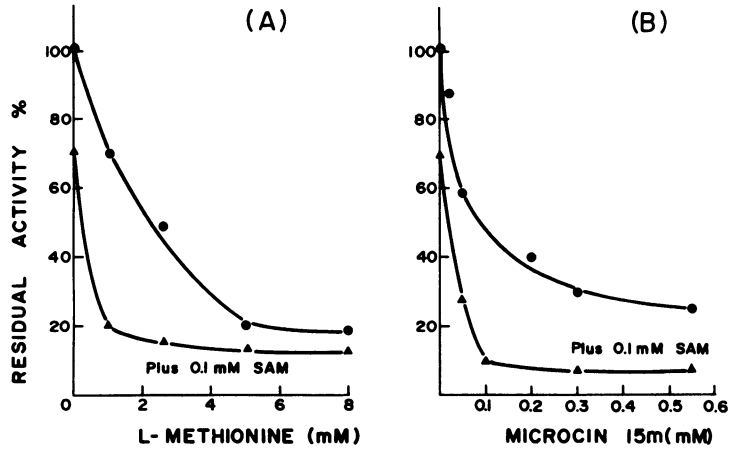

FIG. 3. Inhibition of homoserine-*O*-transsuccinylase activity from *E. coli* 405 by: (A) L-methionine alone (●) or together with 0.1 mM SAM (▲); (B) microcin 15m alone (●) or together with 0.1 mM SAM (▲). The mean specific activity of homoserine-*O*-transsuccinylase in the enzymatic extracts used was 30 nmol of *O*-succinylhomoserine formed per min per mg of protein at 34°C.

cin 15m activity (Fig. 4). L-[*methyl*-^{3}H]methionine, L-[^{35}S]methionine, and L-[*U*-^{14}C]leucine as the control were used separately. The location of the eluted fractions that showed antibiotic activity from either culture correlated well with a pronounced peak of radioactivity when labeled methionine, but not labeled leucine, was used. These results indicate that at least a part of the methionine structure is required for the biosynthesis of microcin 15m by the producer strain, which was confirmed by the structural studies mentioned above.

DISCUSSION

By using minimal media and a simple screening procedure, about 20 apparently different microcins produced by distinct *Enterobacteriaceae* have been identified (1, 2). About 35% of the microcins produced by *E. coli* strains isolated from different specimens of human feces were antagonized by L-methionine and proved to be identical or very similar compounds (2, 11). Therefore, in our investigation, we probably studied a very common type of microcin. Fur-

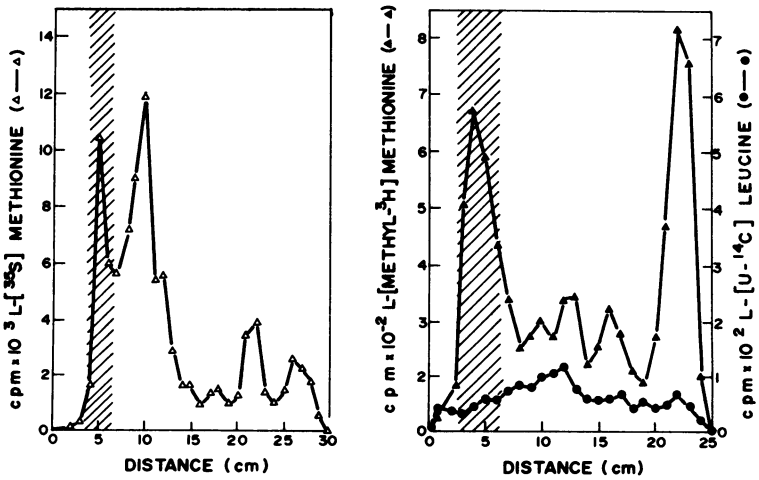

FIG. 4. Chromatographic correlation between microcin 15m activity and labeled L-methionine derivatives in *E. coli* LP15 *met* cells. Hatched bars correspond to the location of microcin activity in ascending paper chromatography of extracts obtained from the culture of the auxotrophic cells grown on ^{35}S (left)- or *methyl*-^{3}H (right)-labeled L-methionine. The curves give the distribution of radioactivity in the same chromatographic samples. On the right can also be seen the chromatographic behavior of cells grown on L-[*U*-^{14}C]leucine (and cold L-methionine) as a control (●). See text for explanation.

thermore, microcin 15m, as well as the other detected microcins, is coded by plasmids (3, 11; F. Sánchez, in preparation), which could facilitate the potential spread of such characters in bacterial populations of the mammalian intestinal tract.

The data given here indicate that microcin 15m, a methionine-containing antibiotic (or at least an analog of this amino acid), blocks protein synthesis, apparently by inhibiting methionine production on the first enzyme of its biosynthetic pathway, homoserine-*O*-transsuccinylase. The finding of a marked inhibition of this enzyme by a low concentration of the microcin (Fig. 3) suggests that it is a site of action for the antibiotic. The inhibition of the homoserine-*O*-transsuccinylase activity by microcin 15m was enhanced in the presence of SAM, which indicates that the microcin exerts its effect by mimicking the concerted allosteric inhibition described for L-methionine and SAM (7). This interpretation is strengthened by the observation that in a mutant of *E. coli* 405 isolated as resistant to microcin 15m, its homoserine-*O*-transsuccinylase had lost susceptibility to either microcin 15m, L-methionine, or SAM when added separately at a concentration range inhibitory for the parental enzyme. As stated before, it was only weakly inhibited by relatively high concentrations of SAM plus either L-methionine or microcin 15m.

The above results do not exclude the possibility that microcin 15m could inhibit other steps more or less related to methionine metabolism. In this regard it is of interest to note that microcin 15m inhibited apparently in a specific manner the incorporation of methionine into Met-tRNA, since the incorporation of labeled phenylalanine to its corresponding tRNA, used as a control, was not affected. However, it was necessary to use a microcin 15m/L-methionine ratio of about 60 to achieve full inhibition of the process. In fact, microcin 15m inhibited methionyl-tRNA synthetase with a K_i of 1.5 mM, whereas the K_m for L-methionine (4; B. Cañas, unpublished data) is as low as 20 μM. Moreover, the *E. coli* 405 derivative resistant to the microcin showed a synthetase activity identical to that obtained from the parental strain in regard to its kinetic behavior for the antibiotic.

Although microcin 15m also inhibited RNA synthesis (approximately 50%; Fig. 2), this may have resulted from the methionine starvation caused by the antibiotic. In this situation, a rather abrupt inhibition of RNA accumulation occurs, among other changes in cellular activity (6).

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