MECHANISM OF ACTION OF BICYCLOMYCIN

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Microscopic examination of cultures of *Escherichia coli* exposed to bicyclomycin revealed elongated or spheroplast-like cells. At the lethal level, bicyclomycin was shown to inhibit the synthesis of RNA and protein in the growing cells of *E. coli* 15 THU, whereas DNA and lipid synthesis were not significantly affected. However, the antibiotic did not block RNA and protein synthesis *in vitro*.

Bicyclomycin was observed to inhibit the synthesis of envelope proteins more markedly than that of cytoplasmic proteins. The synthesis of two major envelope proteins was more sensitive to bicyclomycin than that of the other envelope proteins. One (peak I), which was inhibited to the greatest extent, seemed to be identical with a bound form of lipoprotein, and the other (peak V) with a free form of lipoprotein. Bicyclomycin exhibited inhibitory effects on the exclusive biosynthesis of the lipoprotein in histidine-starved cells of *E. coli* 15 THU. The biosynthesis of the bound form of lipoprotein was more profoundly inhibited by bicyclomycin than that of the free form.

These results indicate that the primary action of bicyclomycin may be due to the interference with the biosynthesis of lipoprotein, and its assembly to peptidoglycan.

Bicyclomycin, discovered by MIYOSHI *et al.*[1], is a new antibiotic with inhibitory activity against Gram-negative bacteria such as *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella*, and *Neisseria*, but inactive against *Proteus*, *Pseudomonas*, and Gram-positive bacteria. Bicyclomycin has a unique chemical structure and exhibits no cross resistance to any other antibacteral drugs.[2,3]

During the course of investigations concerning the effects of bicyclomycin on morphology and on macromolecular synthesis in *E. coli*, it has been found that bicyclomycin inhibits synthesis of the lipoprotein. The lipoprotein exists in the outer membrane of Gram-negative bacteria such as *E. coli* and *Salmonella*, immunologically similar in the two organisms, and has an important function in stabilizing the total structure of the cell wall.[4,5]

The effect of bicyclomycin on the biosynthesis of lipoprotein has been investigated for the purpose of elucidating the biochemical mechanism of its bactericidal action, and the results are presented in this publication.

Materials and Methods

E. coli 15 THU (thy$^-$, his$^-$, ura$^-$) (a gift of Dr. KOMANO, Kyoto University), *E. coli* Q13 and *E. coli* ATCC 27166 were used. The strain of ATCC 27166 is a mutant hypersensitive to bicyclomycin. The former was grown at 37°C in M9-glucose medium[6] supplemented with thymine (4 μg/ml), L-histidine (50 μg/ml), and uracil (20 μg/ml); and the latter two strains in nutrient broth (Difco) at 37°C.

Bicyclomycin was prepared by Fujisawa Research Laboratories. Rifampicin was purchased

from Daiichi Pharmaceutical Co., Ltd. Tetracycline hydrochloride was a product of Takeda Chemical Industries.

Thymine-2-^{14}C (51 mCi/mmole), uracil-2-^{14}C (21.0 mCi/mmole), L-arginine-^{14}C (U) (175 mCi/mmole), L-histidine-1-^{14}C (10.48 mCi/mmole), L-phenylalanine-^{14}C (U) (382 mCi/mmole), acetate-1-^{14}C (54.9 mCi/mmole), and L-leucine-^{14}C (U) (251 mCi/mmole) were purchased from Daiichi Pure Chemicals Co., Ltd. L-Arginine-^{3}H (G) (6.6 mCi/mmole) was a product of New England Nuclear Corp. Uridine-5'-triphosphate(UTP)-5, 6-^{3}H (36.84 Ci/mmole) was purchased from Schwarz/Mann.

In vivo macromolecular synthesis

DNA, RNA and protein synthesis were followed by measuring the incorporation of labeled thymine, uracil and L-histidine into the acid-insoluble precipitates. At intervals after the addition of radioactive precursors, samples were removed to equal volumes of ice-cold 10 % trichloroacetic acid (TCA). After cooling for 30 minutes in an ice bath, the acid-insoluble precipitates were collected on a Millipore filter (0.45 μ porocity) and washed with ice-cold 5 % TCA. The radioactivity was counted in BRAY's solution[7] by a Packard Tri-Carb Liquid Scintillation Spectrometer.

In vivo lipid synthesis

The synthesis of lipids was followed by measuring the incorporation of labeled acetate into the lipid fraction. Samples were removed at intervals and extracted with a mixture of chloroform-methanol (2 : 1), according to the procedure of NUNN[8]. The radioactivity was determined by dissolving the dried extract in toluene scintillation fluid containing 5 g of 2, 5-diphenyloxazole (PPO) and 0.3 g of 1, 4-bis-2(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) per liter of toluene.

Preparation of cell-free extracts and S-30 fraction

E. coli Q13 was grown in nutrient broth containing 5 % glucose, harvested by centrifugation, and stored in a frozen state. The cells were disrupted by grinding with alumina (Nakarai Chemical Co., Ltd.), and suspended in Tris buffer (10 mM, pH 7.0) containing 10 mM $MgCl_2$, 0.1 mM EDTA and 10 mM 2-mercaptoethanol. The cellular debris was removed by centrifugation (20,000 g, 20 minutes). The S-30 fraction was prepared according to the procedure of NIRENBERG[9].

In vitro RNA and protein synthesis

The RNA polymerase activity of cell-free extracts was assayed as described in the legend to Table 1. Cell-free protein synthesis was followed by measuring the incorporation of labeled phenylalanine or labeled leucine into the hot TCA-insoluble material[9].

Biosynthesis of envelope and cytoplasmic proteins

Exponentially growing cultures were labeled with radioactive L-arginine for 2 minutes. The mixtures were immediately chilled in an ice bath and non-radioactive arginine was added. The cells were collected by centrifugation, washed with 10 mM sodium phosphate buffer (pH 7.1), and disrupted in the same buffer by treatment for 5 minutes with a sonic oscillator (20 kc) in an ice bath.

The envelope and cytoplasmic fractions were prepared by the differential centrifugation method as described by INOUYE and his co-workers[10].

For envelope protein synthesis, the envelope fraction (pellet) was solubilized at 70°C for 20 minutes in an appropriate volume of the solubilizing solution (1 % sodium dodecyl sulfate (SDS), 10 % glycerol and 1 % 2-mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.1). After solubilization, the solution was taken to measure the radioactivity incorporated into the envelope proteins, or was subjected to SDS-polyacrylamide gel electrophoresis.

For cytoplasmic protein synthesis, an equal volume of 10 % TCA was added to the cytoplasmic fraction (supernatant). After cooling for 30 minutes in an ice bath, the mixtures were boiled for 30 minutes and chilled for 30 minutes in an ice bath. The hot acid-insoluble precipitates were collected on a membrane filter and washed with 5 % TCA. The radioactivity

was counted in BRAY's solution.

Lipoprotein synthesis in the histidine-starved cells of *E. coli* 15 THU

The cells of *E. coli* 15 THU were grown in M9-glucose medium supplemented with thymine, histidine and uracil, washed and suspended in the above medium without histidine. Radioactive arginine was added to the suspension. After the incubation at 37°C, the mixtures were chilled in an ice bath. The cells were disrupted by sonic oscillation and the envelope fraction was prepared by centrifugation. Half of the envelope fraction was treated with egg white lysozyme (100 μg/ml) at 37°C for 3 hours in 10 mM sodium phosphate buffer, (pH 7.1) to analyze the bound form of the lipoprotein[11]. Lysozyme-treated and untreated fractions were then subjected to SDS-polyacrylamide gel electrophoresis; separate gels contained cytochrome c and Dansyl-insulin as markers. After electrophoresis, the position of lipoprotein was detected by reference to the cytochrome c and Dansyl-insulin and cut out.

After each slice was incubated with 0.5 ml of Soluene 350 (Packard) at 50°C for 3 hours, the radioactivity was counted in toluene scintillation fluid.

Preparation of peptidoglycan

From the envelope fraction, the peptidoglycan was isolated using 4 % SDS as described by BRAUN and SIEGLIN[28]. The insoluble precipitates in hot 4 % SDS were collected by centrifugation at $10^5 \times g$ for 60 minutes at 20°C, and washed twice with water before use.

SDS-polyacrylamide gel electrophoresis

Polyacrylamide gels, 0.6×12 cm, were made with 7.5 % acrylamide and 0.25 % methylenebisacrylamide as cross linking agent in the presence of ammonium persulfate and N, N, N′, N′-tetramethylethylenediamine polymerized in 0.1 M sodium phosphate buffer, pH 7.1, containing 0.5 % SDS. Dansyl(5-dimethylaminonaphthalene-1-sulfonyl)-protein (DANS-protein) as internal moleculra standards were prepared by the method of INOUYE[12]. The procedure of WEBER[13] was employed for staining of the gels.

Results

1. Morphological Changes in Bicyclomycin-treated Cells

The effect of treatment with bicyclomycin on the morphology of *E. coli* strains is shown in Plates 1 and 2. When the culture of *E. coli* 15 THU was exposed to bicyclomycin, elongated and spheroplast-like cells were observed under the microscope. The formation of elongated cells was induced at a level of 12.5 μg/ml of bicyclomycin (Plate 1 (B)). In the medium with 20 % sucrose and 0.2 % $MgSO_4$ as a spheroplast stabilizer, the spheroplast-like cells were observed in the culture treated with a lethal concentration of 25 μg/ml of bicyclomycin (Plate 1 (C)).

In the cells of *E. coli* ATCC 27166, which is a mutant hypersensitive to bicyclomycin, the majority of the cells were elongated many times their normal length at a level of 6 μg/ml of bicyclomycin (Plate 2 (B)). However, the FEULGEN-positive nuclear material in the elongated cells appeared to be uniformly distributed (Plate 2 (C)).

2. Effect of Bicyclomycin on the Synthesis of DNA, RNA and Protein in *E. coli* 15 THU

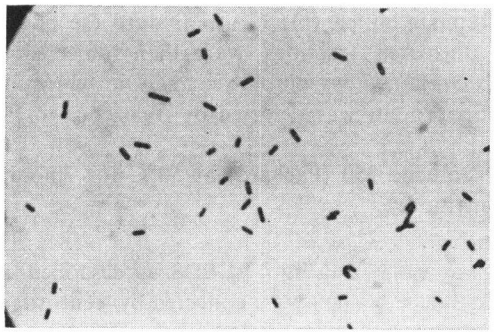
Effects of bicyclomycin on DNA, RNA and protein synthesis are presented in Fig. 1.

The synthesis of DNA, RNA and protein in *E. coli* 15 THU were studied by following the incorporation of radioactive precursors. ^{14}C-Thymine, ^{14}C-uracil or ^{14}C-L-histidine (0.5 μCi/ml, final concentration) was added to separate portions of the exponentially growing culture. Each portion was divided, and bicyclomycin (25 μg/ml or 100 μg/ml, final concentration) was added to one part, the other serving as untreated control. The culture was incubated at 37°C.

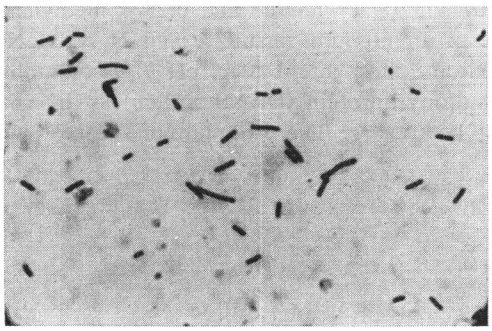
Plate 1. Morphological changes induced in the cells of *E. coli* 15 THU by bicyclomycin

Smears were prepared from 2-hour cultures, stained with phenolic fuchsin except in the case of (C), and photographed with a microscope. Magnification 600×

(A) Control



(B) Bicyclomycin, 25 μg/ml



(C) Bicyclomycin, 25 μg/ml in the medium supplemented with 20 % sucrose and 0.2 % $MgSO_4$

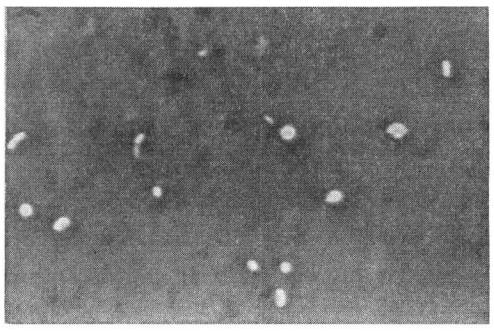
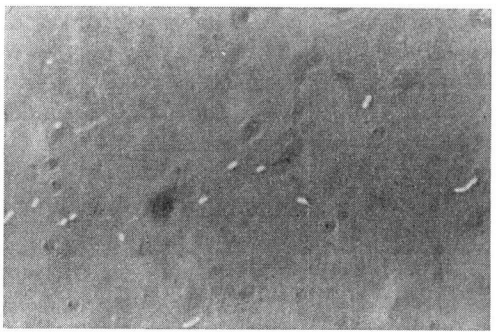


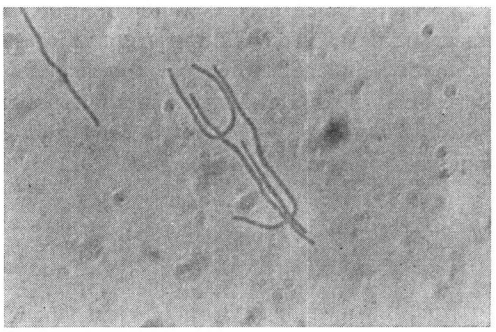
Plate 2. Morphological changes induced in the cells of *E. coli* ATCC 27166 (bicyclomycin-sensitive cells) by bicyclomycin

Smears were prepared from 2-hour cultures and photographed with a microscope. Magnification 600×

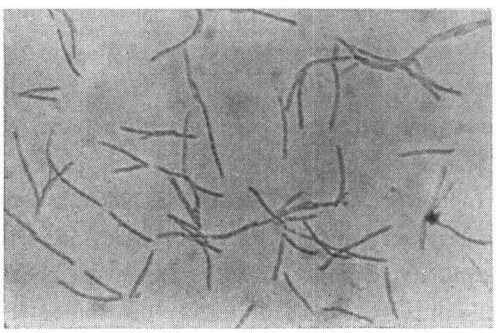
(A) Control



(B) Bicyclomycin, 6 μg/ml



(C) FEULGEN-stained preparation of the elongated cells, bicyclomycin, 6 μg/ml



Samples were removed at intervals for determination of the radioactivity incorporated into the acid-insoluble material.

In the untreated control cultures, the synthesis of DNA, RNA and protein proceeded linearly for 60 minutes (Fig. 1).

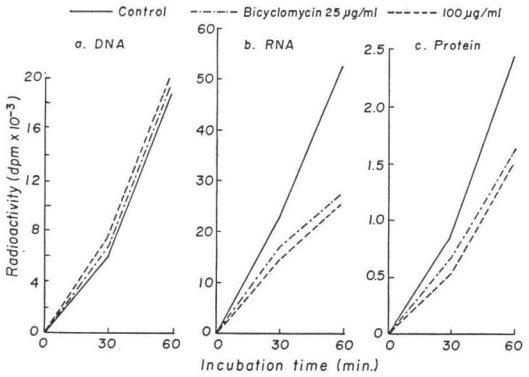
In the cultures treated with bicyclomycin at 25 μg/ml, which is approximately the minimal growth inhibitory concentration, the effects on RNA and protein synthesis during the first 30

minutes of incubation were not very evident. In 60 minutes, however, some inhibition was observed. The incorporation of uracil was blocked approximately 50 %, and L-histidine incorporation was inhibited about 40 %. Both inhibitions were not proportional to the concentration of bicyclomycin. The same grade of inhibition was revealed at 25 μg/ml and at 100 μg/ml (Fig. 1 (b), (c)).

In contrast, the culture exposed to bicyclomycin incorporated ^{14}C-thymine into DNA at the same rate as the untreated control culture (Fig. 1 (a)). It indicated that bicyclomycin did not significantly affect DNA synthesis.

Fig. 1. Effects of bicyclomycin on DNA, RNA and protein synthesis

Bicyclomycin and ^{14}C-labeled precursors were added to the exponentially growing culture of *E. coli* 15 THU. Macromolecular synthesis was measured by incorporation of radioactive precursors into the acid-insoluble material as described in Materials and Methods.



3. The Effect of Bicyclomycin of Lipid Synthesis

Radioactive acetate was added to the exponentially growing culture. It was divided, and bicyclomycin (100 μg/ml, final concentration) was added to one part, the other serving as untreated control. The mixtures were incubated at 37°C. Bicyclomycin (100 μg/ml) did not significantly affect the lipid synthesis.

4. The Effect of Bicyclomycin on *in vitro* RNA Synthesis

Since bicyclomycin was observed to inhibit RNA synthesis *in vivo*, the effect of bicyclomycin on RNA polymerase activity of the cell-free extracts was further examined.

As shown in Table 1, bicyclomycin did not block the RNA polymerase activity of cell-

Table 1. The effect of bicyclomycin on cell-free RNA synthesis

	Treatment	^{3}H-UMP incorporated (dpm)	
		5 minutes	10 minutes
Expt. 1	Complete system	8,320	13,480
	〃 +bicyclomycin, 6.25 μg/ml	11,440	14,720
	〃 +bicyclomycin, 25 μg/ml	10,220	—
	〃 +bicyclomycin, 100 μg/ml	12,890	17,870
	〃 +bicyclomycin, 400 μg/ml	13,700	17,300
	〃 +rifampicin, 20 μg/ml	—	4,020
Expt. 2*	Complete system	9,340	9,740
	〃 +bicyclomycin, 100 μg/ml	13,000	15,140
	〃 +bicyclomycin, 400 μg/ml	12,700	15,000
	〃 +bicyclomycin, 1,000 μg/ml	12,620	15.200

* Template DNA was preincubated with bicyclomycin at 37°C for 30 minutes in the buffer.

The assay system contained 100 mμmoles of ATP, GTP, CTP and UTP (^{3}H-UTP, 0.5 μCi), 1.25 μmoles of magnesium acetate, 0.5 μmole of $MgSO_4$, 1.25 μmoles of 2-mercaptoethanol, 9 μg of herring sperm DNA, and 30 μmoles of Tris buffer (pH 7.8), and cell-free extracts in a total volume of 0.3 ml. The mixtures were incubated at 37°C and the radioactivity of TCA-insoluble material was counted in BRAY's solution.

free extracts at the concentration of 6.25~1,000 μg/ml. The preincubation of bicyclomycin with template DNA showed no effect on the reaction. Bicyclomycin exhibited a slight stimulatory effect on RNA synthesis *in vitro*.

In contrast to bicyclomycin, the addition of 20 μg/ml of rifampicin to the reaction mixture markedly inhibited the RNA polymerase activity by about 70 %.

5. The Effect of Bicyclomycin on Cell-free Protein Synthesis

In order to elucidate the mechanism of bicyclomycin inhibition on protein synthesis *in vivo*, the effect of the antibiotic was examined with the cell-free extracts, synthesizing protein with endogenous mRNA or with poly (U)[9].

Cell-free protein synthesis was not significantly affected by bicyclomycin over a wide range of concentrations (25~1,000 μg/ml).

6. The Effect of Bicyclomycin on the Synthesis of Envelope and Cytoplasmic Proteins

Since protein synthesis *in vitro* was not significantly blocked by bicyclomycin, the effects on envelope and cytoplasmic protein synthesis were examined in order to elucidate the mechanism of the inhibition by the antibiotic of protein synthesis *in vivo*.

Bicyclomycin (25 μg/ml or 100 μg/ml, final concentration) was added to the exponentially growing cultures of *E. coli* 15 THU. The mixtures were incubated at 37°C for 5, 10 and 20 minutes, respectively, as shown in Fig. 2. After the incubation, each cultures was labeled with ^{14}C-L-arginine (0.25 μCi/ml, final concentration) for 2 minutes. The envelope and cytoplasmic fractions were prepared, and the incorporation of ^{14}C-arginine into each fraction was measured as described in Materials and Methods.

As illustrated in Fig. 2, the envelope protein synthesis was more sensitive to bicyclomycin than the cytoplasmic protein synthesis. In the cultures treated with bicyclomycin at the level of 100 μg/ml, the differential inhibitory effect was observed. By 5, 10 and 20 minutes, the envelope protein synthesis was inhibited 35, 40 and 40 %, respectively, and the cytoplasmic protein synthesis was blocked 15 % in each case (Fig. 2 (a)).

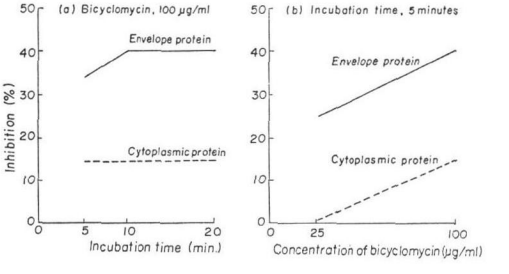


Fig. 2. Effects of bicyclomycin on envelope and cytoplasmic protein synthesis

The exponentially growing cells of *E. coli* 15 THU were treated with bicyclomycin. After the incubation, each culture was labeled with ^{14}C-arginine (0.3 μCi/ml) for 2 minutes. The envelope and cytoplasmic fractions were prepared and these synthesis were measured as described in Materials and Methods.

The control experiment was carried out without the addition of bicyclomycin. All data were expressed as rates of inhibition calculated as percentage of over-all incorporation of the control: (a) as a function of time in the presence of bicyclomycin (100 μg/ml); (b) as a function of bicyclomycin concentration for 5 min-incubation.

At a lower level of 25 μg/ml, the envelope protein synthesis was selectively inhibited 25 %, whereas the cytoplasmic protein synthesis was not significantly affected (Fig. 2 (b)).

7. The Effect of Bicyclomycin on the Synthesis of Individual Envelope Protein

Bicyclomycin was observed to inhibit the envelope protein synthesis more selectively than the cytoplasmic protein synthesis (Fig. 2). The inhibitory effects of the antibiotic on the biosynthesis of individual envelope proteins were further examined. The envelope fraction of *E. coli* 15 THU pulse-labeled with ^{14}C-L-arginine for 2 minutes in the presence of the antibiotic

was prepared, and the envelope proteins were then separated by SDS-polyacrylamide gel electrophoresis. The gel patterns of the envelope proteins labeled in the presence of two different concentrations of the antibiotic, where presented in Fig. 3.

Four major peaks appeared without addition of bicyclomycin, and they were numbered as shown in Figs. 3 and 4.

The biosynthesis of peak II and V proteins was more sensitive to bicyclomycin than that of the other envelope proteins. In the presence of 25 μg/ml of the antibiotic, the incorporation of ^{14}C-arginine into peaks II and V was inhibited 44 % and 48 %, respectively. Similarly, in the presence of 100 μg/ml of the antibiotic, the biosynthesis of peak II and V proteins was inhibited 50 % and 56 %. The inhibition of synthesis of the individual proteins by bicyclomycin is summarized in Table 2.

As illustrated in Fig. 4, the peak V protein was located between cytochrome c and Dansyl-insulin and did not contain L-histidine, as shown by the double label experiment. Thus, the protein at peak V was considered to be identical with the free form of the lipoprotein reported by INOUYE and his co-workers[14].

Fig. 3. The effect of bicyclomycin on the biosynthesis of envelope proteins

The experimental procedures were described in legend to Fig. 2. The envelope fractions were analized by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were sliced with a razor and the radioactivity of each slice was counted as described in Materials and Methods.

Arrows with letters indicate positions of the internal molecular weight standards; a, DANS-serum albumin; b, DANS-trypsin; c, cytochrome c; d, DANS-insulin. The number indicates each peak.

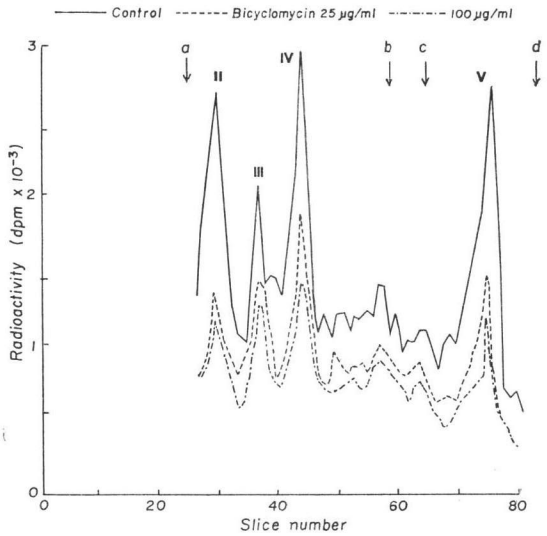


Plate 3 presents the results when the envelope fractions in the bicyclomycin (100 μg/ml)-treated culture were compared with those in normal culture by SDS-polyacrylamide gel electrophoresis. The band at the position of the lipoprotein was scarcely detected with the antibiotic-treated cells.

These results suggest the selective inhibition by bicyclomycin of the biosynthesis of lipoprotein.

Table 2. The effect of bicyclomycin on individual envelope protein synthesis

Bicyclomycin (μg/ml)	Inhibition (%)			
	peak II	peak III	peak IV	peak V
25	44.3	27.0	27.5	47.8
100	50.0	34.7	32.0	56.0

After the treatment with bicyclomycin for 5 minutes, the culture of *E. coli* 15 THU was labeled with ^{14}C-arginine as shown in Fig. 2. The envelope fraction was prepared and subjected to SDS-polyacrylamide gel electrophoresis. For each gel pattern as shown in Fig. 3, the total radioactivities of peak II, III, IV and V were measured, respectively. The rate of inhibition of each peak fraction was calculated as percentage of the over-all incorporation of control.

Fig. 4. Gel electrophoresis of the envelope proteins of *E. coli* 15 THU double-labeled with ^{3}H-L-arginine and ^{14}C-L-histidine

The envelope fraction was prepared and subjected to SDS polyacrylamide gel electrophoresis. After electrophoresis, the gels were sliced with a razor, and the radioactivity of each slice was counted as described in Materials and Methods. Assignments of internal standards are the same as in Fig. 3. The number indicates each peak.

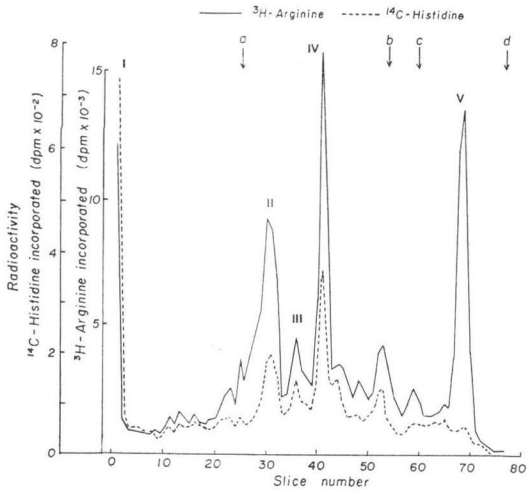
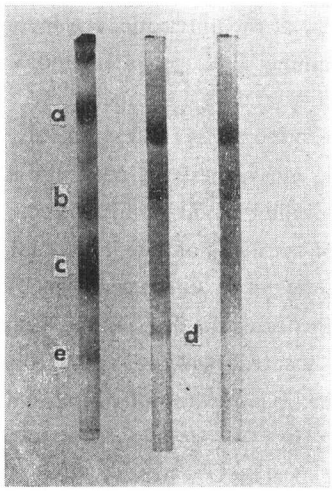


Plate 3. Effects of bicyclomycin on the synthesis of envelope proteins

Bicyclomycin (100 μg/ml, final concentration) was added to the exponentially growing cultures of *E. coli* 15 THU. The mixtures were incubated at 30°C for 60 minutes. The control experiment was carried out without the addition of bicyclomycin. The envelope fractions were prepared and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained by the procedure of WEBER[18].

(A) Internal molecular weight standards; a, DANS-serum albumin; b, DANS-trypsin; c, cytochrome c; e, DANS-insulin (B) Control; d, the free form of lipoprotein (C) Bicyclomycin, 100 μg/ml



(A) (B) (C)

8. The Effect of Bicyclomycin on Lipoprotein Synthesis in Histidine-starved Cells of *E. coli* 15 THU

HIRASHIMA and INOUYE[15] have shown that the lipoprotein, which does not contain histidine, can be synthesized normally, even when the histidine auxotroph of *E. coli* is starved for histidine. Under this condition, the biosynthesis of all the other envelope proteins is almost completely blocked.

Since bicyclomycin inhibited selectively the lipoprotein synthesis, the effect of the antibiotic was examined on the exclusive biosynthesis of lipoprotein of *E. coli* 15 THU in the absence of histidine.

^{14}C-L-Arginine (0.3 μCi/ml, final concentration) and bicyclomycin were added to the cell suspension in the absence of histidine, and the mixtures were incubated at 37°C for 90 minutes. The cytoplasmic and envelope fractions were prepared, and the envelope fraction was subjected to SDS-polyacrylamide gel electrophoresis.

As shown in Fig. 5, the peak I (origin) and V (lipoprotein) appeared but the other peaks had disappeared. ^{14}C-Arginine was incorporated into the cytoplasmic and envelope proteins, and 30~40 % of the radioactivity incorporated into envelope proteins was that of the free form of lipoprotein.

In the cells exposed to bicyclomycin over a wide range of concentrations, the envelope protein synthesis was inhibited 33 %, whereas the cytoplasmic protein synthesis was scarcely affected (Table 3).

Table 3. The effect of bicyclomycin on the biosynthesis of lipoprotein in the histidine-starved cells of *E. coli* 15 THU

Bicyclomycin (μg/ml)	Inhibition (%)			
	Cytoplasmic protein synthesis	Envelope protein synthesis	Peak I protein synthesis	Peak V protein synthesis
25	0	33.5	87.8	52.3
100	0	33.8	88.5	58.9
1,000	0	33.2	88.3	59.9

Bicyclomycin and ^{14}C-arginine were added to the cultures starved for histidine. The mixtures were incubated at 37°C for 90 minutes. The control experiment was carried out without the addition of biocyclomycin. After the incubation, the envelope fractions were prepared as described in Materials and Methods, and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the portions of the gel at the peaks I and V determined by standard markers were sliced with a razor, and then the radioactivity of each slice was counted as described in Materials and Methods.

All data were expressed as rates of inhibition calculated as percentage of the over-all incorporation of controls.

As shown in Table 3, inhibitory effects of bicyclomycin on the biosynthesis of the lipoprotein (peak V) and the peak I protein were observed. The incorporation of ^{14}C-arginine into the lipoprotein was inhibited 52~60 %. The incorporation into the peak I protein, even at a low level of 25 μg/ml, was inhibited 88 %.

Although the strong inhibition of peak I protein was constantly observed, the grade of inhibition of peak V protein varied in the range of 10 % to 60 % (Tables 3, 4, 5 and Figs. 5, 6).

9. The Effect of Bicyclomycin on the Biosynthesis of the Bound Form of Lipoprotein

The results in Table 3 indicate that the primary action of bicyclomycin may be due to the inhibition of the peak I protein synthesis. INOUYE and his co-workers[11] have reported that most of proteins at the origin in SDS-polyacrylamide gel electrophoresis are the bound form of the lipoprotein linked to the peptidoglycan. Therefore, the amount of the bound form of lipoprotein was expressed as the difference of radioactivity at peak I between the lysozyme-treated and untreated envelope fractions.

The effect of bicyclomycin was examined on the biosynthesis of the bound form of lipoprotein, and compared with that of other antibiotics. ^{3}H-L-Arginine (0.25 μCi/ml, final concentration) and the antibiotic were added to the cell suspension of *E. coli* 15 THU in the absence of histidine, and the mixtures were incubated at 37°C for 45 minutes.

As presented in Table 4, in the bicyclomycin-treated cells, the envelope protein synthesis was inhibited 20 %, but the cytoplasmic protein synthesis was not significantly affected. The gel patterns of the envelope fraction in the presence or absence of bicyclomycin were shown in Fig. 5. Bicyclomycin inhibited the synthesis of the peak I protein 80 % and that of the free form of lipoprotein only 14 %.

A half of the envelope fractions was treated with lysozyme and subjected to SDS-polyacrylamide gel electrophoresis. Without addition of the antibiotics, 90 % of the ^{3}H radioactivity, which stayed on top of the gel before the lysozyme treatment, disappeared and shifted to near the position of the free form of the lipoprotein after the lysozyme treatment. Thus, most of the peak I protein were due to the bound form of lipoprotein. In the bicyclomycin-treated

Table 4. Effects of antibiotics on the biosynthesis of lipoportein in the histidine-starved cells of *E. coli* 15 THU

Antibiotic, μg/ml	Inhibition (%)			
	Cytoplasmic protein synthesis	Envelope protein synthesis	Lipoprotein	
			Free form	Bound form*
Bicyclomycin, 100	0	20.0	13.6	91.0
Bicyclomycin, 100 Rifampicin, 50	16.1	33.2	37.9	(81.6)**
Rifampicin, 50	10.0	20.8	27.5	26.2
Bicyclomycin, 100 Tetracycline, 20	85.0	88.0	96.1	91.4
Tetracycline, 20	84.6	87.9	95.9	90.0

* The bound form of the lipoprotein was expressed as the amount of radioactivity released from the peak I protein by the lysozyme treatment.

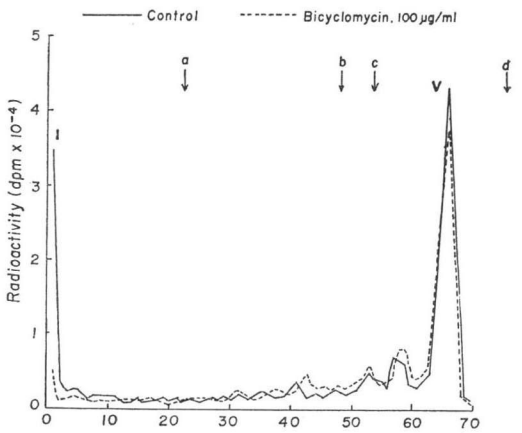
** The experiment was not carried out by the lysozyme treatment. The rate of inhibition was expressed as that of the peak I protein.

The antibiotic and ^{3}H-arginine were added to the culture starved for histidine. The mixtures were incubated at 37°C for 45 minutes. The control experiment was carried out without the addition of antibiotics. After incubation, the envelope fractions were prepared as described in Materials and Methods, and half of them was treated with lysozyme. Each envelope fraction was analyzed by SDS-polyacrylamide gel electrophoresis. All data were expressed as rates of inhibition calculated as percentage of the over-all incorporation of controls.

cells, in which synthesis of peak I was inhibited more than 80 %, the radioactivity of the peak I protein was scarcely transferred from top of the gel by the lysozyme treatment. The biosynthesis of the bound form of lipoprotein was blocked almost completely (91 %) by bicyclomycin (Table 4). Thus, the biosynthesis of the bound form of lipoprotein was more sensitive to inhibitory action of bicyclomycin than that of the free form of lipoprotein.

Fig. 5. The effect of bicyclomycin on the biosynthesis of the lipoprotein in the histidine-starved cells of *E. coli* 15 THU

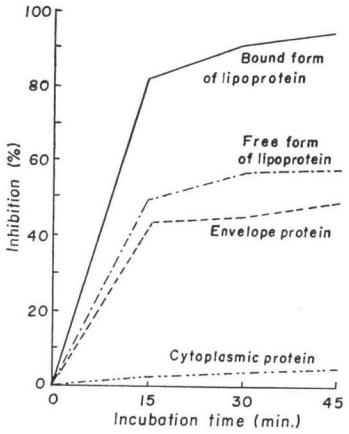
The experiments were carried out as described in the legend to Table 4. The envelope fractions were subjected to SDS polyacrylamide gel electrophoresis. Assignments of internal standards are the same as in Fig. 4. The number indicates each peak.



The inhibitory effect of bicyclomycin on the lipoprotein synthesis was compared with those of rifampicin and tetracycline. HIRASHIMA *et al.*[10,15,16] have reported that the mRNA for the lipoprotein is extraordinary stable, and rifampicin has little effect on the biosynthesis of the free form of lipoprotein in contrast to tetracycline. As shown in Table 4, rifampicin inhibited synthesis of the free form of lipoprotein by only 27 %, and the peak I protein (the bound form) synthesis only 26 %. However, concurrent treatment with both bicyclomycin and rifampicin exhibited additive inhibition of the synthesis of the free form of lipoprotein, whereas synthesis of the peak I

Fig. 6. The effect of bicyclomycin on the biosynthesis of the lipoprotein in the histidine-starved cells of *E. coli* 15 THU as a function of time.

Bicyclomycin and ^{14}C-L-arginine were added to the culture starved for histidine. The mixtures were incubated at 37°C for 15, 30 and 45 minutes. The envelope fraction was prepared, and half of it was treated with lysozyme. Each fraction was subjected to SDS polyacrylamide gel electrophoresis. All data were expressed as rates of inhibition calculated as percentage of the over-all incorporation of controls.



protein (the bound form of lipoprotein) was inhibited 82 %, the same rate as with bicyclomycin alone.

Thus, the selective inhibition by bicyclomycin of the biosynthesis of the bound form of lipoprotein was evident. Tetracycline blocked the synthesis of both forms almost completely.

Furthermore, the effects of bicyclomycin on the incorporation of ^{14}C-L-arginine into the cells were examined as a function of time (Fig. 6).

In the untreated control, the ^{14}C-arginine incorporation into the cytoplasmic proteins, the envelope proteins and the free form of lipoprotein reached a plateau within 15 minutes; on the other hand, that into the bound form of lipoprotein proceeded linearly.

The inhibition of formation of these proteins by bicyclomycin is shown in Fig. 6. Synthesis of the envelope proteins was inhibited by 50 %, whereas that of the cytoplasmic proteins was not significantly affected. As for the lipoprotein, synthesis of the free form and the bound form were blocked approximately 60 % and 95 %, respectively.

For the purpose of confirming the inhibitory effect of bicyclomycin on the biosynthesis of the bound form, the peptidoglycan was prepared using 4 % SDS, treated with lysozyme and analyzed by SDS-polyacrylamide gel electrophoresis to determine the amount of the bound form

Table 5. The effect of bicyclomycin on the biosynthesis of lipoprotein in the histidine-starved cells of *E. coli* 15 THU

	Inhibition (%)			
	Envelope protein synthesis	Lipoprotein		
		the free form	the bound form	
			Method I*	Method II**
Bicyclomycin 100 μg/ml	22.0	10.0	79.9	80.0

* Method I: The bound form of the lipoprotein was expressed as the amount of radioactivity released from the peak I protein by the lysozyme treatment.

** Method II: Peptidoglycan was isolated from the envelope fraction using 4% SDS, and analyzed by SDS-polyacrylamide gel electrophoresis after the lysozyme treatment.

Bicyclomycin and ^{14}C-L-arginine were added to the cultures starved for histidine. The mixtures were incubated at 37°C for 90 minutes. The control experiment was carried out without the addition of the antibiotic. After incubation, envelope fractions were prepared. The synthesis of the bound form of lipoprotein was determined by Method I or II. All data were expressed as rates of inhibition calculated as percentage of the over-all incorporation of controls.

of lipoprotein[14].

^{14}C-L-Arginine (0.75 μCi/ml, final concentration) and bicyclomycin were added to the cell suspension of *E. coli* 15 THU in the absence of histidine, and the mixtures were incubated at 37°C for 90 minutes.

After treatment of the envelope fraction with hot 4 % SDS, most of radioactivity was detected in the soluble fraction and about 30 % remained in the insoluble precipitate (peptidoglycan). The gel patterns of peptidoglycan treated with lysozyme showed that only the peak of the bound form of lipoprotein appeared.

The effects of bicyclomycin on the biosynthesis of the bound form of lipoprotein were observed by isolating the peptidoglycan, which was then treated with lysozyme (Table 5). The biosynthesis of the bound form was inhibited by 80 %. The grade of inhibition was in accordance with that of the peak I protein. In contrast, the synthesis of the free form was inhibited only 10 %.

The above results indicated that bicyclomycin is a potent inhibitor of the biosynthesis of the bound form of lipoprotein.

Discussion

The results suggest that the primary action of bicyclomycin is due to interference with the biosynthesis of lipoprotein and its assembly to peptidoglycan in the cell envelope of *E. coli*.

The lipoprotein has been reported to exist in two forms[11,14] in the outer membrane of *E. coli*: *i.e.* the free form, and the bound form which is covalently bound on average to every 10~12 diaminopimelate residues of peptidoglycan by the C-terminal lysine[4]. About 7.5×10^5 molecules of lipoprotein are assumed to be present in a cell. Thus, it is considered to be the most abundant protein and have an important function in stabilizing the total structure of the cell wall[4,5,21]. The mechanism of biosynthesis and assembly of the lipoprotein has also been investigated. The free form of lipoprotein is first synthesized on the ribosomes directed by mRNA specific for the lipoprotein and converted into the bound form attached to peptidoglycan[10,11,14,15,16,17].

Although the variation (10~60 %) in inhibition is observed as shown in Tables 3, 4 and 5, it is certain that the synthesis of the free form of lipoprotein is sensitive to bicyclomycin. The free form of lipoprotein synthesized in the presence of bicyclomycin may be incomplete and lacking in the capacity of binding with peptidoglycan. The marked inhibition of synthesis of the bound form may probably be a secondary effect caused by the disturbance of the free form synthesis.

From the viewpoint of the mechanism of the lipoprotein synthesis[14], the alternative explanation is that the primary action of bicyclomycin may be the inhibition of the conversion of the free form to the bound form. Tables 4 and 5 show the characteristics of action of bicyclomycin in comparison with that of rifampicin.

The conversion of the free form to the bound form has been shown to be independent upon protein synthesis and the energy production[14]. INOUYE and his co-workers suggest[14] that the reaction is caused by transpeptidase in a similar way to the formation of cross-linkages between two peptide units of peptidoglycan, and the covalent linkage between the ε-amino group of the C-terminal lysine of the lipoprotein and the carboxy group of diaminopimelic acid in the peptidoglycan may be formed by releasing D-alanine or D-alanyl-D-alanine from the peptide unit of peptidoglycan. IZAKI and STROMINGER[18] have described carboxypeptidase II, which releases the second D-alanine residue from the peptide unit of peptidoglycan.

It seems to be of interest to examine the effect of bicyclomycin on the release of D-alanine by carboxypeptidase II in the presence of the free form of lipoprotein.

The inhibition of the bound form of lipoprotein also may be caused by some damage in the structure of peptidoglycan. However, bicyclomycin does not inhibit the cross-link formation in peptidoglycan *in vitro* in spite of inhibiting the incorportion of labeled L-alanine into peptidoglycan of cells in the buffer by approximately 20 % (unpublished data).

It is not well established whether the inhibition by bicyclomycin of the biosynthesis and assembly of lipoprotein is related to the morphological changes in *E. coli* cells. MARTIN *et al.*[19] have reported that some of the spheroplast-type mutants of *E. coli* are defective in lipoprotein. It suggests that bicyclomycin causes the morphological changes in the susceptible cells by disturbing the synthesis and assembly of lipoprotein.

HALEGOUA *et al.*[20] have reported that, although lipoprotein exists in the envelopes of *Salmonella*, *Proteus* and *Pseudomonas*, only the lipoprotein of *Salmonella* is immunologically identical with that of *E. coli*. Gram-positive bacteria do not contain a similar lipoprotein. Bicyclomycin is inhibitory to Gram-negative bacteria such as *E. coli* and *Salmonella*, and is not effective against *Proteus*, *Pseudomonas* and Gram-positive bacteria. These data may explain the characteristic spectrum of bicyclomycin and support the assumption that bicyclomycin may be a specific inhibitor of the biosynthesis and assembly of lipoprotein.

Furthermore, there are other envelope proteins inhibited about 40 % and 27 %, respectively, by bicyclomycin, while the peak V protein is inhibited approximately 48 % (Table 2). The peak II and IV proteins may be identical with the peak 4 and 7 proteins, obtained by INOUYE and his co-workers, respectively, as they show the same mobilities in gel electrophoresis (Fig. 3)[12,14,22]. According to INOUYE[22], the peak 4 and 7 proteins has been considered to be modified by some molecules such as fatty acids (lipoprotein) or by sugars (glycoprotein).

The most plausible conclusion from the available data is that the primary site of action of bicyclomycin may be due to the disturbance in the biosynthesis of lipoprotein, and, more specifically, in its conversion from the free form to the bound form.

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