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Inhibition of the Synthesis of Macromolecules in *Escherichia coli* by Nitrofuran Derivatives. II.[1] Various Nitrofuran Derivatives

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Using 26 nitrofuran derivatives with various chemical structures, the inhibition of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein syntheses was examined in intact cell and protoplast lysate systems of *Escherichia coli* JG181. All the derivatives active against the strain inhibited DNA synthesis in the both systems. However, RNA and protein syntheses were inhibited by some of them in intact cell system and by all but one in protoplast lysate system. The derivatives inactive against the strain but active against staphylococci showed no inhibition in intact cell system although one of them inhibited DNA and RNA syntheses in protoplast lysate system. The derivatives inactive against all bacteria tested did not show inhibition at all in the both systems. Among the syntheses tested, the most inhibited was DNA synthesis and the degree of inhibition in intact cell system was closely correlated to antibacterial activity. The results indicate that the inhibition of DNA synthesis may be a common mechanism of antibacterial action of nitrofuran derivatives.

It has been reported that some [2-(5-nitro-2-furyl)vinyl]pyridine derivatives inhibit the synthesis of macromolecules of *Escherichia coli* K-12 and the inhibition is related to antibacterial activity.[1] To confirm this result and to know whether such inhibition is generally observed in other nitrofuran derivatives, similar experiments were carried out with various nitrofuran derivatives. To avoid the marked degradation of nitrofuran derivatives during an incubation period, a relatively small number of bacterial cells was used in intact cell system of the present experiments. Instead, *Escherichia coli* JG181, which was an auxotrophic mutant of *Escherichia coli* K-12 requiring thymine, uracil and histidine was used with ^{14}C-labeled thymine, uracil and histidine to assure effective incorporation of radioactivity. The results indicate that the inhibition of deoxyribonucleic acid (DNA) synthesis is common to all of the active nitrofuran derivatives tested and is closely related to antibacterial activity, while that of ribonucleic acid (RNA) and protein syntheses is not always observed.

Experimental

(a) **Chemicals**——Nitrofurantoin and furazolidone were extracted with dimethylformamide from commercial products and recrystallized from water-dimethylformamide and dimethylformamide respectively. The other nitrofuran derivatives used were synthesized in Research and Development Division, Dainippon Pharmaceutical Co.[3] Nalidixic acid was extracted from commercial tablets and purified.[4] Chloramphenicol was purchased from Sankyo Co., mitomycin C from Kyowa Hakko Kogyo Co., and lysozyme from Sigma

1) Part I: S. Nakamura and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **21**, 130, (1973).
2) Location: *Enoki 33-94, 564 Suita, Osaka.*
3) S.A. Ciba, Belg. Patent 613604 (1962) [*C.A.*, **58**, 1411 (1963)]; A. Fujita, T. Yamamoto, S. Minami, and H. Takamatsu, *Yakugaku Zasshi*, **85**, 565 (1965); C.F. Boehringer and Soehne G. m. b. H, Belg. Patent 615319 (1962) [*C.A.*, **58**, 11333 (1963)]; A. Fujita, M. Nakata, S. Minami, and H. Takamatsu, *Yakugaku Zasshi*, **86**, 1014 (1966); A. Fujita, T. Yamamoto, S. Minami, and H. Takamatsu, *Chem. Pharm. Bull.* (Tokyo), **13**, 1183 (1965); J. Matsumoto and S. Minami, *ibid.*, **15**, 1806 (1967); Dainippon Pharmaceutical Co., Ltd., Brit. Patent 1105007 (1968) [*C.A.*, **69**, 86809 (1968)]; E. Merck, *Chem. Ber.*, **21**, 2709 (1888); H.J. Sanders, R.T. Edmunds, and W.B. Stillman, *Ind. Eng. Chem.*, **47**, 358 (1955); A. Takai and I. Saikawa, *Yakugaku Zasshi*, **84**, 16 (1964).
4) M. Shimizu, S. Nakamura, and Y. Takase, *Antimicrob. Ag. Chemother.-1970*, **1971**, 117.

Chemical Co. Actinomycin D was a gift from Merck Sharp and Dohme Research Laboratory. ^{14}C-Labeled thymine, uracil and L-histidine were purchased from Radiochemical Centre, whose specific activities were 455, 535 and 373 mCi/mg respectively.

(b) **Organism, Medium and Measurement of Antibacterial Activity**——*Escherichia coli* JG181,[5] an auxotroph requiring thymine, uracil and L-histidine was used throughout the present incorporation experiments. For the culture of JG181, Davis' minimal medium (MM)[6] supplemented with thymine (40 μg/ml), uracil (50 μg/ml) and L-histidine (50 μg/ml) were employed. Antibacterial activity was measured by the broth-dilution method using nutrient broth.[4]

(c) **Preparation of Intact Cell and Protoplast Lysate**——*Escherichia coli* JG181 precultured overnight was diluted 10 times with MM supplemented with thymine, uracil and L-histidine, and incubated at 37° for 4 hours with aeration. The cultured cells were centrifuged, resuspended in the same volume of MM without any supplement, starved by shaking at 37° for 1 hour and immediately used as an intact cell preparation, which contained about 5×10^7 viable cells per ml. For the preparation of protoplast lysate, the cultured cells were washed 3 times with MM, suspended in one fortieth volume of 1/20M tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0 containing 20% sucrose, converted into protoplasts as previously described[2] and suspended in one third volume of MM plus $MgCl_2$ (final 10 mM). The concentration of protoplast lysate was equivalent to about 5×10^9 viable cells per ml.

(d) **Incorporation Experiments of ^{14}C-Labeled Precursors**——Incubation mixtures consisted of the ingredients shown in Table I. One ml of an incubation mixture in a tube was shaken at 37° for 1 hour (intact cell) or 3 hours (protoplast lysate) followed by addition of 1 ml of cold 10% trichloroacetic acid. In the case of intact cell, about 1 mg dry weight of the cells of *Escherichia coli* JG181 was added in a tube as carrier at the same time. The precipitate was washed 4 times with 2 ml of cold 5% trichloroacetic acid, dissolved in 0.5 ml of 0.2N NaOH, mixed with 10 ml of the dioxane scintillator solution[7] and measured for radioactivity by a TEN liquid scintillation counter, model GSL-161 (Kobe Kogyo).

Table I. Incubation Mixture

Radioactive precursor	System	DMM[a]	Thymine 0.8 mg/ml	Uracil 1 mg/ml	Histidine 1 mg/ml	$MgCl_2$ 100 μM	Cell[b] prep.	Isotope[c]	Drug[d]
^{14}C-Thymine	intact cell	0.5	0.025	0.05	0.05	0	0.1	0.05	0.1
	protoplast lysate	0.5	0	0.05	0.05	0.1	0.1	0.05	0.1
^{14}C-Uracil	intact cell	0.5	0.05	0	0.05	0	0.1	0.1	0.1
	protoplast lysate	0.5	0.05	0	0.05	0.1	0.1	0.1	0.1
^{14}C-Histidine	intact cell	0.5	0.05	0.05	0	0	0.1	0.1	0.1
	protoplast lysate	0.5	0.05	0.05	0	0.1	0.1	0.1	0.1

Figures show added volume in ml. Appropriate volume of distilled water was added to adjust final volume to 1 ml. *a*) double strength Davis' minimal medium, *b*) intact cell or protoplast lysate preparation, *c*) ^{14}C-thymine: 2.6×10^6 cpm/ml, ^{14}C-uracil: 6.0×10^5 cpm/ml, ^{14}C-histidine: 6.0×10^5 cpm/ml, *d*) nitrofuran derivatives: 400 μM; mitomycin C and actinomycin D: 100 μg/ml; chloramphenicol and nalidixic acid: 500 μg/ml

Result

(a) Antibacterial Activity

The chemical structures of the nitrofuran derivatives used and their antibacterial activity are shown in Table II and III. As described previously,[2] the derivatives used can be classified into three types according to antibacterial activity. The first type is active against both gram-positive and gram-negative bacteria in which the derivatives No. 1—14, nitrofurazone (NFZ), nitrofurantoin (NFT), furazolidone (FZ) and dihydroxymethylfuratrizine (DMFT) are contained. The second type is active against only gram-positive bacteria and inactive against gram-negative ones to which the derivatives No. 15 and 16 belong. The third type

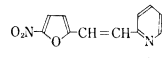
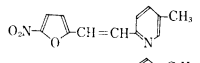
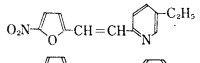
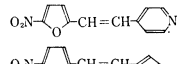
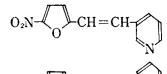
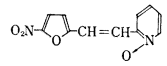
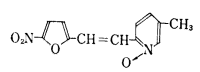
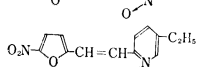
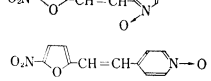
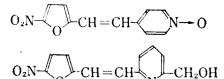
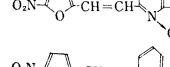
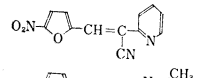
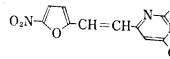
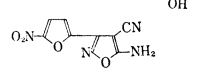
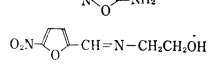
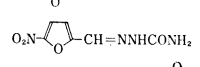
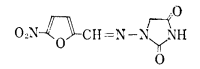
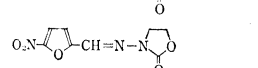
5) M. Ishibashi, *Japan. J. Genetics*, **41**, 75 (1966).
6) B.D. Davis and E.S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).
7) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

is inactive against both gram-positive and gram-negative bacteria, and involves the derivatives No. 17—20 having a 5 nitro group and the derivatives No. 21 and 22 lacking it. In the first type, the minimal inhibitory concentrations against *Escherichia coli* JG181 ranged from 0.03 to 10 μg/ml. In the second and third types, the growth of the strain was not inhibited even at a concentration of 100 μg/ml.

(b) Effect on DNA Synthesis

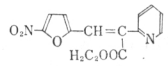
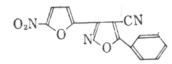
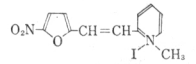
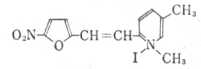
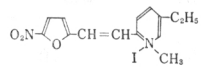
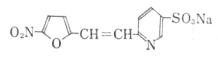
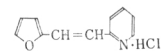
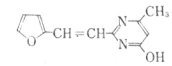
The effect of the nitrofuran derivatives on the incorporation of ^{14}C-thymine into an acid-insoluble fraction is shown in Fig. 1. All nitrofurans in the first type significantly inhibited

Table II. Antibacterial Activity of Nitrofuran Derivatives (first type)

No.	Chemical structure	MIC[a] *E. coli*[b]	*S. flex.*[c]	*S. aureus*[d]	*S. albus*[e]
1		0.3	3	10	1
2		0.3	1	1	0.1
3		1	3	3	0.3
4		0.1	0.3	3	0.3
5		0.03	0.3	1	0.3
6		0.1	0.3	1	0.3
7		0.1	0.3	1	0.3
8		1	1	1	1
9		0.3	1	1	0.3
10		0.03	0.3	0.3	0.1
11		1	1	0.3	1
12		0.1	1	0.3	0.3
13		0.1	0.1	1	0.3
14		1	1	1	0.3
NFZ[f]		10	3	10	10
NFT[g]		10	3	10	10
FZ[h]		1	1	1	1
DMFT[i]		0.03	0.1	0.3	0.03

a) minimal inhibitory concentration (μg/ml), *b*) *Escherichia coli* JG181, *c*) *Shigella flexneri* 2a EW10, *d*) *Staphylococcus aureus* Terajima, *e*) *Staphylococcus albus* AKM, *f*) nitrofurazone, *g*) nitrofurantoin, *h*) furazolidone, *i*) dihydroxymethylfuratrizine

TABLE III. Antibacterial Activity of Nitrofuran Derivatives (second and third types)

No.	Chemical structure	MIC			
		E. coli	*S. flex.*	*S. aureus*	*S. albus*
15		>100	>100	3	3
16		>100	>100	10	10
17		>100	>100	>100	>100
18		>100	>100	>100	>100
19		>100	>100	>100	>100
20		>100	>100	>100	>100
21		>100	>100	>100	>100
22		>100	>100	>100	>100

the incorporation in intact cell system. The degrees of the inhibition were usually as marked as those of mitomycin C (Mt C) and nalidixic acid (NA) except for a few compounds (11, NFZ). All derivatives belonging to the second and third types did not inhibit the incorporation at all. In the protoplast lysate system, the incorporation was inhibited by all of the first type

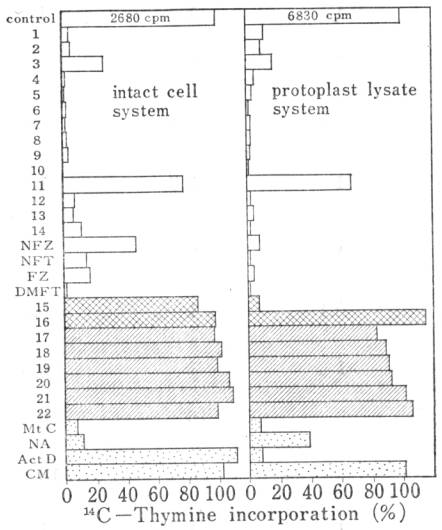


Fig. 1. Effect of Nitrofuran Derivatives on the Incorporation of ^{14}C-Thymine into Acid-insoluble Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* JG181

□: first type, ▨: second type, ▨: third type

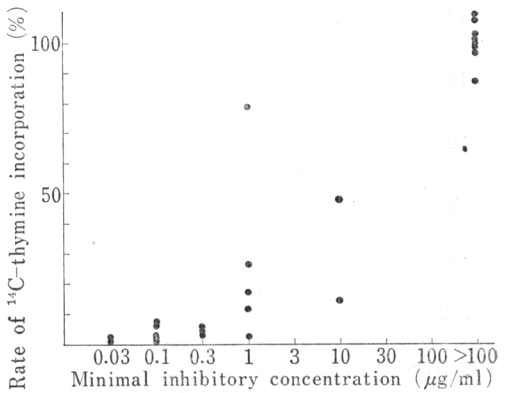


Fig. 2. Correlation of Antibacterial Activity of Nitrofuran Derivatives with the Incorporation Rate of ^{14}C-Thymine in Intact Cell System of *Escherichia coli* JG181

derivatives and one (No. 15) of the second type derivatives. The degrees of the inhibition were as remarkable as those of Mt C, NA and actinomycin D (Act D) except for one first type derivative, No. 11. One second type derivative (No. 16) and all third type derivatives did not show the significant inhibition. Correlation of antibacterial activity with the incorporation rate of ^{14}C-thymine in intact cell system is exhibited in Fig. 2, where antibacterial activity was closely related to the decrease of the incorporation rate.

(c) Effect on RNA Synthesis

The incorporation of ^{14}C-uracil into an acid-insoluble fraction was examined under the effect of nitrofurans. As shown in Fig. 3, the inhibition of the incorporation in intact cell system was considerably observed in some of the first type derivatives, *i.e.* [2-(5-nitro-2-furyl)vinyl]pyridines (No. 1—5), their N-oxide derivatives (No. 6—10), No. 14 and DMFT. The degree of the inhibition was as remarkable as that of Mt C in a few compounds, No. 5 and 10, but relatively less remarkable in the rest. The other derivatives in the first type (No. 11—13, NFZ, NFT, FZ) and all derivatives in the second and third types did not significantly inhibit the incorporation. On the contrary the incorporation was markedly stimulated in the case of NFZ. In protoplast lysate system, all first type derivatives except for No. 11 and one second type derivative, No. 15 showed the inhibition while the other second type derivative, No. 16 and all third type derivatives did not. The inhibition was as strong as that of Mt C or Act D in several derivatives (No. 4—10, 14, DMFT). Correlation of antibacterial activity with the incorporation rate of ^{14}C-uracil in intact cell system is shown in Fig. 4. The reduction of the incorporation rate was related to antibacterial activity to some extent though the correlation was not so clear as observed in ^{14}C-thymine incorporation. Marked reduction of the incorporation rate was observed in a part of nitrofurans with relatively high antibacterial activity.

(d) Effect on Protein Synthesis

Effect of the nitrofuran derivatives on the incorporation of ^{14}C-histidine into an acid-insoluble fraction is shown in Fig. 5. In intact cell system, several nitrofurans (No. 5, 7, 8,

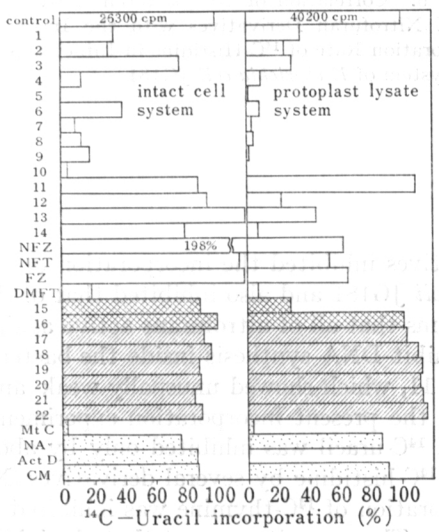


Fig. 3. Effect of Nitrofuran Derivatives on the Incorporation of ^{14}C-Uracil into Acid-insoluble Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* JG181

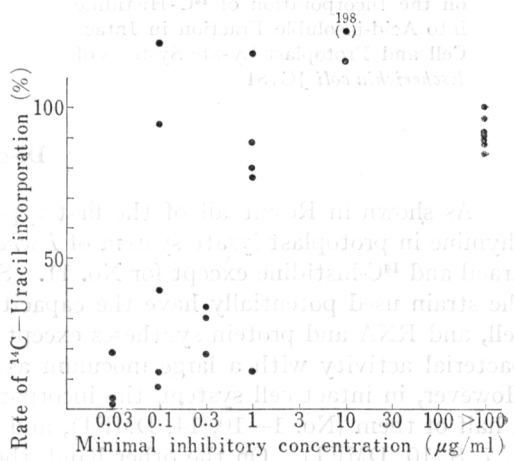


Fig. 4. Correlation of Antibacterial Activity of Nitrofuran Derivatives with the Incorporation Rate of ^{14}C-Uracil in Intact Cell System of *Escherichia coli* JG181

10, DMFT) moderately inhibited the incorporation while the extents of the inhibition were much weaker than that of chloramphenicol (CM). None of the other nitrofurans showed the inhibition in this system. In the protoplast lysate system, all of the first type derivatives except for No. 11 showed the inhibition and the second and third type derivatives did not at all. The inhibition was as strong as that of CM in a few compounds (No. 10, DMFT) but usually weaker in the most. However, the inhibition was comparable with that of Mt C and Act D in most [2-(5-nitro-2-furyl)vinyl]pyridine derivatives (No. 2, 4—9), a 4-[2-(5-nitro-2-furyl)vinyl]pyrimidine derivative (No. 12) and C-(5-nitro-2-furyl)-N-(2-hydroxyethyl) nitrone (No. 14). Correlation of antibacterial activity with the incorporation rate of ^{14}C-histidine in intact cell system was not obvious as shown in Fig. 6. However, the drop of the incorporation rate was only found in a few nitrofurans having relatively strong antibacterial activity.

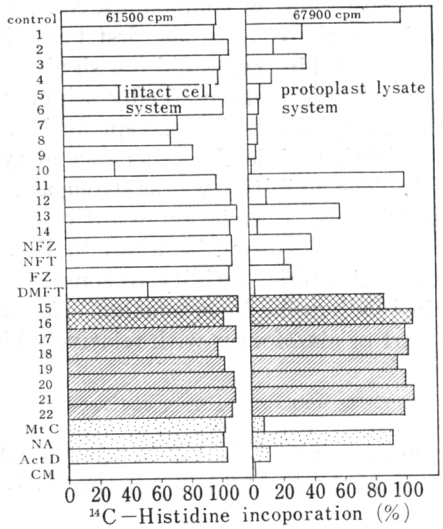


Fig. 5. Effect of Nitrofuran Derivatives on the Incorporation of ^{14}C-Histidine into Acid-insoluble Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* JG181

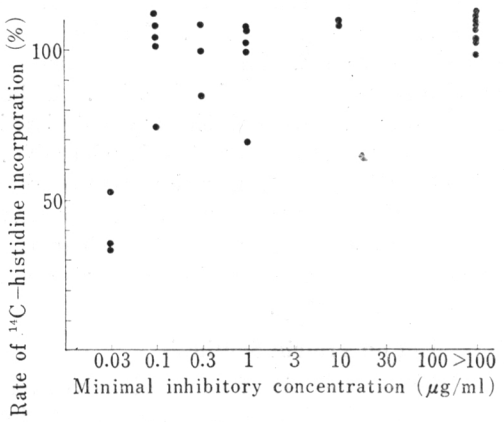


Fig. 6. Correlation of Antibacterial Activity of Nitrofuran Derivatives with the Incorporation Rate of ^{14}C-Histidine in Intact Cell System of *Escherichia coli* JG181

Discussion

As shown in Result all of the first type derivatives inhibited the incorporation of ^{14}C-thymine in protoplast lysate system of *Escherichia coli* JG181 and also inhibited that of ^{14}C-uracil and ^{14}C-histidine except for No. 11. So, it seems that all of nitrofurans active against the strain used potentially have the capacity to inhibit DNA synthesis inside the bacterial cell, and RNA and protein syntheses except for No. 11, which showed unusually weak antibacterial activity with a large inoculum as used in the present incorporation experiments. However, in intact cell system, the incorporation of ^{14}C-uracil was inhibited only by about a half of them (No. 1—10, 14, DMFT), and that of ^{14}C-histidine by several derivatives (No. 5, 7, 8, 10, DMFT). On the other hand, the incorporation of ^{14}C-thymine was inhibited by all of the first type derivatives in intact cell system. These results suggest that the inhibition in intact cell system is affected by the permeability of the nitrofuran derivatives into the bacterial cells and DNA synthesis is relatively sensitive to the derivatives compared with RNA and protein syntheses. The results obtained here almost coincided with the previous ones[1] in protoplast lysate system but did not always coincide in intact cell system also indi-

cating that the permeability of the nitrofuran derivatives into cells may depend on the strain used. The present experiment revealed that the second type derivatives, No. 15 and 16 differed each other in inhibition pattern. No. 15 inhibited the incorporation of ^{14}C-thymine and ^{14}C-uracil in protoplast lysate system but did not in intact cell system, while No. 16 did not show any inhibition in the both systems. This suggests that No. 15 has the capacity to inhibit DNA and RNA syntheses inside the cells but probably impermeable into the strain and No. 16 has intrinsically no effect on the macomolecular syntheses of the strain. The third type derivatives did not inhibit the incorporation at all in the both systems irrespective of the presence or absence of a 5-nitro group. These results indicate that the inhibition of DNA, RNA, and protein syntheses in intact cell system are in some way related to antibacterial activity and that of DNA synthesis is common to the nitrofurans active against the strain used. As the nitrofurans having various chemical structures were used in the present experiment, the inhibition of DNA synthesis may be universal in active nitrofurans. In intact cell system the inhibition of ^{14}C-thymine incorporation was usually marked and comparable with or stronger than that by Mt C or NA, whereas the inhibition of ^{14}C-uracil and ^{14}C-histidine was usually weak compared with that of Mt C or CM. Moreover, the inhibition of ^{14}C-thymine incorporation in intact cell system was closely correlated to antibacterial activity although that of ^{14}C-uracil and ^{14}C-histidine incorporation was not correlated to it so closely. Therefore, the inhibition of DNA synthesis seems to be important for antibacterial activity. These results led us to an idea that the inhibition of DNA synthesis may be a common mechanism of antibacterial action of nitrofurans.

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