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Inhibition of the Synthesis of Macromolecules in *Escherichia coli* by Nitrofuran Derivatives. I. (5-Nitro-2-furyl)vinylpyridines

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It was found that (5-nitro-2-furyl)vinylpyridine derivatives inhibited the synthesis of bacterial macromolecules in intact cell and protoplast lysate systems of *Escherichia coli* K-12. Among the syntheses tested, DNA, RNA and protein syntheses were inhibited correlating to antibacterial activity but lipid synthesis was independent of it. In the derivatives active against the strain, most inhibited DNA and protein syntheses and some additionary RNA synthesis in intact cell system. In protoplast lysate system, these syntheses were markedly inhibited by all of them. The degree of the inhibition was usually more remarkable in DNA synthesis than in protein synthesis. One derivative, which was inactive against the strain but active against *staphylococci*, showed only the slight inhibition of DNA synthesis in intact cell system but did the marked inhibition of DNA and RNA syntheses in protoplast lysate system. The derivatives inactive against all bacteria tested showed no inhibition in intact cell system and no or relatively weak inhibition in protoplast lysate system. The results indicate that the inhibition of DNA synthesis is important for antibacterial activity of (5-nitro-2-furyl)vinylpyridine derivatives and the permeability of the derivatives into bacterial cells may have influence on the activity.

It has been reported that the synthesis of macromolecules of bacteria is inhibited by some nitrofuran derivatives.[2] However, the relation of the inhibition to antibacterial activity has been unclear yet. We found that most nitrofuran derivatives inhibited the synthesis of bacterial macromolecules and the inhibition was related to antibacterial activity. The present report describes the effect of (5-nitro-2-furyl)vinylpyridine derivatives on the synthesis of macromolecules of Escherichia coli in connection with their antibacterial activity.

Experimental

(a) **Chemicals**——All nitrofuran derivatives tested were synthesized in Research and Development Division, Dainippon Pharmaceutical Co.[3] Chloramphenicol was purchased from Sankyo Co., mitomycin C from Kyowa Hakko Kogyo Co., lysozyme, ribonuclease and deoxyribonuclease from Sigma Chemical Co., and ^{32}P-orthophosphate and ^{14}C-labeled amino acids (algal protein hydrolysate), whose specific activities were 94 Ci/mgP and 100 μCi/mg respectively, from Radiochemical Centre. Actinomycin D was a gift from Merck Sharp and Dohme Research Laboratory.

(b) **Measurement of Antibacterial Activity**——*In vitro* antibacterial activity was measured by the broth-dilution method.[4]

(c) **Preparation of Intact Cell and Protoplast Lysate of *Escherichia coli***——*Escherichia coli* K-12, P-5102 precultured overnight in nutrient broth[4] was diluted 10 times with fresh nutrient broth and incubated at 37° for 4 hours with aeration. The cultured cells were chilled in ice-water, centrifuged and resuspended

1) Location: *Enoki 33-94, 564 Suita, Osaka.*
2) A. Taketo, H. Sawada, and Y. Takagi, *Biochem. Biophys. Res. Commun.*, **15**, 256 (1964); D.R. McCalla, *Can. J. Biochem.*, **42**, 1245 (1964); A. Terawaki and J. Greenberg, *Biochim. Biophys. Acta*, **95**, 170 (1965); K. Kato, Y. Sugino, and H. Endo, *ibid.*, **119**, 309 (1966).
3) A. Fujita, T. Yamamoto, S. Minami, and H. Takamatsu, *Yakugaku Zasshi*, **85**, 565 (1965); Ciba S.A., Belg. Patent 613604 (1962) [*C.A.*, **58**, 1411 (1963)]; C.F. Boehringer and Soehne G. m. b. H, *ibid.*, 615319 (1962) [*C.A.*, **58**, 11333 (1963)]; E. Merck, *Chem. Ber.*, **21**, 2709 (1888); H.J. Sanders, R.T. Edmunds, and W.B. Stillman, *Ind. Eng. Chem.*, **47**, 358 (1955).
4) M. Shimizu, S. Nakamura, and Y. Takase, *Antimicrob. Ag. Chemother.*, **1970**, **1971**, 117.

in one fortieth volume of 1/100M tris(hydroxymethyl)aminomethane–HCl buffer (Tris-HCl buffer), pH 7.3. The suspended cells were washed twice with the same buffer and immediately used as an intact cell preparation, which usually contained about 5×10^9 viable cells per ml. For the preparation of protoplast lysate, the suspended cells were centrifuged and resuspended in the same volume of 1/20M Tris-HCl buffer, pH 8.0 containing 20% sucrose (Tris-Sucrose buffer). To 15 ml of the suspension were added 0.75 ml of a lysozyme solution (2 mg/ml) and 1.5 ml of a disodium ethylenediaminetetraacetate solution (40 mg/ml) followed by incubation at 37° for about 15 minutes until more than 99% of cells were transformed into spheres under microscopic observation. Lysozyme action was stopped by dilution with the cold Tris-Sucrose buffer. The protoplasts formed were washed once with the Tris-Sucrose buffer, suspended in one third volume of 1/20M Tris-HCl buffer, pH 7.3 containing 10 mM $MgCl_2$, freezed in dry ice–acetone, stored at −70° and thawed just before use. The concentration of the protoplast lysate thus prepared was usually equivalent to about 1.5×10^{10} viable cells per ml.

(d) **Incorporation Experiments**——An Incubation Mixture contained per ml: 0.5 ml 1/20M Tris-HCl buffer, pH 7.3, 0.05 ml Casamino Acids (Difco) containing 10% tryptophan (20 mg/ml), 0.05 ml 100 mM $MgCl_2$, 0.05 ml glucose (200 mg/ml), 0.1 ml drug solution (nitrofuran derivatives; 400 μM, the other chemicals; concentrations are described in Result), 0.05 ml isotope (^{14}C-amino acids; about 10^6 cpm/ml, ^{32}P-orthophosphate; about 10^7 cpm/ml), 0.1 ml intact cell or protoplast lysate preparation and 0.1 ml distilled water. One ml of an incubation mixture in a tube (1.8 cm in diameter) was shaken at 37° for 30 minutes and 3 hr followed by addition of 1 ml 10% cold trichloroacetic acid. The resultant precipitate was fractionated according to Schmidt-Thanhauser's method.[5] ^{14}C-Protein fraction was dissolved in 0.2 ml 1N NaOH, mixed with 3 ml Hyamine Hydroxide[6] and 10 ml toluene scintillator solution,[7] acidified with several drops of concentrated H_2SO_4 and assayed for radioactivity by a Tri Carb liquid scintillation spectrometer, model 314 (Packard). ^{32}P-Labeled fractions were dried in planchets and radioactivity was measured by a TEN GM counter, model GM-132A (Kobe Kogyo).

Result

(a) Comparison between Intact Cell and Protoplast Lysate Systems

As shown in Table I, one of the intact cell and protoplast lysate systems prepared as described in Experimental contained 5.3×10^8 and less than 2.5×10^6 viable cells per ml incubation mixture respectively. The ratio of protoplast lysate system/intact cell system in viable cell number was less than 0.0047. The ^{32}P-incorporation activity of the both systems is shown in the same Table. In intact cell system radioactivity was incorporated into DNA, RNA, and lipid fractions to a fairly large extent by incubation for 30 minutes or 3 hours. However, protoplast lysate system did not show significant incorporation into DNA and RNA fractions for first 30 minutes, although a small amount of incorporation was observed in lipid fraction during this period. But the prolongation of incubation time up to 3 hours resulted in small but obvious incorporation of radioactivity into DNA and RNA fractions. The ratios of

Table I. ^{32}P-Incorporation Activity of Intact Cell and Protoplast Lysate Systems of *Escherichia coli* K-12

System	Viable cell No./ml	^{32}P-Incorporation (cpm) into					
		DNA fraction		RNA fraction		Lipid fraction	
		30 min	3 hr	30 min	3 hr	30 min	3 hr
Intact cell	5.3×10^8	1960	9760	2540	6570	12380	26340
Protoplast lysate	$<2.5\times10^6$	17	450	35	1150	410	650
Protoplast lysate / Intact cell	<0.0047	0.0087	0.046	0.014	0.175	0.033	0.025

5) E. Volkin and W.E. Cohn, "Method of Biochemical Analysis," Vol. 1, ed. by D. Glick, Interscience Publishers, Inc., New York, N.Y., 1954, p. 287.
6) R.J. Herberg, *Anal. Chem.*, **32**, 42 (1960); W.O. Brown and H.G. Badman, *Biochem. J.*, **78**, 571 (1961).
7) T.C. Hall and E.C. Cocking, *Biochem. J.*, **96**, 626 (1965).

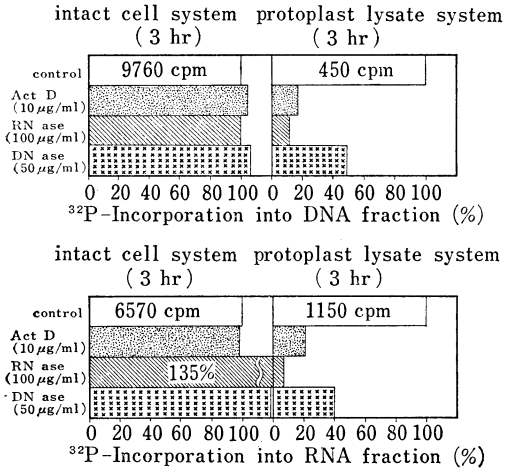


Fig. 1. Inhibition of ^{32}P-Incorporation into DNA and RNA Fractions by Actinomycin D, Ribonuclease and Deoxyribonuclease

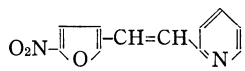
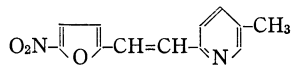
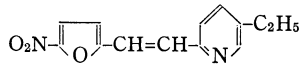
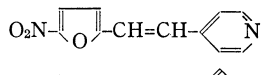
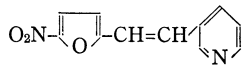
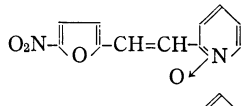
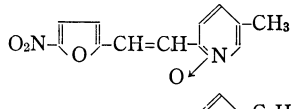
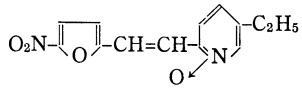
Act D: actinomycin D, RNase: ribonuclease, DNase: deoxyribonuclease

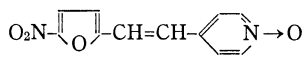
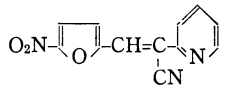
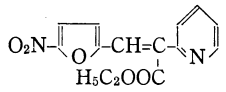
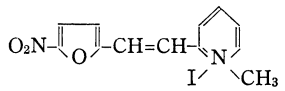
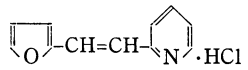
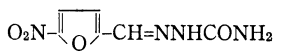
protoplast lysate system/intact cell system in ^{32}P-incorporation at 3 hours were 0.046 in DNA fraction, 0.175 in RNA fraction and 0.025 in lipid fraction, which were evidently higher than the ratio in viable cell number. Moreover, ^{32}P-incorporation into DNA and RNA fractions in protoplast lysate system was significantly inhibited by actinomycin D, ribonuclease and deoxyribonuclease as shown in Fig. 1 while that in intact cell system was not. These results indicate that the ^{32}P-incorporation activity in protoplast lysate sytem is not due to the contaminated intact cells but due to protoplast lysate itself.

(b) Antibacterial Activity

In vitro antibacterial activity of (5-nitro-2-furyl)vinylpyridine derivatives used is shown in Table II compared with nitrofurazone. The most derivatives, No. 1—10, were active against all the bacteria tested and their minimal inhibitory concentrations were lower than those of nitrofurazone. No. 11 was active against staphylococci but inactive against gram-negative bacilli. No. 12, a derivative having a 5-nitro group, was inactive as well as No. 13, a derivative lacking a 5-nitro group.

Table II. *In Vitro* Antibacterial Activity of (5-Nitro-2-furyl)vinylpyridine Derivatives

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	1	3	0.3
5		0.1	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

No.	Chemical structure	MIC[a] *E. coli*[b]	*S. flex.*[c]	*S. aureus*[d]	*S. albus*[e]
9		0.3	1	1	0.3
10		1[f]	1	0.3	1
11		>100	>100	3	3
12		>100	>100	>100	>100
13		>100	>100	>100	>100
NFZ[g]		10[f]	3	10	10

a) minimal inhibitory concentration (μg/ml) *b*) *Escherichia coli* K-12
c) *Shigella flexneri* 2a EW10 *d*) *Staphylococcus aureus* TERAJIMA
e) *Staphylococcus albus* AKM *f*) higher than 10 with a large inoculum
g) nitrofurazone

(c) ^{32}P-Incorporation into DNA Fraction

The effect of the nitrofuran derivatives on ^{32}P-incorporation into DNA fraction is shown in Fig. 2. In intact cell system, a control incorporated 1960 cpm of radioactivity for 30 minutes of incubation. In the presence of the nitrofuran derivatives, No. 1—9, the incorporation of radioactivity was markedly inhibited and the degree of the inhibition was comparable with that of mitomycin C. The derivatives, No. 10, 11 and nitrofurazone slightly inhibited the incorporation while the derivatives No. 12 and 13 did not. In protoplast lysate system, a control incorporated 450 cpm of radioactivity for 3 hours. Under the effect of the active nitrofuran derivatives, No. 1—11 and nitrofurazone, the incorporation of radioactivity was markedly inhibited, even if they were inactive against *Escherichia coli* like the derivative No. 11. In contrast with it, inhibition of the incorporation by the inactive nitrofuran derivatives No. 12 and 13 was relatively very weak.

(d) ^{32}P-Incorporation into RNA Fraction

The effect of the nitrofuran derivatives on ^{32}P-incorporation into RNA fraction was similarly investigated as shown in Fig. 3. In intact cell stysem, the incorporated radioactivity was 2530 cpm for 30 minutes of incubation in a control. Some of the active nitrofuran derivatives such as No. 4, 5, 7, 8 and 9 showed the moderate inhibition of the incorporation, while the other derivatives did not irrespective of their antibacterial activity. Actinomycin D did not reduce the incorporation as well in this system. In protoplast lysate system, where 1150 cpm of radioactivity was incorporated after 3-hour incubation in a control, all the nitrofuran derivatives tested except for the derivatives No. 12 and 13 markedly inhibited the incorporation as well as actinomycin D.

(e) ^{32}P-Incorporation into Lipid Fraction

The effect of the nitrofuran derivatives on ^{32}P-incorporation into lipid fraction is shown in Fig. 4. A control incorporated 12300 cpm of radioactivity in intact cell system and 410 cpm in protoplast lysate system following 30-minute incubation. In intact cell system,

none of the derivatives tested affected ^{32}P-incorporation into lipid fraction. In protoplast lysate system, the inhibition was marked in the derivatives No. 1, 2, 3, and 13 but was weak or not significant in the others.

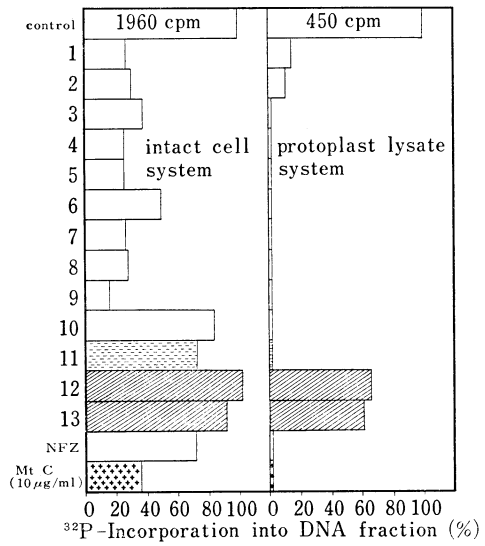


Fig. 2. Effect of (5-Nitro-2-furyl)vinylpyridine Derivatives on the Incorporation of ^{32}P-Orthophosphate into DNA Fraction in Intact Cell and Protoplast Lysate Systems of *Eschericiha coli* K-12

Mt C: mitomycin C

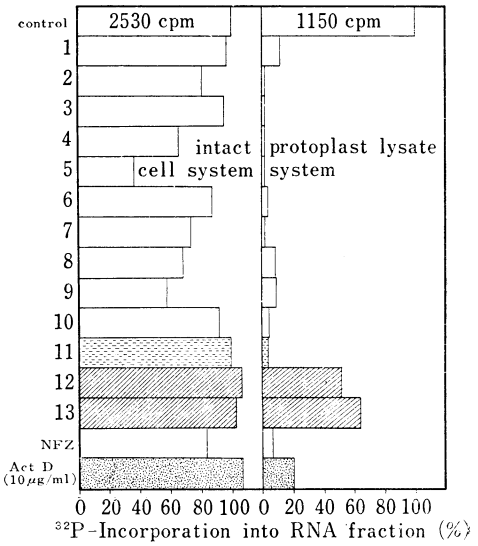


Fig. 3. Effect of (5-Nitro-2-furyl)vinylpyridine Derivatives on the Incorporation of ^{32}P-Orthophosphate into RNA Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* K-12

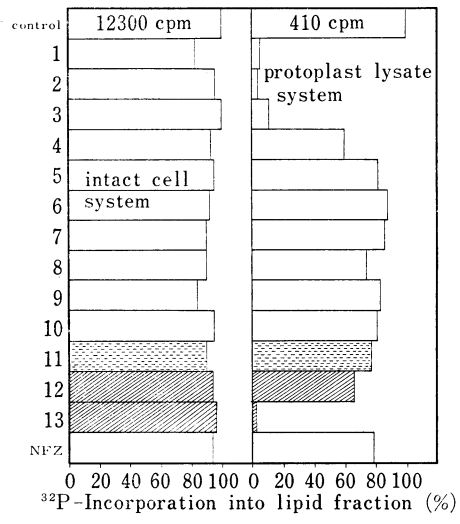


Fig. 4. Effect of (5-Nitro-2-furyl)vinylpyridine Derivatives on the Incorporation of ^{32}P-Orthophosphate into Lipid Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* K-12

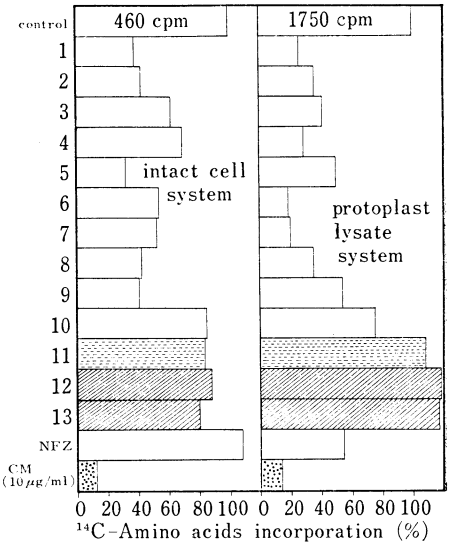


Fig. 5. Effect of (5-Nitro-2-furyl)vinylpyridine Detivatives on the Incorporation of ^{14}C-Amino acids into Protein Fraction in Intact Cell and Protoplast Lysate Systems of *Escherichia coli* K-12

CM: chloramphenicol

(f) ^{14}C-Amino Acid Incorporation into Protein Fraction

The incorporation of ^{14}C-amino acids into protein fraction was examined as shown in Fig. 5. Radioactivity incorporated in a control was 460 cpm in intact cell system and 1750 cpm in protoplast lysate system for 30 minutes of incubation. In the former system, the incorporation was significantly inhibited under the effect of the nitrofuran derivatives No. 1—9 and was not in the presence of the derivatives No. 10—13 and nitrofurazone. The degree of the inhibition was not so remarkable as that of chloramphenicol. In the latter system, the inhibition was also observed in the derivative No. 10 and nitrofurazone in addition to the derivatives showing the inhibition in intact cell system.

Discussion

As described in Result protoplast lysate system seems to be a cell-free system losing permeability barriers because ^{32}P-incorporation was not ascribed to contaminated intact cells and was sensitive to actinomycin D, ribonuclease and deoxyribonuclease which were considered to be impermeable into intact cells. (5-Nitro-2-furyl)vinylpyridine derivatives tested can be divided into three types according to their antibacterial activity. First type is active against both gram-positive and gram-negative bacteria, and includes the deribatives No. 1—10. Second type is active against gram-positive bacteria and inactive against gram-negative ones to which the derivative No. 11 belongs. Third type is inactive against all bacteria tested, in which No. 12, a derivative having a 5-nitro group and No. 13, a derivative lacking a 5-nitro group are contained. In the first type, most derivatives inhibited the incorporation of ^{32}P-orthophosphate and ^{14}C-amino acids into DNA and protein fractions respectively and some of them additionally inhibited ^{32}P-incorporation into RNA fraction in intact cell system. All of them inhibited the incorporation into these fractions in protoplast lysate system. The second type derivative showed only slight inhibition of ^{32}P-incorporation into DNA fraction in intact cell system but did the marked inhibition with ^{32}P-incorporation into DNA and RNA fractions in protoplast lysate system. The derivatives in the third type showed no inhibition in intact cell system and weak inhibition with ^{32}P-incorporation into DNA and RNA fractions in protoplast lysate system. The incorporation into lipid fraction was not inhibited by any of the nitrofuran derivatives tested in intact cell system but did by a few 2-[2-(2-furyl)vinyl]pyridine derivatives in protoplast lysate system irrespective of antibacterial activity. These inhibition observed should be either due to the inhibition of the synthesis of the macromolecules or due to the stimulation of the degradation of them. However, the former seems to be the case, for it has been reported that the exposure of bacteria to nitrofuran derivatives for 0.5—2 hours does not reduce the amount of preexisting DNA, RNA and protein by chemical analysis, and inhibits constantly the incorporation of radioactive precursors into the macromolecules without showing a peak in radioactivity incorporation.[2,8] So, the results obtained indicate that the inhibition of DNA, RNA and protein syntheses is in some way related to antibacterial activity of the nitrofuran derivatives whereas that of lipid synthesis is not. Among DNA, RNA and protein syntheses, RNA and protein syntheses in intact cell system were inhibited only by a part of the active nitrofuran derivatives which usually showed high antibacterial activity. However, DNA synthesis in the same system was markedly inhibited by all active ones except for No. 10, 11 and nitrofurazone which showed relatively weak inhibition. The derivative No. 11 is active against *staphylococci* but inactive against gram-negative bacilli like *Escherichia coli* as described before, and the minimal inhibitory concentrations of the derivative No. 10 and nitrofurazone were more than 40 μM (a concentration used) with a large inoculum as used in the present incorporation experiments. This is possibly due to the rapid degradation of the nitrofuran derivatives

8) Y. Takase, S. Nakamura, M. Ishiyama, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **21**, 114, (1973).

by a large number of intact bacterial cells.[9] So, all the nitrofuran derivatives active against *Escherichia coli* under the experimental conditions used seems to inhibit DNA synthesis. The degrees of the inhibition in DNA synthesis by the nitrofuran derivatives were marked and comparable with those by mitomycin C, suggesting that the inhibition was important for antibacterial activity. The inhibition was usually marked in protoplast lysate system compared with that in intact cell system. This was notable in the second type derivative, No. 11. The result indicates that the strength of antibacterial activity and difference in antibacterial spectrum of the nitrofuran derivatives at least in part depend on the permeability of the derivatives into bacterial cells. Although the present study revealed that (5-nitro-2-furyl)-vinylpyridine derivatives inhibited the synthesis of macromolecules in intact cell and protoplast lysate systems of *Escherichia coli*, and the inhibitions were related to antibacterial activity especially in DNA synthesis, it remains in future to know whether these inhibitions are observed in the other nitrofuran derivatives and are really responsible for antibacterial activity.

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9) D.L. Cramer, *J. Bacteriol.*, **54**, 119 (1947).