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Studies on the Stability of Drugs in Biological Media. IV.[1] Disappearance of Nitrofurans in Culture Media inoculated with *Staphylococci*[2]

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Disappearance of nitrofuran derivatives in pH 7.0 culture media and in the media inoculated with *Staphylococcus aureus* was investigated at 37°.

From the media inoculated with the bacteria, rapid disappearance which followed a biexponential profile was observed in contrast to the monoexponential one observed with the media excluding the bacteria. Apparent rate of disappearance was dependent on the bacterial contents, concentration of the drugs, and the compositions of the media. Generally, vinyl derivatives were more susceptible to the process than azomethine ones.

Equilibrium dialysis behavior, extent of the binding of the drugs to the crude membrane fraction of the bacteria, and the effect of medium compositions on the disappearance as well as the binding tendency of the drugs together with the inhibitory effect of *p*-chloromercurybenzoate suggest the possible involvement of a specialized transport process in the disappearance of nitrofurans from the culture media inoculated with the bacteria. The implications of these results from *in vitro* biological activity standpoint are discussed.

Nitrofurans have been widely used in clinical and veterinary medicine. Uriah and Pozo have reported the effect of light and temperature on the stability of nitrofurans employing several types of pharmaceutical preparations.[4] Microbial reduction of 5-nitro-2-furaldehyde semicarbazone (nitrofurazone) by cell free extracts of *E. coli* has been demonstrated by Asnis[5] and studies along similar lines have been carried out so far with respect to their inactivation or metabolism by microorganisms.[6] These works, however, are largely of qualitative nature and lack in their correspondence to the antibacterial activity especially against *Staphylococci*.

The primary purpose of this work is to estimate the stability and the disappearance profile of nitrofuran derivatives in various culture media containing *Staphylococci* in order to get further insight in a rational approach to the evaluation of their *in vitro* antibacterial activity.

Experimental

Materials——2-(5-Nitro-2-furyl)vinylpyridine derivatives (NF-148, -253, -305, 323), 2-(5-Nitro-2-furyl) vinyl azole derivatives (NF-153, -320), and 5-Nitro-2-furfurylidene amino derivatives (Nitrofurantoin =NFT, Monafuracin=MF, NF-501) were the gift from Dainippon Seiyaku Co., Ltd., Osaka, Japan. Struc-

1) Part III: K. Kakemi, H. Sezaki, K. Iwamoto, H. Kobayashi, and K. Inui, *Chem. Pharm. Bull.* (Tokyo), **19**, 730 (1971).
2) Part of this work was presented at the 91th Annual Meeting of the Pharmaceutical Society of Japan, Fukuoka, April 1971.
3) Location: *a*) *3-1, Tanabe-dori, Mizuho-ku, Nagoya*; *b*) *Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
4) J. Uriah and A. del Pozo, *Galenicia Acta* (Madrid), **19**, 137 (1966).
5) R.E. Asnis, *Arch. Biochem. Biophys.*, **66**, 208 (1957).
6) *a*) S. Oda, *Ag. Bicl. Chem.* (Tokyo), **26**, 250 (1962); *b*) T. Okutsu, T. Kawabata, and T. Kozima, *Nippon Suisan Gakkaishi*, **30**, 147 (1964); *c*) P.M. Theus, *Arch. Pharm.* (Weinheim), **301**, 412 (1968); *d*) K. Kakemi, H. Sezaki, and H. Fujioka, *Yakuzaigaku*, **27**, 301 (1967).

Nitrofurans

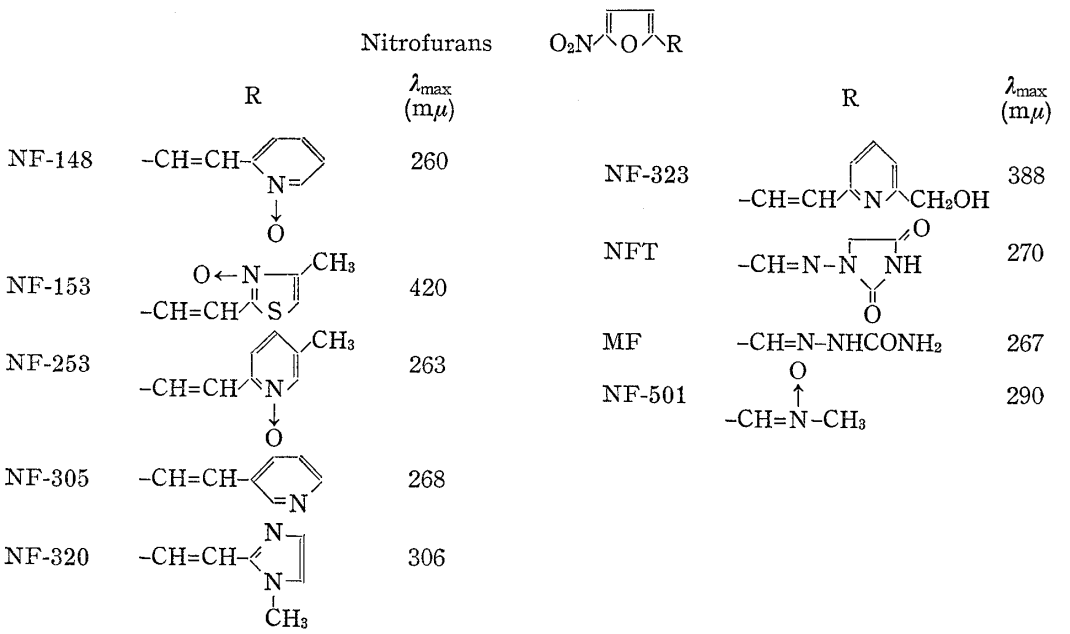

Chart 1. Structure and Maximum Absorption Wave Length of Nitrofurans

TABLE I. Compositions of Culture Media

Component	Media[a]		
	Phosphate broth[b]	Phosphate buffer medium	Synthetic medium
Phosphate broth(Nissan)	6.0 g		
K_2HPO_4	4.0 g		
KH_2PO_4		4.54 g	4.54 g
NaCl	4.0 g	5.00 g	5.00 g
Glucose			2.25 g
L-Arginine hydrochloride			1.91 g
Distd. water	1000 ml	1000 ml	1000 ml

a) all adjusted at pH 7.0
b) corresponding to 5-fold diluted one

tures of these compounds are shown in Chart 1. All other chemicals were of reagent grade. Media and their compositions are listed in Table I.

Kinetic Procedure in Phosphate Broth——Appropriate amount of nitrofuran derivatives (NF) were dissolved in 5-fold diluted phosphate broth (Nissan) which maintained the same concentration of phosphate moiety as non-diluted one and was adjusted at pH 7.0. Initial concentration of NF was adjusted at 4×10^{-5}M unless otherwise specified. Kinetic run was carried out in a volumetric flask which was immersed in a constant temperature bath kept at 37°. Aliquots were withdrawn periodically and mixed with appropriate volume of extracting solvents, chloroform and ethyl acetate, for vinyl and azomethine derivatives, respectively. These organic phases were separated after extraction of NF and were analyzed spectrophotometrically against a similarly prepared blank at each wave length as indicated in Chart 1. All the procedures were carried out under the condition of shielding from the light. Effect of L-cysteine on the stability of NF was tested similarly in the phosphate broth.

Kinetic Procedure in the Presence of Staphylococci——Various amounts (wet weight) of 24-hour-old culture of *Staphylococcus aureus* FAD 209P were inoculated in the phosphate broth which contains appropriate amount of NF. Kinetic run and assay of NF were conducted in the same manner as described elesewhere in this report. Effects of initial concentration of NF and addition of 0.1 mM *p*-chloromercurybenzoate (PCMB) were tested keeping the initial quantity of the microbial cells constant.

Effect of Medium Components on the Disappearance of NF——In addition to the phosphate broth, two types of pH 7.0 synthetic phosphate media were prepared so as to be the modified ones of Surgalla medium which is conventionally employed in the incubation test of *Staphylococci*. Kinetic run was carried out similarly to that of phosphate broth except adjusting the initial amount of microorganisms at 0.20 mg per ml.

cell suspension of 48-hour-old culture (10 g wet weight per 10 ml of 0.067M phosphate buffer solution, pH 7.8)

↓ added with 10 g of glass beads (100 to 150 mesh) and disintegrated by ultrasonic oscillation with Kubota Ultrasonic Generator KMS-250 for 20 min (output voltage: 120)

disintegrated cell suspension

↓ centrifuged 2 times at $400 \times g$ for 20 min after decanting the suspension which was added later with the washings

supernatant fluid — precipitate (unbroken cells of clumps of cell walls)

↓ centrifuged at $4200 \times g$ for 60 min

precipitate — supernatant (soluble or particle fraction)

↓ resuspended in 40 ml of the buffer solution containing crystalline trypsin (0.1 mg per 1.0 of turbidity reading at 550 mμ) and incubated at 40° for 60 min before the centrifugation at $9500 \times g$ for 20 min

precipitate

↓ washed 2 times with the buffer solution and distilled water and centrifuged at $9500 \times g$ for 30 min

cell wall fraction (crude membrane fraction)

Chart 2. Method of Fractionation of *Staphylococcal* Cell Walls

Binding of NF to the Crude Membrane Fraction——Cell wall of the test microorganisms was fractionated by the partly modified method of Cummins, Kotani, and their co-workers.[7] An outline of the procedure is illustrated in Chart 2. Disappearance of NF was measured periodically in the presence of crude membrane fraction (0.8 mg per ml) in the phosphate broth by adjusting the initial concentration of NF at 1.2×10^{-5}M. Assay of NF were conducted for the supernatant solution obtained by centrifugation of the test suspension. Binding test of NF was carried out by a conventional equilibrium dialysis method using Visking cellulose tube. Concentration of crude membrane fraction applied was 2.5 mg per ml. After 16 to 33 hours incubation at 37°, NF was assayed against a similarly treated blanks which exclude crude membrane fraction. Effect of PCMB as well as medium components on the binding of NF was tested on NF-148.

Antibacterial Activity Tests——These were carried out in the same manner as reported previously[1] except inoculation with 0.1 ml of supsension of the test organisms (1 mg per 1 ml), incubation for 48 hours at 37°, and the omission of cupric ion.

Table II. Rate Constants and Half-Lives for the Degradation of Nitrofurans in pH 7.0 Phosphate Broth at 37°

Nitrofurans	k_{app} (10^{-2} hr^{-1})	$t_{1/2}$ (hr)
NF-148	0.498	139
NF-153	0.0780	888
NF-253	0.0512	1350
NF-305	0.258	269
NF-320	0.624	111
NF-323	0.144	481
NFT	1.370	50.6
MF	1.220	56.8
NF-501	0.216	321

7) *a*) C.S. Cummnis and H. Harris, *J. Gen. Microbiol.*, **14**, 583 (1956); *b*) S. Kotani, T. Kitaura, T. Hirano, and A. Tanaka, *Biken's J.*, **2**, 129 (1959).

Result

Stability of NF in the Phosphate Broth

The degradation of NF followed apparent first-order kinetics as shown in Fig. 1 and the rate constants are summarized in Table II together with the half-lives. Aqueous solution of NF has been demonstrated to be ralatively stable at higher temperatures[4] and in the pH range of 4 to 8.[6b] Though vinyl derivatives were proved to be more stable than azomethine ones except NF-501 which possesses N-oxide structure, all the drugs investigated were considerably stable in the phosphate broth of pH 7.0.

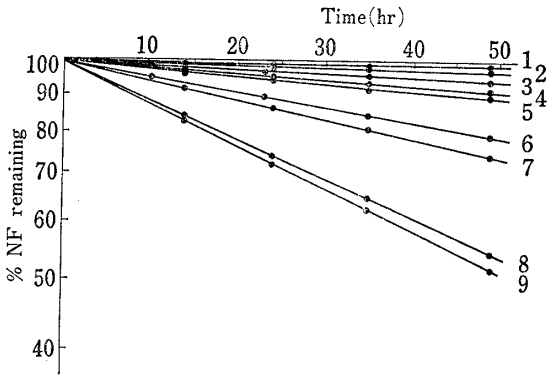

Fig. 1. Stability of Nitrofurans in pH 7.0 Phosphate Broth at 37°

1: NF-253, 2: NF-153, 3: NF-323, 4: NF-501, 5: NF-305, 6: NF-148, 7: NF-320, 8: MF, 9: NFT

Disappearance of NF in the Presence of *Staphylococci*

Since there was no difference between absorption spectra of the extracts from the test suspension and the supernatant samples obtained by immediate centrifugation, all the spectrophotometric analyses were conducted for the former unless otherwise specified. As illustrated in Fig. 2, enhanced bi-exponential disappearance curves were obtained contrary to the results in Fig. 1. Generally vinyl derivatives showed considerably rapid disappearance. The rate of disapperance, investigated as a function of the initial concentrations of NF, are shown in Fig. 3. The rate was apparently suppressed with the increase of the NF concentration, suggesting possible contribution of enzymic and/or catalytic interaction between NF

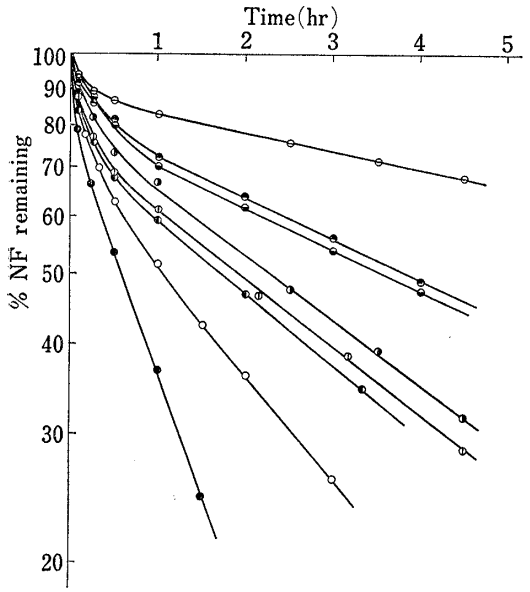

Fig. 2. Disappearance Profile of Nitrofurans in pH 7.0 Phosphate Broth Suspended with *St. aureus* (0.2 mg/ml) at 37°

○: NF-148, ●: NF-153, ◑: NF-253, ◑: NF-320, ◐: NF-323, ⊖: NFT, ◒: MF, ◓: NF-501

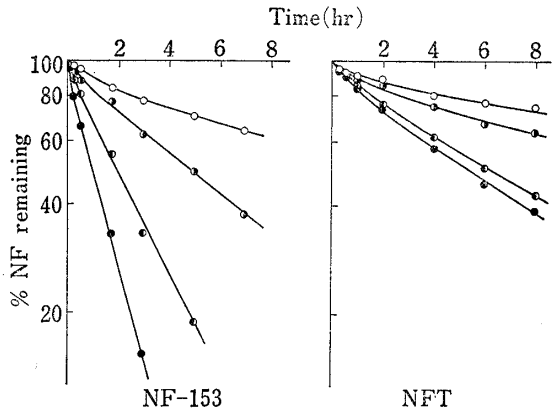

Fig. 3. Effect of Initial Concentration of Nitrofurans on the Disappearance in pH 7.0 Phosphate Broth Suspended with *St. aureus* (0.1 mg/ml) at 37°

●: 2×10^{-5}M, ◐: 4×10^{-5}M, ◑: 8×10^{-5}M, ○: 1.6×10^{-4}M

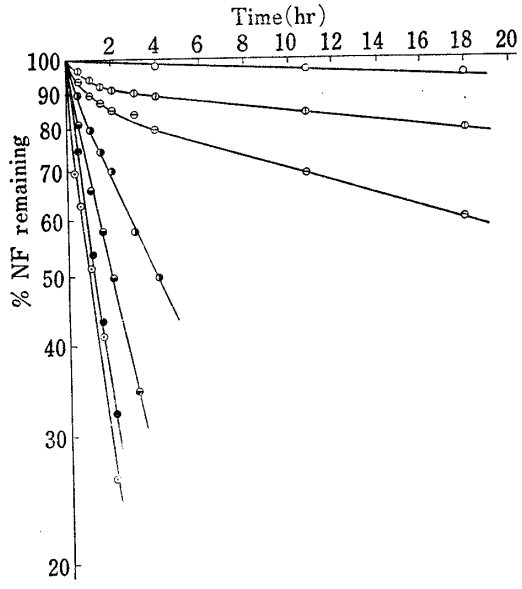

Fig. 4. Effect of Varying the Quantity of *St. aureus* Suspended in pH 7.0 Phosphate Broth on the Disappearance of NF-153 at 37°

○: 0 mg/ml, ◔: 0.01 mg/ml, ⊖: 0.02 mg/ml, ◑: 0.05 mg/ml, ◒: 0.10 mg/ml, ●: 0.15 mg/ml, ⊙: 0.20 mg/ml

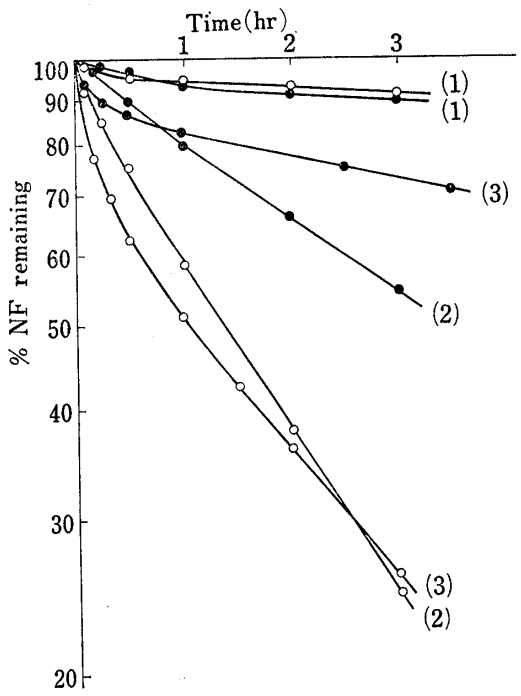

Fig. 5. Effect of Medium Components on the Disappearance of NF-148 (○) and NFT (●) in pH 7.0 Suspension of *St. aureus* (0.2 mg/ml)

1: phosphate buffer solution 2: synthetic medium, 3: phosphate broth

and the microbial constituents. Effect of microbial cell concentration was examined with NF-153 and the results are shown in Fig. 4. With the increase of the cell concentrations in the broth, bi-exponential disapperance features became more conspicuous. All other drugs showed similar tendency, indicating the microorganisms' role in the disappearance of NF from the media.

Effect of Medium Components and PCMB on the Disappearnace of NF

Effect of medium conponents on the disappearance of NF was tested with NF-148 and NFT as model compounds. The results are presented in Fig. 5, in which phosphate broth is included as a comparison. The approximate extent of disappearance in the phosphate buffer medium was merely 10% in three hours, whereas 75 and 30 to 45% for NF-148 and NFT, respectively, in the phosphate broth and in the synthetic phosphate medium which contains glucose and L-arginine as crabon and nitrogen sources, respectively. Such disappearance was inhibited dramatically by the simultaneous addition of PCMB. This inhibitory effect was more pronounced when the microorganisms were preloaded with PCMB 35 minutes before the run (Fig. 6).

Disappearance and Binding of NF in the Cell Wall Suspensions

Time course of NF disappearance in the phosphate broth suspended with crude membrane fraction is indicated in Fig. 7. Though the disappearance rates were estimated to be relatively small as compared with those of intact cells shown in Fig. 2, similar rank-order in the rates was obtained. Disappearance rate was found to decline progressively and cease after a further exposure of NF to the crude membrane fraction. Binding of NF to the crude membrane fraction was examined in various media using conventional equilibrium dialysis method. As shown in Table III, binding of NF-148 was very small in the phosphate buffer

medium as compared with other media. In the synthetic media, binding was decreased to an appreciable extent with the decrease of the concentration of essential medium components such as glucose and L-arginine. The marked fall by the addition of PCMB demonstrates a possible involvement of thiol groups in the binding process.

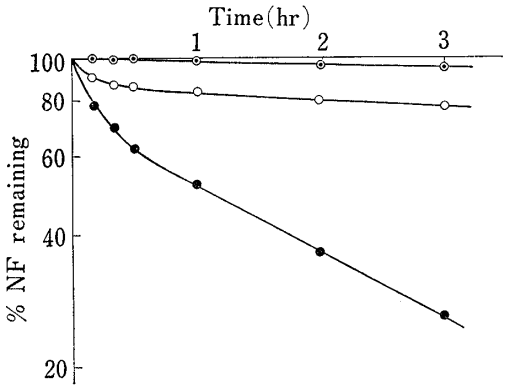

Fig. 6. Effect of PCMB on the Disappearance of NF-148 in pH 7.0 Phosphate Broth Suspended with *St. aureus* (0.2 mg/ml) at 37°

⊙: preloaded for 35 min before the run
○: added at 0 time
●: not added

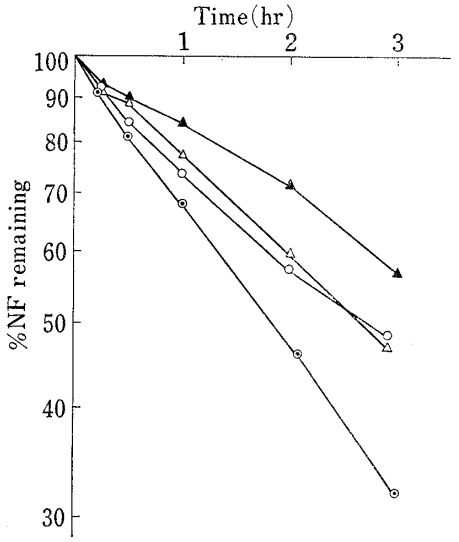

Fig. 7. Disappearance Profile of Nitrofurans in pH 7.0 Phosphate Broth Suspended with Cell Wall Fraction of *St. aureus* (0.8 mg/ml) at 37°

○: NF-148, ⊙: NF-153, △: NF-323, ▲: NFT

TABLE III. Effect of Medium Components on the Binding of NF-148 to Cell Wall Fraction of *St. aureus* at pH 7.0 and 37°

Media[a)]	% bound[b)]
Phosphate buffer medium	1.88
1/5 Synthetic medium	19.8
Synthetic medium	43.3
Synthetic medium+PCMB[c)]	0.785
1/5 Phosphate broth	50.7

a) see Table I
b) tested by equilibrium dialysis method employing initial concentration of 8×10^{-5}M
c) concentration of 1×10^{-4}M

TABLE IV. Comparison of Minimal Inhibitory Concentrations (MIC) of Nitrofurans against *St. aureus*

Nitrofurans	MIC (M)
NF 148	4×10^{-6}
NF 153	4×10^{-6}
NF 320	1.6×10^{-5}
NF 323	1.6×10^{-5}
NFT	4×10^{-5}
MF	4×10^{-5}
NF 501	2×10^{-5}

Antibacterial Activity Tests

The results of the activity testing are shown in Table IV, which agree considerably well with those of Fujita.[8]

Effect of L-Cysteine on the Stability of NF

Effect of cysteine was investigated in pH 7.0 phosphate broth at 37° and the results are summarized in Fig. 8. Good correlationship between the concentration of cysteine and the apparent first-order rate constant of the degradation reaction of NF is evident.

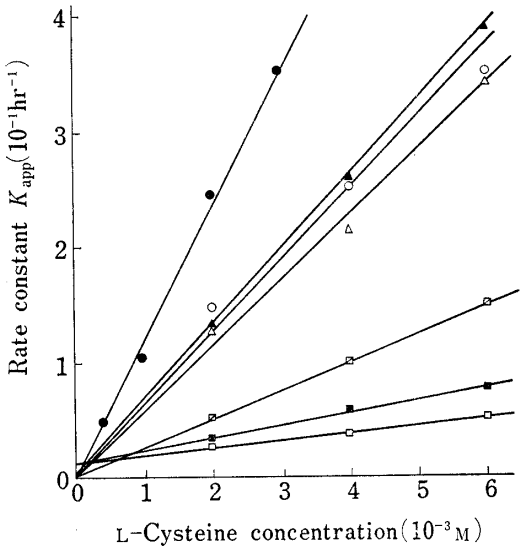

Fig. 8. Effect of L-Cysteine on the Stability of Nitrofurans in pH 7.0 Phosphate Broth at 37°

○: NF-148, ●: NF-153, △: NF-253, ▲: NF-320
□: NFT, ■: MF, ▨: NF-501

Discussion

The *in vitro* and *in vivo* antibacterial activities of NF have been extensively investigated by a number of workers. However, little has been known about the possible relevance of their disappearance behavior to the antibacterial activities when exposed to bacterial cells.

The activity data shown in Table IV suggest that activities seem to be better related to the rate of disappearance of NF from the media. Compounds having higher *in vitro* activity have a tendency to disappear faster from the media than those having less activity. Nitrofurans in their dilute concentrations disappear much faster than in the higher concentrations and it is worthy to note that the rate of such disappearance is definitely influenced by the composition of incubation media. Incubation in the synthetic medium or in the phosphate broth caused much faster disappearance of NF than from the simple phosphate buffer solution. Such remarkable effect of medium components is also reflected upon the binding of NF-148 to the curde membrane fraction obtained from the cell suspension of 48-hour-old culture of *Staphylococcus aureus*. Results of the equilibrium dialysis experiments indicate that in the synthetic medium or in the phosphate broth, NF-148, at an initial concentration of 8×10^{-5}, was bound to the extent of nearly 50% in the presence of the crude membrane fraction.

In the diluted medium and in the phosphate buffer medium which is devoid of nutrients, such binding was reduced to a considerable extent. In the presence of PCMB, binding was almost completely inhibited.

Among a number of studies with respect to the binding property of bacterial cells, Galdiero and co-worker have investigated metal and hydrogen ion binding properties of *Staphylococcal* cell wall and have proposed their mechanisms and sites,[9] whereas transport in *Staphlococci* and accumulation by *E. coli* of tetracycline have been demonstrated to follow saturation kinetics at low antibiotic concentrations and to be bi-exponential, respectively.[10,11]

Recently Franklin and Higginson have discussed on the probable mechanisms of active accumulation of tetracycline by *E. coli*, showing the acceleration of tetracycline efflux by 2,4-dinitrophenol and inhibition of its accumulation by N-ethylmaleimide.[12] So far, however,

8) A. Fujita, J. Aritomi, S. Minami, and H. Takamatsu, *Yakugaku Zasshi*, **86**, 427 (1966).
9) *a*) F. Galdiero, *Experientia*, **24**, 352 (1968); *b*) F. Galdiero and C. Cutinelli, *J. Bacteriol.*, **93**, 2022 (1967).
10) B.L. Hutchings, *Biochem. Biophys. Acta*, **174**, 734 (1969).
11) J.R. DeZeeuw, *J. Bacteriol.*, **95**, 498 (1968).
12) T.J. Franklin and B. Higginson, *Biochem. J.*, **116**, 287 (1970).

no evidence for the direct involvement of thiol groups in the transport or uptake process has been reported in *Staphyloccoci*. Our data suggest the possible mediation of a specialized process in the binding or the uptake of NF to the crude membrane fraction in which thiol groups might be involved. The rate of disappearance of NF from pH 7.0 phosphate broth containing the crude membrane fraction suspension was found to be well correlated with the *in vitro* antibacterial activities of the compounds. This suggest the possibility that the specialized transport process in or on the cell membrane susceptible to the compositions of culture media might be one of the limiting factors governing the active concentration of NF in the bacterial cells although the catalyzing reaction by thiol groups within the cells as demonstrated by the effect of L-cysteine on the stability of NF (Fig. 8) may well be another contributory factor.