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SECTION MEETINGS

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School of Hygiene and Public Health	December 19, 1951
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Resistance and Cross Resistance of Bacteria to Nitrofurans. (19321)

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It has been shown that many nitrofurans have a broad antibacterial spectrum(1-4). Green(5) has demonstrated that *Micrococcus pyogenes var. aureus* can develop limited resistance to 5-nitro-2-furaldehyde semicarbazone (Furacin*). The present study is concerned with development of bacterial resistance and cross resistance to nitrofurans of varied chemical structure.

The nitrofurans used in this study were characterized by a nitro group in the 5-position of the furan ring and a semicarbazone, semioxamazone, or closely related side chain in the 2-position.† Most of these nitrofurans have a basic structure correlated with chemotherapeutic activity(4).

Experimental. For the preliminary phase of the study of resistance development, three organisms: *Escherichia coli*—A.T.C. No. 6522, *M. pyogenes var. aureus*—F.D.A No. 209, and *Streptococcus faecalis*—A.T.C. No. 6057, were habituated to a selected nitrofuran by exposing the bacteria to 2-fold increasing concentrations in Difco nutrient broth using smaller increments as limiting resistance was approached. The starting inoculum was one loopful (4 mm loop of 23 gauge platinum wire) of the 24-hour culture of the test bacteria grown in broth. Subsequently, a loop inoculum was taken from the tube containing the highest concentration of the nitrofuran in which definite visible growth occurred. Density of growth for all 3 organisms was reduced considerably after the first few subcultures under the influence of the nitrofuran and 24-hour transfers were changed

* Brand of nitrofurazone N.N.R.

† The compounds were synthesized by the Division of Chemistry of Eaton Laboratories.

TABLE I. Development of Resistance to 5-Nitro-2-Furaldehyde 2-(2-Hydroxyethyl) Semicarbazone (NF-67) in Broth.

Test organism	Conc. NF-67 mg/l in which visible growth occurred* at start	at end	Degree of resistance development	No. of subcultures
S. faecalis	31	400	13x	6
M. aureus	9	200	22x	8
E. coli	19	400	21x	9

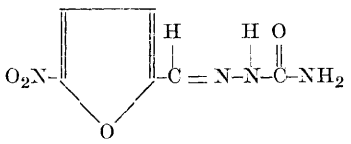
* Loop inocula.

to 48 hours to allow suitable growth. The resistance thus obtained by 3 organisms against a selected nitrofuran (NF-67) is shown in Table I.

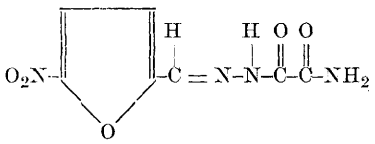
After the limiting resistance had been reached by the loop method, substitution of a larger inoculum (0.1 ml) in place of the loop inoculum (about 0.01 ml) resulted in attenuated growth in the case of *E. coli* and *M. aureus* up to twice the above concentrations. Therefore 0.1 ml inocula were used in all subsequent studies in developing resistant organisms.

Although resistance to NF-67 occurred in the 3 bacteria tested, its development was definitely slower (about 20x in 8 subcultures) than observed by Klimek *et al.*(6) in the development of resistance of *M. aureus* to penicillin (about 80x in 8 subcultures) and to streptomycin (about 200x in 8 subcultures).

E. coli. The development of resistance by *E. coli* to various nitrofurans was investigated next. The nitrofurans studied and their structure appear in Table II. The water-solubility of the nitrofurans studied is given since it is a limiting factor in some cases. The data in columns 4 and 5 show that *E. coli* can develop resistance to all nitrofurans studied. Of importance is the fact that, where solubility permits, a limiting concentration is reached to which bacteria cannot readily develop resistance. It was found that bacteria resistant to single semicarbazones such as NF-7 (Furacin), NF-61, and NF-67 were cross resistant to other semicarbazones. However these organisms were still susceptible to the semioxamazones, NF-84 and NF-89. The structure of the simplest member of each class is as follows:



Semicarbazone (NF-7)

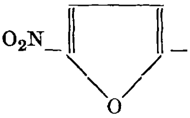
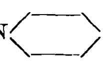


Semioxamazone (NF-84)

A strain of *E. coli* highly resistant to a typical semicarbazone (NF-67) and a strain highly resistant to a typical semioxamazone (NF-84) were now titrated for possible cross resistance to a series of nitrofurans chosen for varying structure, suitable solubility and degree of antibacterial activity. The last two columns of Table II indicate, on the basis of cross resistance, that the nitrofurans studied fall into two groups. *E. coli* resistant to the semicarbazone NF-67 is cross resistant to a series of nitrofurans which we have arbitrarily classified as Type I compounds. This same NF-67-resistant *E. coli* is, however, susceptible to another series of nitrofurans which we term Type II compounds.

From inspection of the structural formulas of the nitrofurans in Table II it will be noted that the Type II nitrofurans all differ from the Type I nitrofurans by having a carbon atom between the first carbonyl carbon and the terminal group of the side chain. On the basis of this observation the study of cross resistance was extended by developing strains of *E. coli* which were highly resistant to each of 4 individual nitrofurans of the Type I series and to 3 of the Type II series, as shown in the

TABLE II. Resistance and Cross Resistance of *E. coli* to Nitrofurans of Varied Structure.

Nitrofuran No. (NF)	Structure R = O_2N–  –	Solubility in water, mg/l¶	Resistance* Parent strain	Resistant strain	Cross resistance* Strain resistant to NF-67†	Strain resistant to NF-84‡
	Type I					
7	R—CH = N·NH·CO·NH_2	210	12.5	75	100 C	100 C§
61	R—CH = N·N·CO·NH_2 │ CH_3	317	6.5	100	100 C	100 C
62	R—CH = N·NH·CO·NH·CH_3	670	25		200 C	200 C
64	R—CH = N·NH·CO·CH_3	135	11.2		45 C	
65	R—CH = N·NH·CO·NH·CO·NH_2	60	8.75		70 C	
67	R—CH = N·N·CO·NH_2 │ CH_2CH_2OH	1250	12.5	600	—	200 C
106	R—C = N·NH·CO·NH_2 │ CH_2OH	275	12.2		196 C	
131	R—C·CH_2·CH_2–N  ·HCl ‖ N·NH·CO·NH_2	12100	50		400 C	200 C
145	R—CH = N·NH·CO·NH·CH_2·CH_2·OH	1030	38.3		307 C	
147	R—C = N·NH·CO·NH·CH_2·CH_2·OH │ CH_3	157	58.6		234 C§	234 C§
148	R—CH = N·N·CO·NH·CH_3 │ CH_3	600	24.6		395 C	
153	R—CH = N·N·CO·NH │ │ CH_2·CO	125‖	4.2	200	162 C	137 C
156	R—CH = N·N·CO·NH_2 │ CH_2·CHOH·CH_3	420	19.3		620 C§	
	Type II					
69	R—CH = N·NH·CO·CH_2·NH_2·HCl	60400	12.1	80	24 N	100 C§
82	R—CH = N·NH·CO·CO·OC_2H_5	220	62.5		125 N	
84	R—CH = N·NH·CO·CO·NH_2	120	9.37	100	12.5 N	
89	R—CH = N·NH·CO·CO·NH │ CH_2CH_2OH	140	11.2		22.4 N	
114	R—CH = N·NH·CO·CH_2·N$(CH_3)_3$ │ Cl	89000	23.9	200	23.9 N	200 C

* Highest concentration in mg/l of nitrofuran permitting visible growth in 24-48 hr. Median value used where determination was run repeatedly.

† Organisms in which resistance to 600 mg NF-67 per 1 had been developed. C = cross resistance, N = non-cross resistance.

‡ Organisms in which resistance to 100 mg NF-84 per 1 had been developed.

§ Highest conc. tried.

‖ At pH 5 for this solubility. There is a marked increase in solubility with an increase of pH with this compound: Furadantin, brand of nitrofurantoin.

¶ Usually higher in culture media.

vertical column of Table III, grouped according to type. This tabular form of presentation is similar to that used by McIntosh and Selbie(7) for convenience in evaluating the entire resistance picture. These seven resistant strains were then individually titrated

TABLE III. Degree of Resistance and of Cross Resistance of *E. coli* to Certain Nitrofurans.

E. coli resistant to nitrofurans below	Degree of resistance*						
	Type I				Type II		
	NF-7	NF-61	NF-67	NF-153	NF-69	NF-84	NF-114
			Type I				
NF- 7	*6*	16	8	32	2	1	1
61	4	*10*	32	16	2	1	1
67	8	16	*32*	32	2	1	1
153	4	4	8	*37*	2	1	1
			Type II				
69	2	8	16	32	*8*	2	1
84	16	16	32	32	16	*8*	8
114	2	2	4	4	8	2	*8*

* Values = highest nitrofuran conc. in which resistant strain grew divided by highest concentration parent strain grew tested concurrently.

against each of the nitrofurans in the top horizontal column. For the experiments presented in this table, titrations were done by serial dilution except in a few cases where intermediate dilutions were used. All values in the table were obtained by dividing the highest nitrofuran concentration in which the resistant strain could grow, by the highest concentration in which the parent susceptible strain grew when tested concurrently. The seven italicized figures on the diagonal represent the degree of resistance displayed by the resistant *E. coli* to the specific nitrofuran to which it had become habituated, as compared to the susceptible parent organism at the time of the study.

Data in the upper left quadrant show that a strain of *E. coli* resistant to any Type I nitrofuran is cross resistant to all other Type I nitrofurans (all numbers being 4 or greater). This phenomenon, which we term *reciprocal cross resistance,* occurs with all compounds of Type I studied to date. Examination of the upper right quadrant of Table III shows in a clear-cut manner that *E. coli* strains resistant to Type I nitrofurans are not resistant to Type II nitrofurans, since regardless of the strength of resistance that *E. coli* had developed to a Type I nitrofuran, it was still about as susceptible to a Type II nitrofuran as the parent non-resistant organism. However, from the lower left quadrant is can be readily observed that *E. coli* strains resistant to Type II compounds are, in most cases, also resistant to Type I compounds. Three exceptions may be noted: NF-69-resistant strain is susceptible to NF-7, and the NF-114-resistant strain to NF-7, and NF-114-resistant strain to NF-61. These 3 pairs are also reciprocally non-cross resistant as may be noted from data in the upper right quadrant—*i.e., E. coli* resistant to either member of these pairs is susceptible to the other member. Careful examination of the lower right quadrant indicates that, although there are cases of cross resistance and non-cross resistance among compounds of Type II, in no case does reciprocal cross resistance or reciprocal non-cross resistance occur within this type. Upon examination of the table as a whole, it is to be noted that *E. coli* resistant to any of the other nitrofurans is susceptible to NF-84; moreover, *E. coli* resistant to NF-84 is cross resistant to all other nitrofurans studied.

Streptococcus pyogenes. Supplementary studies of the resistance behavior of a gram-positive organism, *Strep. pyogenes,* were now made. This organism was chosen since it has been used in our laboratories for *in vivo* chemotherapy studies with the nitrofurans. A different culture medium, brain heart infusion, was required. In all other respects the technic for this work was the same as described for the investigation of *E. coli.* These studies were carried out using representative nitrofurans NF-7 and NF-67 from Type I, and NF-84 and NF-114 from Type II. The data from this study are presented in Table IV. Examination of this table in the same manner as Table III indicates that the following generalizations can be made for the organisms studied. 1. Bacteria resistant to any one member of Type I nitrofurans are cross resistant to all nitrofurans of Type I.

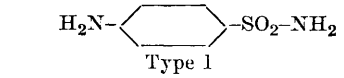
TABLE IV. Degree of Resistance and of Cross Resistance of *Strep. pyogenes* to Certain Nitrofurans.

Strep. pyogenes resistant to nitrofurans below	Degree of resistance*			
	Type I		Type II	
	NF-7	NF-67	NF-84	NF-114
	Type I			
NF- 7	*8*	20	1	1
67	8	*20*	1	1
	Type II			
84	8	8	*3*	8
114	8	5	2	*4*

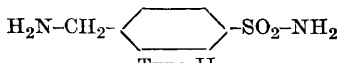
* See footnote Table III.

2. Bacteria resistant to any one member of Type I nitrofurans are susceptible to any member of Type II nitrofurans. 3. Only nitrofurans of Type I show reciprocal cross resistance. It is also indicated that cases of reciprocal non-cross resistance may occur between Type I and Type II nitrofurans. No suitable nitrofuran was available to determine whether the presence of two carbon atoms between the carbonyl carbon and the terminal group would produce a class of nitrofurans differing in resistance behavior from those of Type I and Type II.

Clostridium perfringens. A structural analogy has been noted between our two nitrofuran types and the sulfonamides (Type I) and a closely related class of compounds (Type II) studied by both Klarer(8) and Domagk(9).



Type 1



Type II

In their Type II compounds, the amino group (or substituted amino group) is separated from the benzene ring by a CH_2 group. Their Type II compounds differ markedly from the sulfonamides in being highly active against the anaerobic organisms of the Clostridium group and in not being inhibited by p-aminobenzoic acid. While it was known that Furacin (NF-7) was moderately active against the Clostridium group of anaerobic bacteria, no such data were available on our Type II nitrofurans. It was therefore planned to titrate 2 typical Type I nitrofurans concurrently with 2 typical Type II nitrofurans against *Cl. perfringens*. The common method of using thioglycollate in the medium for the maintenance of anaerobic conditions was not permissible since the concentration required led to nitrofuran destruction, probably by the reducing action of thioglycollate. The use of freshly prepared brain-heart infusion broth with 0.1% agar coupled with vacuum or nitrogen gas for maintenance of anaerobic conditions permitted an evaluation of these two types of nitrofurans. Type I nitrofurans NF-7 and NF-67 prevented growth from a heavy inoculum (0.1 cc of 20-hour culture) of *Cl. perfringens* at 50 and 25 mg per liter respectively. Comparatively, Type II nitrofurans NF-84 and NF-69 prevented growth only at levels greater than 50 mg and 150 mg per liter respectively. Contrary to our expectation, it is clearly shown that the Type II nitrofurans have no greater antibacterial activity than has the Type I group against this anaerobe.

Nitrofuran-antibiotic cross resistance. A study by Green and Mudd(10) indicates that bacteria with developed resistance to sulfonamides, streptomycin, and penicillin, remain susceptible to Furacin (NF-7). In their work *M. aureus* strains resistant to either sulfathiazole or penicillin were as susceptible to Furacin as the parent strains. *E. coli* and several other organisms resistant to streptomycin were not resistant to Furacin. The present study covers similar observations with chloramphenicol and aureomycin. A chloramphenicol-resistant strain of the *E. coli* used throughout these studies was developed in the usual manner (from 2 mg up to 100 mg per liter) and tested for resistance to the individual nitrofurans NF-7, NF-67, NF-84, and NF-114. *E. coli* resistant to chloramphenicol remained as susceptible to our series of four representative nitrofurans as the parent strain. An aureomycin-resistant strain of the same organism was then developed (from 0.6 mg per liter up to 10 mg per liter) and tested for resistance to this same series of nitrofurans. This aureomycin-resistant strain was also as susceptible to the nitrofurans as the parent strain. Strains of *E. coli* resistant to NF-7, NF-67, NF-84, and NF-114 were tested for

concomitant resistance to chloramphenicol and to aureomycin. No cross resistance was indicated, with the possible exception of a slight cross resistance (4x) of the 2-(2-hydroxy ethyl) substituted semicarbazone (NF-67)-resistant strain to chloramphenicol.

These studies, added to those of Green and Mudd(10), indicate that those bacteria studied, with developed resistance to chloramphenicol, aureomycin, penicillin, streptomycin, and sulfonamides, retain unaltered their susceptibility to the nitrofurans.

Summary. (1) Bacteria can develop only a limited resistance to the nitrofurans *in vitro*. On the basis of a bacterial cross resistance study of a series of eighteen nitrofurans, it was found that the compounds investigated could be divided into two classes. The second class differs in chemical structure by having a carbon atom between the carbonyl group and the terminal group of the nitrofuran side-chain. Bacteria becoming resistant to the first class are reciprocally cross resistant to all members of this class but remain susceptible to nitrofurans of the second class. (2) Bacteria in which resistance was developed to chloramphenicol or to aureomycin remain susceptible to the nitrofurans. The converse is also true.

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Particulate Component of the Plasma of Fowls with Avian Lymphomatosis.* (19322)

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The avian leukosis complex consists in two chief pathological types of neoplastic disease (1). One of these is an "intravascular" process characterized by the occurrence in the blood stream of large numbers of primitive cells of erythroblastic or myeloblastic characters. The other type, lymphomatosis, is manifested in various forms dependent on localized or widespread overgrowths of primitive cells of the lymphoid type which may reach the circulation in only small numbers or not at all. Evidence of the viral etiology of both types of the complex has been demonstrated repeatedly, and a principal problem in the study of the complex is the possible relationships of the respective causative agents.

One approach to this problem is the purification of the viruses associated with the diverse forms of the complex and the direct comparison of their physical, chemical, and immunological properties. Progress in this direction has been made in recent work(2,3) in the purification and study of the virus of erythromyeloblastic leukosis which has been found to be present in large amounts in the plasma of affected chicks. It has been shown (4), also, that the infectious agent of some

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