

On the Antibacterial Action and Mechanism of Nitrofuran Derivatives

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The antibacterial activity of 68 nitrofuran derivatives and 18 thiophene derivatives was investigated in vitro. The effects of the nitrofuran derivatives on the bacterial growth were studied by the turbidimetric method, and the fate of the 5-position nitro radical, especially the reduction of nitro radical, caused by the bacterial metabolism or multiplication was demonstrated by means of spectrophotometry and polarography, using the sensitive, resistant or non-sensitive organisms. Finally the development of bacterial resistance to nitrofuran derivatives was studied by several procedures.

Introduction

The antibacterial nitrofuran derivatives form a series of chemotherapeutic agents which were discovered in 1944 by M. C. Dodd and W. B. Stillman¹⁾ of the Eaton Laboratory, U.S.A., 2-(5-Nitro)-furfural semicarbozone, called furacin, because of its remarkable antibacterial action and low toxicity, was used in the World War II as a local antibacterial agent in wound infections on which neither the sulfamin preparations nor penicillin was efficacious, and showed high promise. With the promising action of this agent a new field in chemotherapy has been opened and attention has been drawn to these agents.

H. Saikachi²⁾ succeeded in synthesizing furacin in Japan. His success was a result of his investigations since 1943 in the nature of the ether bonds which hold oxygen to the furan nucleus. Since then more than three hundred additional derivatives have been synthesized by H. Saikachi and his coworkers, and the other agents have been extensively studied in our laboratory by R. Kimura,^{3) 7) 8)} N. Higashi,⁹⁾ D. Mizuno,¹⁰⁾ Z. Goi,¹¹⁾ K. Ikegaki,^{12) -18)} M. Fujimoto,^{19) 20)} H. Iwasaki^{21) 22) 23)} and others. The systematic studies have included the antibacterial action in vitro and in vivo, antiviral action, diffusibility, toxicity in tissue cultures, animal experimentation and the development of bacterial resistance, etc. H. Saikachi *et al.*^{4) 5)} have kept on synthesizing new derivatives and discovered the 2-(5-Nitro)-furyl-acrolein as an antibacterial group. He has also studied the erythro and threo type compounds of the nitrofuryl series from a stereochemical standpoint. The antibacterial action of these compounds was studied by T. Toda and his collaborators. From these studies an excellent derivative was discovered which exhibited excellent action against *Staphylococcus aureus* and *Escherichia coli*

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groups and even against *Mycobacterium tuberculosis*.

Many experiments concerning the antibacterial activity of these agents have been performed by T. Fujino *et al.*,²⁴⁾²⁵⁾ K. Shibata,²⁶⁾⁻³¹⁾ S. Hatta,³²⁾³³⁾ H. Miyamoto,³⁴⁾ S. Kuwabara,³⁵⁾ Z. Asakura³⁶⁾ and others.

Those persons who studied the antibacterial mechanism of these agents were D. L. Cramer,³⁷⁾³⁸⁾ M. C. Dodd,³⁷⁾ H. Tamiya *et al.*,³⁹⁾ A. Kasuga⁴⁰⁾ and K. Shibata.³¹⁾ The observations on the resistant strain of bacteria to these agents were made by K. Ishigami⁴¹⁾ from the enzymological standpoint. The investigations for substances antagonistic to these derivatives in the metabolic processes of bacteria were carried out by M. N. Green.⁴²⁾

Analytical studies employing the polarography were made by I. Tachi⁴³⁾ and T. Sasaki.⁴⁴⁾⁴⁵⁾ S. Takagi studied the synthesis of several reduction products employing electrolytic reduction.

D. L. Cramer³⁸⁾ measured the oxidation-reduction potentials attributable to the reagents in the process of bacterial growth. H. E. Paul *et al.*,⁴⁶⁾⁴⁷⁾ and others⁴⁸⁾⁻⁵² investigated the fate of these derivatives employing the ultraviolet absorption spectrography or the enzymological procedure, and also their effect on the metabolism in various organs and tissues.

During this period 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride and 2-(5-Nitro)-furylacrolein semicarbazone as well as the above mentioned 2-(5-Nitro)-furfural semicarbazone was found to have definite antibacterial action and comparatively low toxicity. These are used with success in surgical procedures of otorhinology and dermatology as local chemotherapeutic agents. Since then these agents have found wider use,⁵³⁾ even in internal medicine in the treatment of sulfamin resistant bacillary dysenteriae. These agents are also being used in the field of veterinary medicine in the treatment of coccidiosis and as a disinfectant in foods.⁵⁴⁾⁵⁵⁾

M. C. Dodd and W. B. Stillman,¹⁾ in 1944, reported the definite antibacterial activity of forty two nitrofur derivatives. Similar results were obtained in our laboratory.⁸⁾¹⁴⁾¹⁶⁾ According to such studies, it is clear that the presence of a nitro radical at position 5 is essential for antibacterial activity. Furfural semicarbazone has no antibacterial activity, whereas 2-(5-Nitro)-furfural semicarbazone has an excellent one. 2-(5-Chloro)-furfural semicarbazone has a definitely reduced antibacterial action. To further corroboration of this belief, 2-(3-Nitro)-furoic acid methylester has an antibacterial activity of only 1 : 1,000 against *Staphylococcus aureus* whereas 2-(5-Nitro)-furoic acid methylester has a much higher activity against the same organism *i.e.* 1 : 10,000. In addition to the necessity of the 5-position nitro radical the antibacterial spectrum varies with the 2-position side chain.

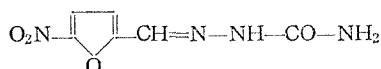
However, it is interesting that there is as yet no certain correlation between the chemical structure of the synthetic chemotherapeutics and their antibacterial activity in spite of numerous reports published on this subject.

Antibacterial Nitrofuran Derivatives

The author has compared and examined the antibacterial activity of several derivatives newly synthesized by the coworkers T. Sasaki⁵⁶⁾⁵⁷⁾ and K. Suzuki,⁵⁸⁾ and has investigated the correlation between the chemical structure and antibacterial activity. Simultaneously, in order to study the mode of action of these derivatives, using the ultraviolet absorption or the polarography, the chemical changes occurring in these derivatives due to bacterial metabolism and multiplication were investigated, and also various aspects of the development of bacterial resistance to these agents was studied.

The chemical structures of 2-(5-Nitro)-furfural semicarbazone and 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride, which were the main agents employed in the following experiments, are as follows:

1. 2-(5-Nitro)-furfural semicarbazone

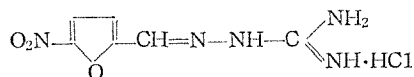


A crystalline powder of lemon yellow.

m.p. 236-240°C.

Solubility in water 1 : 4,200.

2. 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride



Fine, short cylindrical, yellow crystals.

m.p. 248-250° C.

Solubility in water 1 : 100.

I. The Antibacterial Activity of Various Nitrofuran Derivatives

Several published reports have indicated that a variety of nitrofuran derivatives having a 5-position nitro radical show various grades of antibacterial activity according to the character of the 2-position side chain.

In order to discover derivatives having more powerful antibacterial action, and to investigate the relationship between chemical structure and antibacterial activity, the author studied the antibacterial action, in vitro, of the 2-(5-Nitro)-furfurylidene series, -furylacrolein series, -furyl acrylic amide series, -furoic amide series and other furan derivatives, as well as thiophene nucleus possessing compounds, i. e., heterocyclic compounds as furan nucleus, which were among the compounds newly synthesized by the above-mentioned coworkers.⁵⁶⁾⁵⁷⁾⁵⁸⁾

EXPERIMENTAL METHODS

Various methods and techniques for the microbiological assay of antibacterial

substances have been used and discussed regarding to the reagent and its usages. As for the nitrofurans derivatives, their antibacterial activities were measured by the dilution method using broth which had been used with success in our laboratory.¹²⁾ The test reagent was prepared by making a 1 : 5,000 dilution. This solution was diluted by a two-fold serial dilution method and was inoculated with the organisms. After incubating at 37° C. for a definite time the culture tubes were checked for turbidity, as a response of growth, and the minimum concentration for complete inhibition of bacterial growth was checked with the unaided eye.

Since nitrofurans derivatives exert antibacterial activity against both Gram positive and negative organisms, which is one of their notable characteristics not shown in other chemotherapeutics, a screening test was carried out using *Staphylococcus aureus* (FDA 209P) and *Escherichia coli* (*E. coli communior*, Kyoto Univ. strain), and reagents showing the most promising antibacterial action were selected. The antibacterial spectra of these agents were widely investigated. The strains used were usually prepared from the preservation in our laboratory. The bacteria which had been cultured for 18 hr were diluted with 5 % broth containing physiological saline and 5×10^4 to 5×10^6 organisms were inoculated.

RESULTS

The data in Table 1 to 6 show the minimum concentration at which complete inhibition of bacterial growth in 72 hr-cultures occurred. (unit 1 : 10,000)

The results of 2-(5-Nitro)-furfural semicarbazone and 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride are seen in Table 7. * ; insoluble in 5,000-fold volume of broth.

1. The 2-(5-Nitro)-furfurylidene series compounds (Table 1).

All of these were very difficult to dissolve and few showed striking antibacterial action. Only (3) inhibited the growth of *St. aureus* at the concentration of 1 : 80,000.

2. The 2-(5-Nitro)-furylacrolein series compounds (Table 2).

2-(5-Nitro)-furylacrolein as an antibacterial group showed a striking antibacterial action, (7), (9) and (10) inhibited the growth of *St. aureus* at the concentration of 1 : 160,000 and (10) inhibited the growth of *E. coli* at 1 : 160,000. However, these were all difficult to dissolve.

3. The 2-(5-Nitro)-furyl acrylic amide series compounds (Table 3).

(11), (13) and (27) inhibited the growth of *St. aureus* and *E. coli* at the concentration of 1 : 160,000 and 1 : 320,000, (11), (13), (30) and (31) showed a striking antibacterial action on *Salmonella typhi* (No. 58), *Shigella dysenteriae* and *Shigella paradysenteriae* (Komagome BI). (26) did not show remarkable antibacterial activity although it did inhibit growth of *St. aureus* at 1 : 160,000.

Antibacterial Nitrofuran Derivatives

4. The 2-(5-Nitro)-furoic amide series compounds (Table 4).

Although the solubility in broth was excellent, the antibacterial activity was low. Only (48) inhibited the growth of *St. aureus* at 1 : 80,000. However, this compound did not show, as did (26), antibacterial activity against other organisms.

5. The other furan derivatives (Table 5).

In the study of the antibacterial activity of furyl alcohol and furylactone only 2-(5-Nitro)-furyl allyl alcohol inhibited the growth of *St. aureus* at 1 : 80,000 and *E. coli* at 1 : 160,000.

6. The thiophene compounds (Table 6).

All had no antibacterial activity.

Table. 1.

I) 2-(5-Nitro)-furfurylidene series compounds.		(R=O ₂ N- )				
		St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(1)	$R-CH=C \begin{matrix} \text{CONH} \\ \text{NHCO} \end{matrix} C=CH-R$	* 1	—			
(2)	$R-CH=C \begin{matrix} \text{CO-NH} \\ \text{CO} \\ \text{CO-NH} \end{matrix}$	* <1	<1			
(3)	$R-CH=N-NHCOOC_2H_5$	* 8	4	2	<2	<2
(4)	$R-CH=N-NHCH_2-COOC_2H_5$	* <1	<1			
(5)	$(R-CH=N-NHCO)_2NH$	* —	—			
(6)	$R-CH=N-N \begin{matrix} \text{CH=N} \\ \text{CH=N} \end{matrix}$	<.5	<.5			

Table 2.

II) 2-(5-Nitro)-furylacrolein series compounds.		St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(7)	$R-CH=CH-CH=N \cdot NHCOOC_2H_5$	* 16	8	4	4	4
(8)	$R-CH=CH-CH=N \cdot NHCH_2COOC_2H_5$	* 2	1			
(9)	$(R-CH-CH-CH=NNHCO)_2NH$	* 16	16			
(10)	$R-CH=CH-CH=N-N \begin{matrix} \text{CH=N} \\ \text{CH-N} \end{matrix}$	* 16	8	2	1	2

Table 3.

III) 2-(5-Nitro)-furyl acrylic amide series compounds.

	St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(11) $R-CH=CH-CO-NHCH_3$	16	32	16	4	16
(12) $R-CH=CH-CO-N < \begin{matrix} CH_3 \\ CH_3 \end{matrix}$	4	4			

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(13)	$R-CH=CH-CO-NH-C_2H_5$	8	8	16	4	8
(14)	$R-CH=CH-CO-N < \begin{smallmatrix} C_2H_5 \\ C_2H_5 \end{smallmatrix}$	2	<.5			
(15)	$R-CH=CH-CO-NH-C_3H_7$ (n)	* 16	32	8	4	4
(16)	$R-CH=CH-CO-NH-C_3H_7$ (iso)	4	2			
(17)	$R-CH=CH-CO-N < \begin{smallmatrix} C_3H_7 & (n) \\ C_3H_7 & (n) \end{smallmatrix}$	4	<.5			
(18)	$R-CH=CH-CO-N < \begin{smallmatrix} C_3H_7 & (iso) \\ C_3H_7 & (iso) \end{smallmatrix}$	* 4	<1			
(19)	$R-CH=CH-CO-NH-C_4H_9$ (n)	4	1			
(20)	$R-CH=CH-CO-N < \begin{smallmatrix} C_4H_9 & (n) \\ C_4H_9 & (n) \end{smallmatrix}$	* 4	<1			
(21)	$R-CH=CH-CO-NH-C_4H_9$ (iso)	* 4	<1			
(22)	$R-CH=CH-CO-N < \begin{smallmatrix} C_4H_9 & (iso) \\ C_4H_9 & (iso) \end{smallmatrix}$	2	<.5			
(23)	$R-CH=CH-CO-NH-C_4H_9$ (sec)	* 4	<1			
(24)	$R-CH=CH-CO-NH-C_8H_{11}$ (n)	* <1	<1			
(25)	$R-CH=CH-CO-N < \begin{smallmatrix} C_5H_{11} & (iso) \\ C_5H_{11} & (iso) \end{smallmatrix}$	* 2	<1			
(26)	$R-CH=CH-CO-NH-C_8H_{17}$ (n)	16	<2	<2	<2	<2
(27)	$R-CH=CH-CO-NH-CH_2-CH=CH_2$ *	16	16	8	4	4
(28)	$R-CH=CH-CO-NH-C_2H_4OH$	4	4	8	4	4
(29)	$R-CH=CH-CO-N < \begin{smallmatrix} C_2H_4OH \\ C_2H_4OH \end{smallmatrix}$	2	<.5			
(30)	$R-CH=CH-CO-NH-CH_2-\underset{\substack{ \\ OH}}{CH}-CH_3$	8	8	16	8	16
(31)	$R-CH=CH-CO-NH-CH_2-\underset{\substack{ \\ OH}}{CH}-\underset{\substack{ \\ OH}}{CH}-CH_2$	4	8	8	8	16
(32)	$R-CH=CH-CO-NH \cdot NH_2$	* —	—			
(33)	$R-CH=CH-CO-NH \cdot NH_2 \cdot HCl$	<.5	<.5			
(34)	$R-CH=CH-CO-NH-N=C < \begin{smallmatrix} CH_3 \\ CH_3 \end{smallmatrix}$	* —	—			
(35)	$R-CH=CH-CO-NH-NH-CO-\langle \text{cyclohexyl} \rangle$	* —	—			

Table 4.

IV) 2-(5-Nitro)-furoic amide series compounds.

	St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(36) $R-CO-NH_2$	1	1			
(37) $R-CO-NHCH_3$	.5	4			
(38) $R-CO-N < \begin{smallmatrix} CH_3 \\ CH_3 \end{smallmatrix}$	<.5	2			
(39) $R-CO-NH-C_2H_5$	<.5	4			

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(40)	$R-CO-N < \begin{smallmatrix} C_2H_5 \\ C_2H_5 \end{smallmatrix}$	<.5	<.5			
(41)	$R-CO-NH-C_3H_7$ (n)	<.5	1			
(42)	$R-CO-NH-C_3H_7$ (iso)	.5	.5			
(43)	$R-CO-NH-C_3H_9$ (n)	2	1			
(44)	$R-CO-NH-C_4H_9$ (iso)	1	1			
(45)	$R-CO-N < \begin{smallmatrix} C_4H_9 \text{ (iso)} \\ C_4H_9 \text{ (iso)} \end{smallmatrix}$	1	<.5			
(46)	$R-CO-NH-C_4H_9$ (sec)	.5	1			
(47)	$R-CO-NH-C_5H_{11}$ (iso)	1	<.5			
(48)	$R-CO-NH-C_8H_{17}$ (in) *	8	<2	>2	>2	>2
(49)	$R-CO-NH-CH_2-CH=CH_2$	.5	1			
(50)	$R-CO-NH-C_2H_4OH$	1	4			
(51)	$R-CO-N < \begin{smallmatrix} C_2H_4OH \\ C_2H_4OH \end{smallmatrix}$	<.5	1			
(52)	$R-CO-NH-CH_2-\underset{\substack{ \\ OH}}{CH}-CH_3$	<.5	2			
(53)	$R-CO-NH-CH_2-\underset{\substack{ \\ OH}}{CH}-\underset{\substack{ \\ OH}}{CH_2}$	<.5	2			
(54)	$R-CO-NH(CH_2)_6-NH-OC-R$	—	—			
(55)	$R-CO-NH-NH_2$	<.5	<.5			
(56)	$R-CO-NH \cdot NHCOCH_3$	<.5	<.5			
(57)	$R-CO-NH \cdot NHCO$ 	1	4			
(58)	$R-CONH-HNOC-R$	—	—			

Table 5.

V) The other furan derivatives. ($R' =$ )

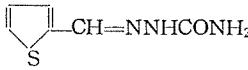
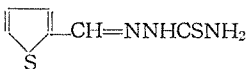
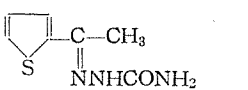
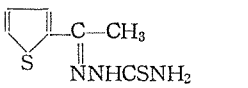
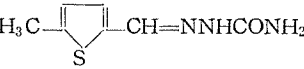
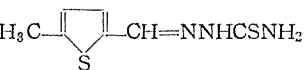
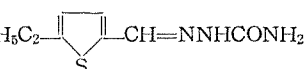
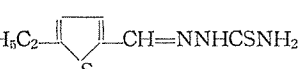
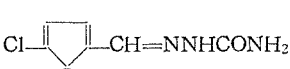
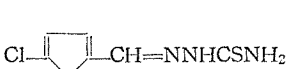
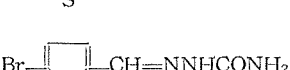
	St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(59) $R'-CH=CHCH_2OH$	<.5	<.5			
(60) $R-CH=CHCH_2OH$	8	16	2	4	
(61) $R'-\underset{\substack{ \\ OH}}{CH}-CH_3$	<.5	<.5			
(62) $R-\underset{\substack{ \\ OH}}{CH}-CH_3$	<.5	1			
(63) $R-CH_2OCOCH_3$	<.5	1			

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(64)	$\text{R}-\text{CH}=\text{CHCOCH}=\text{CH}-\text{R}$	<.5	<.5
(65)	$\text{R}-\underset{\text{OH}}{\text{CH}}-\text{CH}_2-\text{CO}-\text{CH}_2-\underset{\text{OH}}{\text{CH}}-\text{R}$	1	.5
(66)	$\text{R}-\text{COCH}_2\text{COOC}_2\text{H}_5$	<.5	<.5
(67)	$\text{R}'-\underset{\text{OH}}{\text{CH}}-\underset{\text{NH}_2}{\text{CH}}-\text{COOH}$	<.5	<.5
(68)	$\text{R}-\underset{\text{OH}}{\text{CH}}-\underset{\text{NH}_2\cdot\text{HCl}}{\text{CH}}-\text{COOC}_2\text{H}_5$	<.5	<.5

Table 6.

VI) The thiophene compounds.

		St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
(69)		<.5	<.5			
(70)		.5	<.5			
(71)		<.5	<.5			
(72)		.5	<.5			
(73)		<.5	<.5			
(74)		2	<.5			
(75)		* .5	<.5			
(76)		1	.5			
(77)		<.5	<.5			
(78)		* 2	<.5			
(79)		* <.5	<.5			

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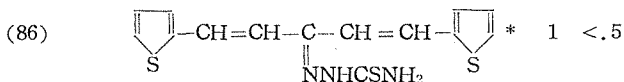
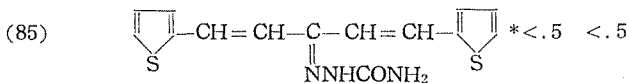
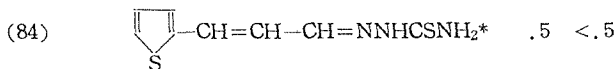
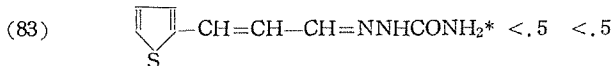
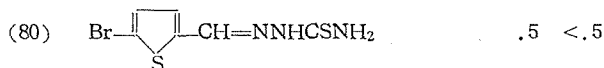


Table 7

		St. aureus	E. coli	S. typhi	Sh. dysent.	Sh. paradysent. Komagome B 1
K-1	$R-CH=N-NH-CO-NH_2$	14	14	10	8	8
K-2	$R-CH=N-NH-C \begin{matrix} \nearrow NH_2 \\ \searrow NH \cdot HCl \end{matrix}$	8	4	4	6	6

DISCUSSION AND SUMMARY

The investigation of the antibacterial activity of these 68 compounds of the 6 series among nitrofurans shows that 2-(5-Nitro)-furylacrolein compounds have a striking antibacterial action as H. Saikachi and others⁽⁴⁾⁽⁵⁾⁽¹⁴⁾⁽¹⁵⁾ have reported previously. These are only slightly soluble in water as the previously synthesized nitrofurans derivatives.

Comparison between 2-(5-Nitro)-furyl acrylic amides and -furoic amides shows that the former are more effective than the latter against *St. aureus* and *E. coli*. There is a tendency that the compounds which have less than 3 carbon atoms, i. e., ethyl, methyl and propyl, are more potent, and more so if the configuration is normal rather than an iso-type. This is seen in (11), (13), (15) and (28). The mono-amides seem to be more potent than the diamides. Comparison between (30) and (36) which has been tried by the addition of one or two moieties of OH to (15) to increase their solubilities in water, shows that compounds which are not combined with OH has excellent

antibacterial action.

It is of interest that (26) and (48) inhibit the growth of *St. aureus* at 1 : 80,000 and 1 : 160,000 although it does not show activity against other bacteria.

The antibacterial action of thiophene derivatives which are similar heterocyclic compounds to those of furan derivatives were low.

CONCLUSION

The results of the investigation concerning the antibacterial activity of 68 nitrofuran derivatives of the 2-(5-Nitro)-furfurylidene series, furylacrolein series, furyl acrylic amideseries, -furoic amide series, other furan derivatives and thiophene derivatives using the serial dilution method of broth are as follows.

1. Several derivatives which have more excellent antibacterial action than furacin were discovered in the furylacrolein and furyl acrylic amide series.
2. In comparison of furyl acrylic amides (25 compounds) with furoic amides (23 compounds) the former were shown to be superior in antibacterial activity.
3. Among the furyl acrylic amide compounds, the following showed excellent antibacterial activity.
 - a. Amides which have less than 3 carbon atoms, i. e., ethyl, methyl and propyl,
 - b. Amides of normal-type.
 - c. Mono-amides.
 - d. Those without OH moieties.
4. The thiophene derivatives had no antibacterial activity.

II. The Sensitive, Resistant or Non-sensitive Organisms in Relation to the Nitrofuran Derivatives

It is of importance to investigate the mode of action of antibacterial substances, i. e., the bacteriostatic, bacteriocidal or bacteriolytic action, and to study the role that the concentration of the reagent plays and also to inquire into the relationship of the bacterial amount to the antibacterial activity and to study the antibacterial action itself of these agents. The mode of action and its application to medical treatment must be studied as well.

The possibility of inhibition of bacterial growth due to various antibacterial substances was discussed and studied by P. Fildes⁵⁹⁾, S. A. Waksman⁶⁰⁾, H. Tamiya³⁹⁾ and A. Goldstein⁶¹⁾. The antibacterial mode and mechanism of the nitrofuran derivatives were also studied by many investigators. However, these observations were mainly concerned with the response of various organisms to the agents, i. e., the growth inhibition or abnormal metabolic processes of bacteria due to the presence of the reagent,

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or the enzymological study of resistant organisms, etc. As for the fate of the reagent caused by living organisms there are only reports by R.C. Bender⁴⁸⁾, H. E. Paul^{46) 47)}, and H. Tsukamoto who studied *in vivo*, in organs and in tissues.

It is known that among nitrofuran derivatives those which are readily reduced usually possess an antibacterial action. However, further investigation will be necessary to determine whether the "reduction of the nitro radical" is primarily or secondarily related to the antibacterial activity, i. e., whether the nitro radical acts antibacterially in the process of reduction or acts as a nitro radical itself and is then reduced secondarily.

The author investigated the effects of the nitrofuran derivatives on the bacterial growth with the turbidimetric method and also studied the fate and effect of the 5-position nitro radical, which is an antibacterial body, on the bacterial metabolism or multiplication with the ultraviolet absorption spectrum or polarogram.

A. MODE OF ANTIBACTERIAL ACTION

The application of turbidimetry on the observation of the mode of action of various antibacterial substances on bacterial growth was first devised by J.W. Foster^{62) 63)}. This method was tested and improved by S.W. Lee et al⁶⁴⁾, J.R. Mahan⁶⁵⁾, D.A. Joslyn and M. Goldbraith^{67) 68)} as a technique for the microbiological assay of the antibacterial substances as well as the serial dilution method. In addition, H. Tamiya et al.,³⁹⁾ T. Yanagida⁶⁶⁾ and others classified similar modes of action according to the changes of the growth curve due to the addition of a variety of agents as well as the above mentioned quantitative experiments. These experiments on the nitrofuran derivatives were partially studied by D.L. Cramer³⁷⁾, K. Shibata³¹⁾, H. Tamiya³⁹⁾ and others. In order to promote a further systematic study on the mode of action on bacterial growth, the author carried an experiment using synthetic media and aeration culture.

EXPERIMENTAL METHODS

In order to observe the inhibition of bacterial growth due to various reagents and the growth curves by using the turbidimetric method, the shaking, agitation and aeration culture methods were devised.

In order to fulfil the following requisites:

1. to promote the bacterial growth,
2. to equalize the turbidity of the media due to bacterial growth,
3. to simplify the aseptic procedure to remove the materials,
4. to avoid colours in the media,

the special flask (Fig. 1) was devised and incubated at 37°C. using aeration technique with a synthetic media. (Table 8)

The procedure with this flask was as follows. The air for the aeration was passed

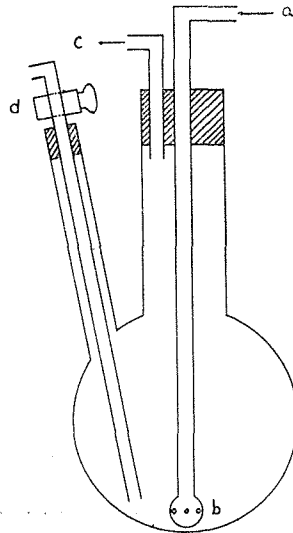


Fig. 1. Culture flask.

Table 8.

Na_2HPO_4	2.5 g	KH_2PO_4	0.35 g
$(\text{NH}_4)_2\text{SO}_4$	4.5 g	NH_4Cl	0.5 g
FeSO_4	0.01 g	MgSO_4	0.01 g
Glucose	2.0 g	Water	1,000 ml

twice through 500 ml of 0.1 % sublimate solution and then through 5 cm of sterilized cotton and then through (b) bubbled into the medium at approximately 300 ml per min. The air was then led out through (c). The valve (d) was used to withdraw material. When the material was taken out the valves were kept in negative pressure as compared with the inside of the flask to prevent bacterial contamination.

The bacterial suspensions were taken out and the turbidity was measured by a photoelectric colorimeter (AKA 5B Photoelectric colorimeter, Filter B, Antimony photoelectric bulb).

2-(5-Nitro)-furfural semicarbazone was used as the test reagent, *E. coli* as the sensitive organism and *Pseudomonas pyocyanea* as the non-sensitive organism.

RESULTS

1. The Sensitive Organism (Fig. 2)

(a) The reagent was added during the lag phase. In the culture of *E. coli*, in the media containing the reagent at the concentrations of 1 : 250,000 and 1 : 200,000, a bacterial growth was recognized at 2.5 hr after inoculation in the control, at 4 hr in the 1 : 250,000 and at 5 hr in the 1 : 200,000 media. The initial growth was delay-

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ed 1.5 hr and 2.5 hr respectively. However, the rate of growth did not largely change during the logarithmic phase as compared with that of the control.

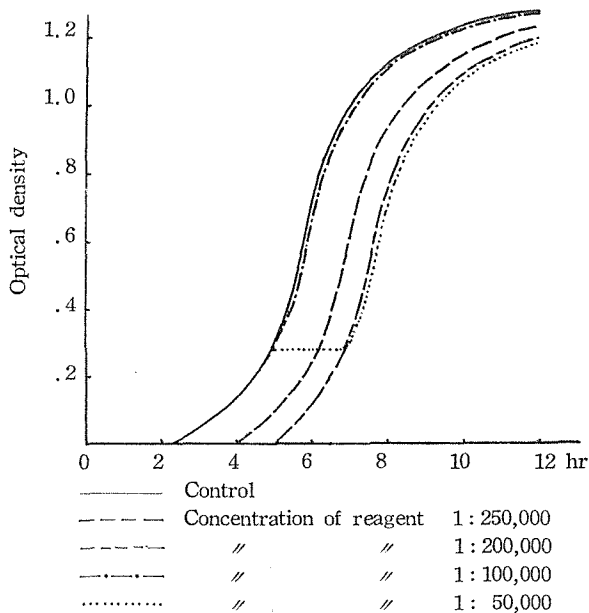


Fig. 2. Growth curves of *E. coli* in 2-(5-Nitro)-furfural semicarbazone.

(b) The reagent was added during the logarithmic phase. After five hour-growth in a reagent-free media growth was stopped temporarily by the addition of reagents of 1 : 100,000 and 1 : 50,000. Growth recommenced after 2 hr in the 1 : 50,000, but was merely delayed 30 min in the 1 : 100,000 and then grew similarly to the control in both media.

2. The Non-sensitive Organism (Fig. 3)

(a) The reagent was added during the lag phase. In the culture of *Ps. pyocyanea* in the medium containing a 1 : 100,000 concentration of the reagent the initial growth was delayed about 30 min in comparison with that of the control. But the growth in the logarithmic phase was similar to that of the control.

(b) The reagent added during the logarithmic phase. The addition of the reagent was made after the culture grew uninhibitedly for 6 hr in a reagent-free medium. After introduction of the reagent in the concentration of 1 : 50,000 a slight inhibition occurred but after 1.5 hr the bacteria grew normally at a rate equal to that of the control.

B. SPECTROPHOTOMETRICAL OBSERVATION

The fate of the nitrofuran derivatives in vivo has been investigated by H. E. Paul *et al.*^{(46) (48) (49)} and others by measuring the ultraviolet absorption spectra. The

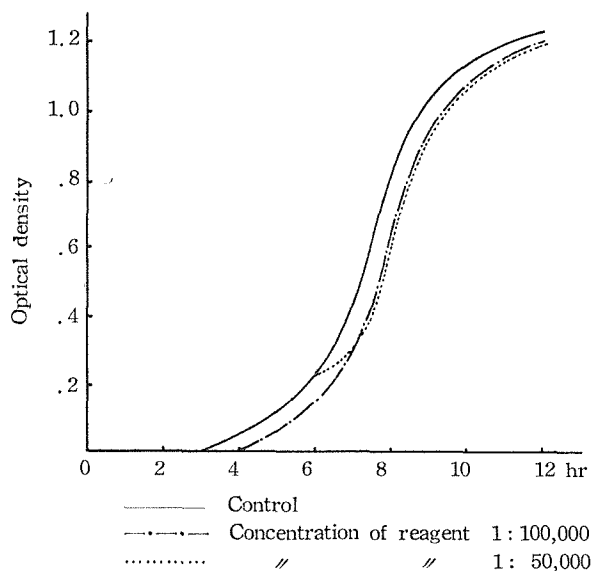


Fig. 3. Growth curves of *Ps. pyocyanea* in 2-(5-Nitro)-furfural semicarbazone.

ultraviolet absorption spectra of 2-(5-Nitro)-furfural semicarbazone and 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride have their absorption peak, due to the 5-position nitro radical, at 375 $m\mu$ and 368 $m\mu$ respectively. The fate of the nitro radical in the case of suspensions or cultures of the sensitive, resistant or non-sensitive organisms were studied with these absorption spectra.

1. The Case of Bacterial Suspension

The sensitive, resistant or non-sensitive organisms was suspended in reagents containing buffer solutions and was incubated for a proportional time and then the fate of the nitro radical was studied.

EXPERIMENTAL METHODS

St. aureus, *E. coli*, *Bacillus subtilis* (PCI 219), *S. typhi* and *Sh. dysenteriae* were used as the sensitive organisms and *Ps. pyocyanea* as the non-sensitive, and the resistant strains of *St. aureus* which had the resistance 16 times the original strain, and *E. coli* which had 20 times the original resistance to 2-(5-Nitro)-furfural semicarbazone was used as the resistant organisms.

The nitrofurran derivatives used were 2-(5-Nitro)-furfural semicarbazone and 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride. The optimal concentration of the reagent in its antibacterial activity and ultraviolet absorption intensity was 1:50,000. In order to harvest a large quantity of organisms the bacteria were cultured on agar in Kolle's flasks and incubated for 18 to 24 hr and gathered and thoroughly washed three times with Sørensen's phosphate buffer solution (pH 7.2) and centrifuged at 3,000

r.p.m. for 20 min. The bacteria were measured and suspended in the phosphate buffer only or in the reagent containing phosphate buffer. The washed suspension was then incubated at 37°C. for a definite time. In order to equalize the conditions approximately, each 5–10 ml was taken out from the bacterial samples at definite intervals and was completely freed of organisms by centrifuging at 3,000 r.p.m. for 25–30 min. After the supernant became clear they were held in ice-box and then their absorption spectra were studied. The absorption spectrum was measured in the range of the ultraviolet, *i.e.*, from 250–400 $m\mu$, using Beckman Model DU photoelectric quartz spectrophotometer.

RESULTS

When 2-(5-Nitro)-furfural semicarbazone or 2-(5-Nitro)-furfurylidene aminoguanidine hydrochloride was dissolved in Sørensen's phosphate buffer solution, in the concentration of 1 : 50,000, the maximum spectral absorption due to the nitro radical was at 375 $m\mu$ or 368 $m\mu$ respectively. The spectral absorption of the buffer solution alone was not recognizable.

(a) **The case of a suspension containing a relatively large quantity of organisms.** In all cases of the sensitive, resistant or non-sensitive organisms the maximum peaks became low with time. At the same time the peak shifted to the shorter wavelength and the presence of Paul's reduced furacin, which has a maximum peak at 335 $m\mu$, was proved. With further incubation the maximum peak disappeared completely within about one hour. In this phenomenon there was no remarkable difference between different organisms. The rate of the diminution of the peak also was not proportional to the quantity of organisms present.

(b) **The case of a suspension containing a relatively small quantity of organisms.** The experiment was performed similarly to a). The curve was almost the same as that of the control, even after 6 hr. The maximum peak merely shifted down slightly.

Then an intermediate experiment was performed in which there was bacterial suspension of 3 mg per ml.

(c) **The case of a suspension containing 3 mg of organisms per ml.**

i) The sensitive organisms. (Fig. 4)

Within one hour-incubation the peak for *St. aureus* fell remarkably. Passing through the reduced furacin, the curve became flat after 2 hr and the maximum peak vanished. The results for all the previously mentioned sensitive organisms, *i.e.*, *E. coli*, *B. subtilis*, *S. typhi* and *Sh. dysenteriae* were similar to that of *St. aureus*.

ii) The non-sensitive organisms. (Fig. 5)

In the case of *Ps. pyocyanea*, a non-sensitive organisms, a 3 hr-incubation yielded only a small decline of the maximum peak.

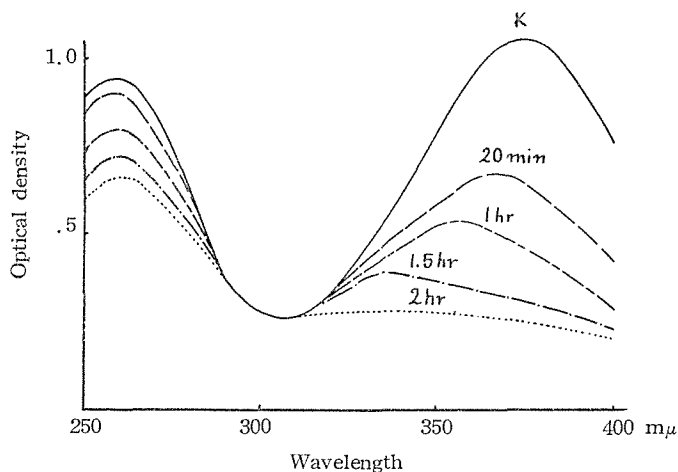


Fig. 4. Ultraviolet absorption curves of incubation of 2-(5-Nitro)-furfural semicarbazone with *St. aureus*.

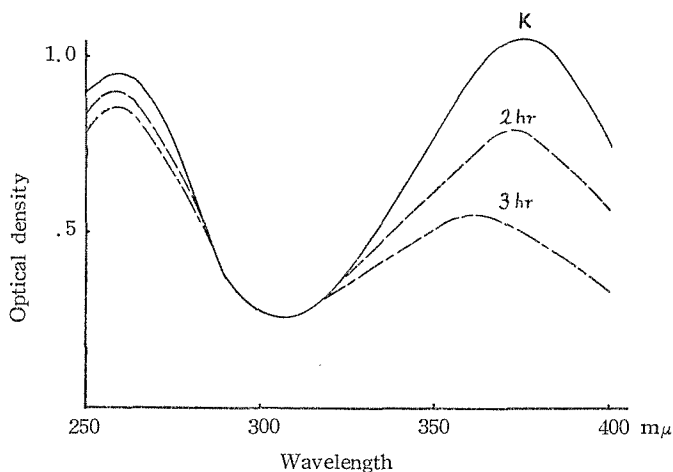


Fig. 5. Ultraviolet absorption curves of incubation of 2-(5-Nitro)-furfural semicarbazone with *Ps. pyocyanea*.

iii) The resistant strains of the sensitive organisms.

In the case of a resistant strain of *St. aureus*, as in the case of non-sensitive ones, even after 4 hr-incubation the change was difficult to distinguish. The experiment with the resistant strain of *E. coli* showed similar results.

(d) **The effect of pH.** This experiment was performed in a manner similar to c), in Sørensen's phosphate buffer solutions which were adjusted to pH 6.5, 7.2 and 8.0. There were no differences due to pH.

(e) **In the anaerobic conditions.** This experiment was performed similarly to c) only under anaerobic conditions using Thunberg's tubes. But nothing remarkable was noted.

(f) **The dead organisms.** In order to clarify the possibility that this phenomenon was only a passive one due to the reagent adhering to the surface of the bacterial cells. The *St. aureus* and *E. coli* were heated at 60°C. for one hour prior to their use and then examined as in c). The maximum peaks in both cases did not fall.

2. The Case of Bacterial Culture

The sensitive or non-sensitive organisms was cultured and the reagent was added in each growth phase. Thus the fate of the nitro radical due to the bacterial multiplication was studied.

EXPERIMENTAL METHODS

E. coli and *B. subtilis* were used as the sensitive organisms and *Ps. pyocyanea* as the non-sensitive one.

The synthetic media used in experiment (II) A) was used, because of its ultra-violet absorption quality. Since broth has an intense spectral absorption in the region of shorter wave lengths (300 $m\mu$) it was found unsuitable even in 1 : 20 dilutions. The cultural methods and experimental procedures were identical with (II) A).

RESULTS

When 2-(5-Nitro)-furfural semicarbazone was dissolved in the above mentioned synthetic media, the maximum peak of the nitro radical was at 375 $m\mu$, as in the case where it was dissolved in Sørensen's phosphate buffer solution. The absorption bands due to the synthetic medium alone was hardly recognizable.

(a) **In the culture of the sensitive organisms.** When the reagent of 1 : 50,000

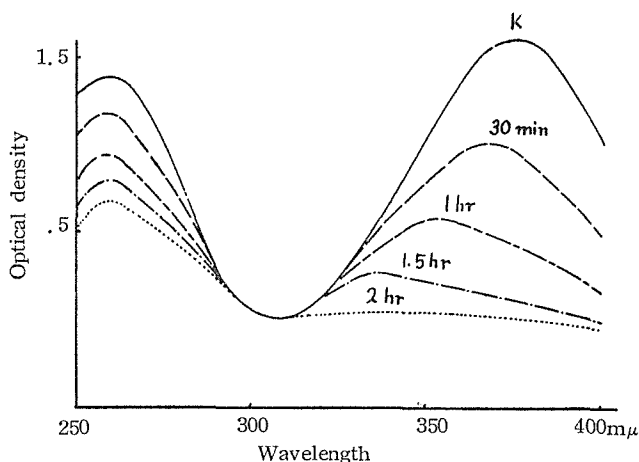


Fig. 6. Ultraviolet absorption curves of 2-(5-Nitro)-furfural semicarbazone after cultivation of *E. coli*.

was added in the logarithmic phase, i.e., after culturing *E. coli* in a reagent-free medium for 5 hr, the growth stopped but recommenced after 2 hr and it began to grow normally again (Fig. 2). During this period, the bacterial samples were removed at intervals of 30 min and examined for changes in the nitro radical using the method in the previous section.

The result was that the maximum peak fell clearly in 30 min and shifted further to the shorter wavelength at the end of one hour. (Fig. 6) The existence of the reduced furacin which has a maximum peak at 335 m μ was once again shown, and 2 hr later the curve became flat and the maximum peak vanished, i.e., the organism did not begin to grow again till the maximum peak had vanished.

An identical experiment with *B. subtilis* showed similar results.

(b) In the culture of non-sensitive organisms.

i) The reagent was added during the lag phase.

When *Ps. pyocyanea* was cultured in a medium containing the reagent at a concentration of 1 : 100,000, the initial growth was delayed approximately one hour longer than the control. During this period, samples were taken out at the instant of cultivation, at 30 min and one hour and examined for the absorption spectrum. Then the vanishing of the nitro radical was proportional through the lag phase into the logarithmic phase to the increase in the bacterial number which accounted for the entrance from the lag phase into the lagarithmic phase.

ii) The reagent was added during the logarithmic phase.

Ps. pyocyanea was cultured in a reagent-free medium for 6 hrs, and reagent was added in the concentration of 1 : 50,000, then the growth stopped temporarily. After 30 min it recommenced at the same rate as the control. (Fig. 3) During the period the absorption spectrum was observed 30 min. Even after one hour the maximum peak

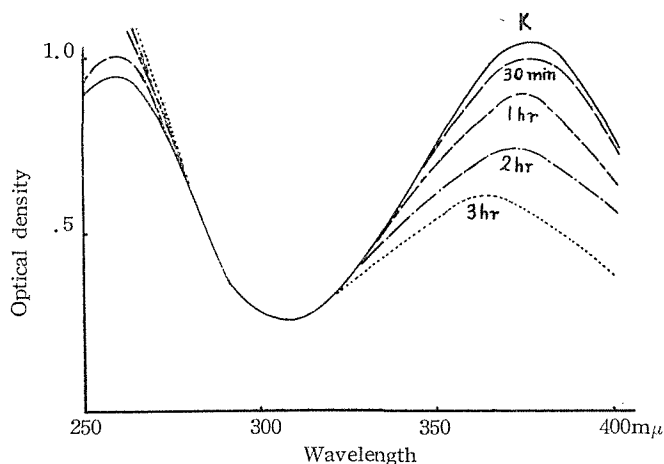


Fig. 7. Ultraviolet absorption curves of 2-(5-Nitro)-furfural semicarbazone after cultivation of *Ps. pyocyanea*.

of the nitro radical declined only slightly. Three hours later the maximum peak diminished only slightly. (Fig. 7)

C. POLAROGRAPHICAL OBSERVATION

D.L. Cramer³⁸⁾ cultured *St. aureus* in media containing 2-(5-Nitro)-furfural semicarbazone and measured the oxidation-reduction potentials. I. Tachi⁴³⁾ and T. Sasaki⁴⁴⁾⁴⁵⁾ investigated analytically the polarograms of a variety of nitrofuran derivatives. Those reports show that the reduction of the nitro radical plays an important role in its antibacterial activity. The author studied the changes of the nitro radical caused by bacteria, in parallel to the original study, the response of sensitive, resistant or non-sensitive organisms to the nitro radical. As the reagent 2-(5-Nitro)-furfural semicarbazone was used at a concentration of 1 : 50,000.

1. The Case of Bacterial Suspension

In a spectrophotometric study of the nitro radical in a suspension of bacteria of 3 mg per ml marked changes were observed in the sensitive, resistant or non-sensitive organisms. The differences were investigated polarographically.

EXPERIMENTAL METHODS

For the test organisms *St. aureus*, *E. coli*, and *B. subtilis* were used as the sensitive organisms, *Ps. pyocyanea* as the non-sensitive, and for the resistant strains those of *St. aureus* and *E. coli* were used which have a resistance to 2-(5-Nitro)-furfural semicarbazone.

The method of preparation was identical with the preceding parts. The bacterial samples were adjusted to pH 7.0-7.2 and were placed in the cells and hydrogen gas was passed for more than 20 min. The measurement was carried out by using the photo-recording polarograph at 20-25°C.

RESULTS

When 2-(5-Nitro)-furfural semicarbazone was dissolved at the concentration of 1 : 50,000 in Sørensen's phosphate buffer solution two reduction waves appeared coincident with I. Tachi and T. Sasaki. The first reduction potential which expresses the nitro radical was -0.347v and the second which represents the semicarbazone side chain appeared at -1.351v.

When *St. aureus*, sensitive organism, was suspended, both waves of the nitro radical and of the semicarbazone declined with time and were completely reduced at 2 hr. (Fig. 8 a) Similarly, when *E. coli* or *B. subtilis* was suspended both reduction waves vanished rapidly.

However, in the case of *Ps. pyocyanea*, non-sensitive organism, and the resistant

strain of *St. aureus* and of *E. coli* neither reduction waves appeared even after 2 hr. (Fig. 8 b)

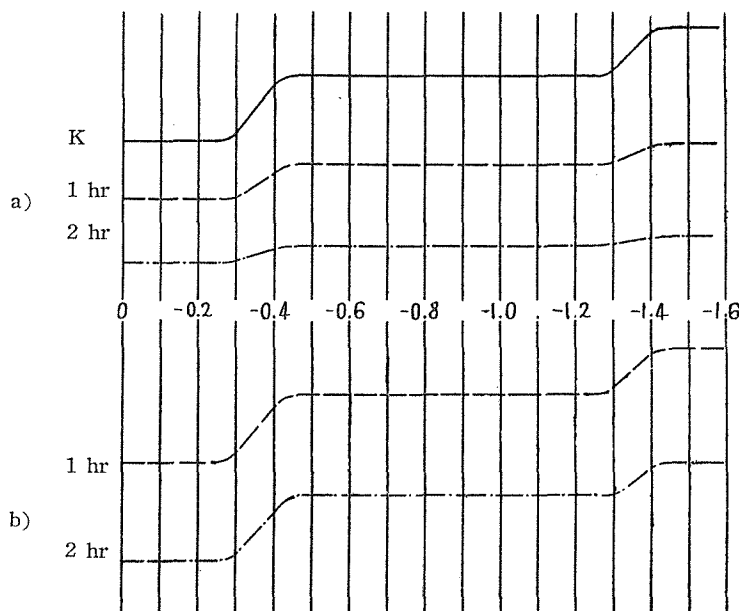


Fig. 8. Polarograms of incubation of 2-(5-Nitro)-furfural semicarbazone with *St. aureus* (a) and *Ps. pyocyanea* (b)

2. The Case of Bacterial Culture

As for the fate of the nitro radical due to bacterial multiplication it has already been established by turbidimetric investigations that the sensitive organisms did not multiply until the nitro radical was reduced but that non-sensitive organisms began to multiply regardless of the state of reduction of the nitro radical. The author has carried out further investigations concerning these fates employing the polarograph.

EXPERIMENTAL METHODS

The experiment was carried out in a manner similar to those in the preceding section. *E. coli* and *B. subtilis*, as the sensitive organisms, and *Ps. pyocyanea*, as the non-sensitive organisms, were cultured in synthetic media, and into which 2-(5-Nitro)-furfural semicarbazone was added in the concentration of 1 : 50,000, during the lag phase or the logarithmic phase. The bacterial samples were taken out for a definite time and polarographic record was taken.

RESULTS

1. The case of the culture of sensitive organisms

E. coli was cultured in reagent-free media with the aeration method as mentioned

above and to it the reagent of 1 : 50,000 dilution was added during the logarithmic phase. By this addition the growth was delayed for 2 hr. (Fig. 2) The organisms then resumed normal growth. The result of the polarographic investigation of the materials, which were removed every 30 min, showed that normal growth did not begin again until the reduction waves due to the nitro radical and semicarbazone had disappeared

The identical experiment with *B. subtilis* gave similar results.

2. The case of the culture of non-sensitive organisms

When *Ps. pyocyanea* was cultured in a similar manner and the reagent was added to it in the concentration of 1 : 50,000 during the lag phase or the logarithmic phase, the reduction waves disappeared according to the bacterial growth in all cases.

DISCUSSION AND SUMMARY

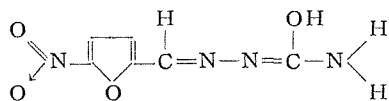
Cultivation of organisms in synthetic media containing 2-(5-Nitro)-furfural semicarbazone shows that the lag phase is prolonged while the logarithmic phase is not. This was also evidenced in reports by D. L. Cramer,³⁷⁾ K. Shibata³¹⁾ and H. Tamiya.³⁹⁾ The lag phase is prolonged in proportion to the concentration of the reagent present in the media. The logarithmic phase is not influenced. After a certain amount of bacteria is reached, in the logarithmic phase, the multiplication ceases for a certain period of time, which is proportional to the concentration of the reagent added, growth is then resumed at the same rate as that of the control. In this case, the later the reagent is added, i.e., larger amount of bacteria present at the time, the shorter becomes the period of delay. The height of the stationary phase of the curve was identical with that of the control and unaffected in both cases. H. Tamiya³⁹⁾ included this mode of action in his type I (Prolongation of the lag phase) which is due to the action of mercuric chloride, mercurochrome and homosulfamine. In contrast, sulfamin preparation exerts no action during the lag phase but does exert an antibacterial action in the logarithmic phase. This indicates a striking difference in the mode of action between these two groups. Even at concentrations at which antibacterial action is not demonstrable by serial dilution of the reagent, the changes in the growth curve suggest an inhibition proportional to the concentration of the reagent. Attention should therefore be given to this fact when antibacterial potency is assayed by the serial dilution method only. This fact is also interesting from the mode of action and from the viewpoints of the development of bacterial resistance.

Spectrophotometric and polarographic investigations indicate that bacterial metabolism reduces the nitro radical, much more so in the sensitive organisms than in the non-sensitive organisms. The resistant strains of the sensitive organisms show similar tendency in reducing the nitro radical to those of the non-sensitive groups.

The experiment using the resting cell shows that these differences become very

definite at the concentration of 3 mg organisms per ml and that there is a proportionality between bacteria and reagent. That this is not attributable to adhesion is clearly shown in the experiment using dead bacteria. Similar results were obtained under both aerobic and anaerobic conditions. The cultivation of bacteria showed that sensitive organisms do not grow till the nitro radical has been reduced and that the non-sensitive organisms grow in proportion to the reduction of the nitro radical. The maximum peak of the nitro radical is not valuable as a quantitative index but is more valuable as a qualitative one as it shifts to the shorter wave length. Reduced furacin which has a maximum peak at $335\text{ m}\mu$ is proved to be a reduction product. This coincides with the results H. E. Paul obtained in his animal tissue experiments. S. Takagi et al. showed the lowering of the maximum peak of 2-(5-Nitro)-furfural semicarbazone by electrolytic reduction but this may be a different reduction process and the production of reduced furacin is not necessarily proved.

Spectrophotometric observations suggest mainly the change in the nitro radical, and the polarographic studies suggest, in addition, that in the semicarbazone, 2-position side chain is also proportionally reduced. It seems that from these facts the chemical nature, especially the electrical characteristics of the 2-position side chain also plays an important role in the manifestation of antibacterial activity as well as the 5-position nitro radical as advocated by C. E. Hoffman and O. Rabin, H. Saikachi and others. As is easily seen from the structural formula of 2-(5-Nitro)-furfural semicarbazone,



the nitro radical is readily oxidized and since it contains the conjugate double bonds, it is easily reduced. From this fact it appears likely that this molecule plays an important part, as an hydrogen-acceptor, in biological oxidative processes. In the presence of 2-(5-Nitro)-furfural semicarbazone the oxidation-reduction system of sensitive organisms is disturbed and they cannot function normally until the reagent has been reduced. Accordingly, it might be postulated that the bacteriocidal ability of the nitrofuran derivatives should be in accordance with the grade of the nitro radical reduction. The growth processes of non-sensitive organisms are not disturbed and bacteria grow normally reducing this agent secondarily.

CONCLUSION

The investigation of the sensitive, resistant or non-sensitive organisms in relation to the nitrofuran derivatives, especially 2-(5-Nitro)-furfural semicarbazone, in vitro, gives the following results.

1. The investigation of the mode of action shows that the reagent prolonged the

lag phase but does not affect the logarithmic phase.

2. The spectrophotometric investigation for the fate of the nitro radical in the metabolism of bacteria, using the resting cell, indicates that there are striking differences between sensitive organisms, their resistant strain or non-sensitive organisms at the concentration of 1 : 50,000 of the reagent, and in a bacterial concentration of 3 mg per ml the sensitive organisms reduces the nitro radical much more rapidly than the others.

3. Spectrophotometric studies revealed that the sensitive organisms do not multiply until all the nitro radicals are reduced whereas the nitro radical is reduced proportionally to the growth by the non-sensitive bacteria.

4. Polarographic investigation on the fate of the nitro radical and the semicarbazone using the resting cell indicates that sensitive organisms rapidly eliminate both reduction waves but its resistant strain, or the non-sensitive organisms do so only slowly.

5. Polarographic investigation on the fate of both the nitro radical and the semicarbazone side chain due to bacterial growth shows that sensitive organisms do not grow until both reduction waves have been eliminated whereas in non-sensitive organisms the reduction waves are eliminated parallel to the bacterial growth.

III. The Mechanism of the Development of Bacterial Resistance

The problem of the development of resistant strains arose in the days of P. Ehrlich but has come to the fore with the extensive use of chemotherapeutics. Recently this problem has come into the spotlight as the interest in and importance of the sulfamin preparations and antibiotics increased, and has been the object of the investigation of many scholars. However, the exact mechanism of the development of the bacterial resistance is still only conjectured. There are many theories concerning it, *i. e.*, the spontaneous mutation theory which was advanced by M. Demerec⁷⁰⁾ and S. E. Luria,⁷¹⁾ the adaptation theory advanced by C. N. Hinshelwood⁷²⁾ and others and combinations of these theories.

Although numerous experiments were conducted concerning the development of bacterial resistance to the nitrofurán derivatives by K. Ikegaki,¹⁷⁾ K. Shibata,³¹⁾ H. E. Paul *et al.*⁷³⁾ and Y. Morimura⁷⁴⁾ these were all descriptive works concerning the development of bacterial resistance and not investigation of the actual mechanism concerned. At first the author carried out experiment designed to either confirm or modify these previous experimental results and then in order to inquire into the mode of the mechanism of bacterial resistance, the resistance of *St. aureus* and *E. coli* was investigated from many standpoints employing 2-(5-Nitro)-furfural semicarbazone.

A. THE RELATIONSHIP OF ANTIBACTERIAL ACTION TO THE QUANTITY OF BACTERIA PRESENT

In the observation of the development of bacterial resistance it is important to

determine the concentration, at which the growth of the original strain is inhibited, to know the limit of selective action by the reagent used as well as in the study of a bacterial resistance. That is to say that the antibacterial activity is related to the ratio of the reagent concentration to the quantity of the bacterial inoculum and there is a striking difference in the antibacterial activity with different amount of inoculums of bacteria. The relationship between a known inoculum and the minimum concentration of nitrofurantoin derivatives for growth inhibition was previously reported by K. Ikegaki. The author investigated further into the relationship of the bacterial amount to the antibacterial activity employing turbidimetry and investigated further growth inhibition in this experiment.

EXPERIMENTAL METHODS

2-(5-Nitro)-furfural semicarbazone was serially diluted from 1:80,000 to 1:600,000 at intervals of 1:20,000 in broth of pH 7.2 and to these diluted media 18 to 20 hr-cultured *St. aureus* or *E. coli* were inoculated which were diluted so that a five per cent broth containing physiological saline contains approximately 10^5 , 10^4 , 10^3 , or 10^2 organisms respectively. After 24 hr-incubation at 37°C. in the normal stationary method these cultures were homogenized and their turbidities were read with a photoelectric colorimeter.

RESULTS

In the case of *St. aureus* the minimum concentration at which complete inhibition of the bacterial growth occurred was 1:100,000 for 10^5 organisms, 1:120,000 for 10^4 , 1:140,000 for 10^3 , 1:180,000 for 10^2 and the minimum concentration for relative inhibition of the bacterial growth was one half of these values.

In the case of *E. coli* the minimum concentration for complete inhibition of the bacterial growth was 1:120,000 for 10^5 organisms, 1:140,000 for 10^4 , 1:160,000 for 10^3 and 1:200,000 for 10^2 , and the minimum concentration for relative inhibition was one half of these values also.

B. THE INHIBITION OF THE SURVIVING ORGANISMS AT VARIOUS CONCENTRATIONS OF REAGENT

Since the bacterial growth has been measured turbidimetrically up to this point the changes recorded are for the total quantity of bacteria present. As the next step the author investigated the distribution of the survivors at different concentrations of reagent in the cases of known quantities of inoculated organisms and also whether the bacterial population was uniformly sensitive to the reagent.

EXPERIMENTAL METHODS

2-(5-Nitro)-furfural semicarbazone was serially diluted from 1:20,000 to 1:200,000

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at intervals of 1:20,000 with 2 % agar media of pH 7.2. Three ml of each concentration were poured into Petri dishes and allowed to solidify. Agar media, of the same concentration with that containing the reagent which had been previously cooled to 48°C. was thoroughly mixed with a dilution of *St. aureus* or *E. coli* which had been so prepared as to contain 10^3 organisms in a 5% broth containing physiological saline. After incubation at 37°C. for 24 or 48 hr the colonies were counted in each dish of the various concentrations and the figure attained was presumed to be the number of the surviving bacteria.

RESULTS

In the case of *St. aureus* in the 24 hr-culture, the number of survived bacteria was nearly equal to that of the control at the concentration of 1:140,000 but only 40 at 1:120,000 and about 20 at 1:100,000. (Fig. 9) However, in the 48 hr-cultures the number increased to 950 at 1:120,000, i. e., it was nearly equal to the control, and

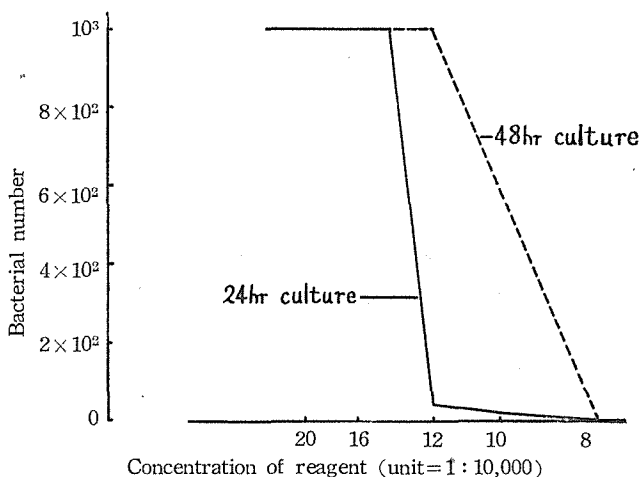


Fig. 9. Survival curves of *St. aureus* in 2-(5-Nitro)-furfural semicarbazone.

about 600 at 1:100,000.

In the case of *E. coli* of the 24 hr-culture the number of survived bacteria was nearly equal to that of the control at 1:140,000 as it was with *St. aureus*, about 300 at 1:120,000 and 170 at 1:100,000. In the 48 hr-culture the number was equal to the control at 1:120,000, 450 at 1:100,000 and 200 even at 1:180,000. (Fig. 10) In the latter the colonies could not be seen at 24 hr with the unaided eye.

C. THE PASSAGE THROUGH MEDIA OF THE INCREASING REAGENT CONCENTRATION FOR THE DEVELOPMENT OF BACTERIAL RESISTANCE

The method was carried out by the repeated transplanting of bacteria on the media of increasing reagent concentrations with many steps in between. This is the most

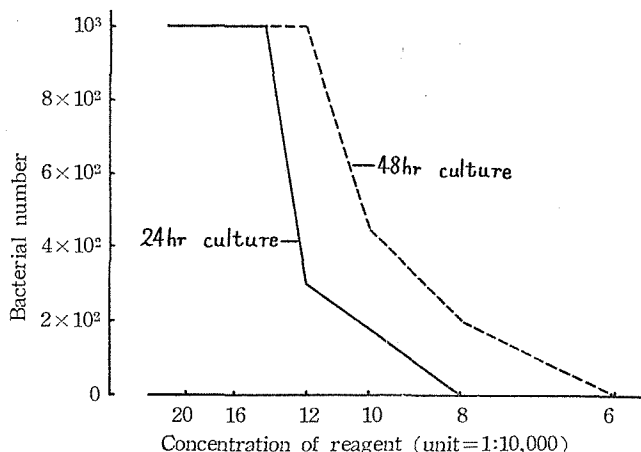


Fig. 10. Survival curves of *E. coli* in 2-(5-Nitro)-furfural semicarbazone.

common method to observe the development of bacterial resistance both with the nitro-furan derivatives and other antibacterial substances. In this method the selective activity of the reagent is strong and the development of spontaneous mutant strains was considered.

EXPERIMENTAL METHODS

2-(5-Nitro)-furfural semicarbazone was made a serial dilution with broth of pH 7.2 as usual and was inoculated with *St. aureus* or *E. coli* and then transplanted from the tube, containing growth somewhat poorer than that of the control of the 24 hr-culture but not inferior to that of the control of the 48 hr-culture, to the tube containing the next higher concentration of the reagent.

RESULTS

In the case of *St. aureus* the resistance increased stepwise to the fifth generation then stopped till the twelfth generation and then increased again. The resistance was fourteen times that of the original strain at the fourteenth generation and twenty times at the sixteenth generation. (Fig. 11 a)

In the case of *E. coli* the resistance increased stepwise to the fourteenth generation at which it was twenty times that of the original strain. (Fig. 11 b)

D. THE PASSAGE THROUGH MEDIA OF THE CONSTANT REAGENT CONCENTRATION FOR THE DEVELOPMENT OF BACTERIAL RESISTANCE

In order to minimize the possible selective action of the reagent the organisms were successively transplanted on media containing reagent of a concentration less than the minimum concentration necessary for relative inhibition of the bacterial growth. The degrees in the development of bacterial resistance were checked by the measurement

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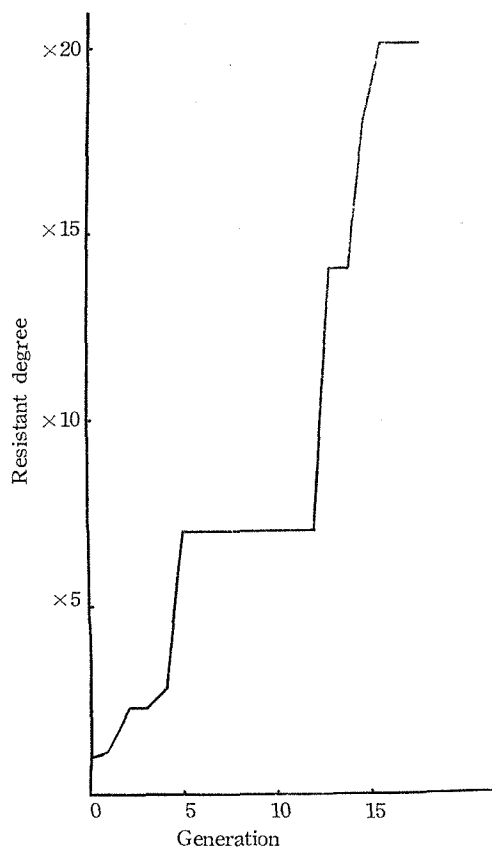


Fig. 11, a. Resistant degree of *St. aureus* to 2-(5-Nitro)-furfural semicarbazone.

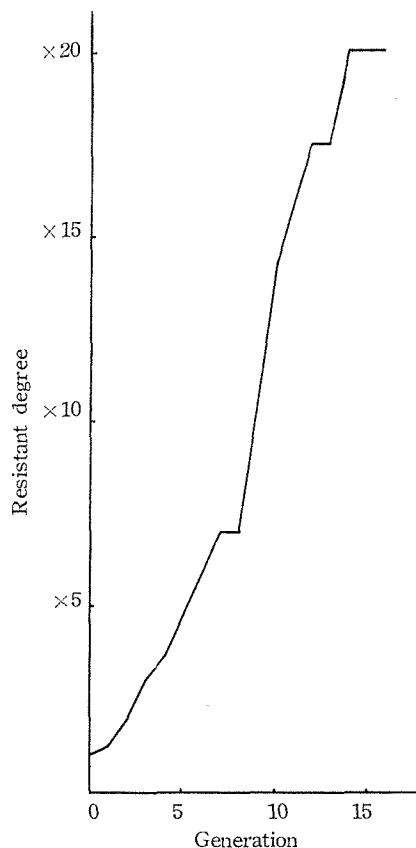


Fig. 11, b. Resistant degree of *E. coli* to 2-(5-Nitro)-furfural semicarbazone.

of the antibacterial activity at each generation.

EXPERIMENTAL METHODS

Since the minimum concentration for the relative inhibition of the growth of *E. coli* with 2-(5-Nitro)-furfural semicarbazone was 1:140,000, the bacteria were transplanted daily into broth of pH 7.2 containing 2-(5-Nitro)-furfural semicarbazone in concentrations of 1:200,000, 1:400,000 and 1:600,000 respectively. The antibacterial activity of the first, fourth, tenth and fifteenth generations was measured by the dilution method. The organisms, which were transplanted successively for twenty generations in the media containing 1:200,000 concentration of reagent, were cultured pouring the culture on reagent containing agar media, which had been previously prepared by a serial dilution, and the number of survived bacteria was counted as in B).

RESULT

By transplanting every day in the 2-(5-Nitro)-furfural semicarbazone-containing

media, the resistance became twice that of the original strain. This occurred at the first generation in the 1 : 200,000 and 1 : 400,00 media and at the fourth generation in the 1 : 600,000. In the seventh generation a resistance of seven times the original was procured in all reagent concentration. (Table 9)

Table 9

Sensitivity of resistant strain of *E. coli* to 2-(5-Nitro)-furfural semicarbazone. (unit=1:10,000)

concentration of reagent	generation : original	I	IV	VII	V	XV
1 : 200,000	14	6	4	2	2	2
1 : 400,000	14	6	4	2	2	2
1 : 600,000	14	8	6	2	2	2

The numbers of survived bacteria in all concentrations after the twentieth transplant were nearly the same, i. e., after incubation for 24 hr in the broth containing the reagent at a concentration of 1 : 200,000, there were 10^3 organisms which were the same as the control at 1 : 100,000, 700 at 1 : 80,000, 400 at 1 : 600,000, and 50 at 1 : 20,000 in contrast to those of the original strain. (Fig. 12)

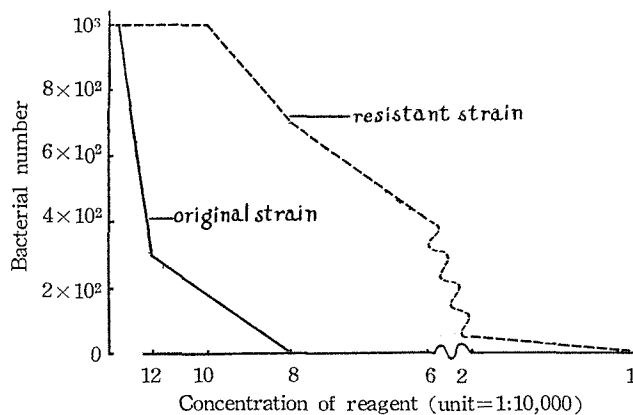


Fig. 12. Survival curves of the original and resistant strain of *E. coli* in 2-(5-Nitro)-furfural semicarbazone.

DISCUSSION AND SUMMARY

It has been shown in the investigations of other antibacterial substances that the antibacterial activity varies according to the quantity of the bacterial inoculum. With the nitrofur derivatives, K. Ikegaki¹⁷⁾ also investigated this phenomenon using 2-(5-Nitro)-furfural semicarbazone. However, in investigating the development of bacterial resistance, it is necessary to know the extent at which the reagent exerts a selective action. The results of the turbidimetric studies show that the growth was inhibited at a concentration more than one half of the minimum concentration necessary for the complete inhibition.

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However, in a study of the survival curve, about 600 out of 10^8 *St. aureus* grew in the reagent of 1 : 100,000 and 200 of *E. coli* grew at a concentration of 1 : 80,000. If the amount of the bacterial inoculum was larger, the creation of more resistant strain might be found. The inclination of the survival curve of *St. aureus*, which is steeper than that of *E. coli*, indicates the uniformity of the sensitivity to 2-(5-Nitro)-furfural semicarbazone. This uniformity is seen in the graph for the passage through media of increasing test reagent concentration in the resistance development experiment. That is, the development of resistance stopped once at the point of seven times the original resistance and then increased again in the case of *St. aureus* and increased rapidly in the case of *E. coli*.

The development of bacterial resistance to 2-(5-Nitro)-furfural semicarbazone is difficult as is generally agreed. The development stops at the resistance of twenty times that of the control in both *St. aureus* and *E. coli*. K. Ikegaki reported many facts concerning adaptation as the mechanism for the development of bacterial resistance to nitrofurane derivatives and stated in conclusion that he could not determine whether the resistance was a matter of adaptation or spontaneous mutation. If the development of resistance was induced by adaptation only a uniformity of reaction to the reagent should be recognizable. But, even in the case in which organisms were successively transplanted on the media of 1 : 600,000, which is one fifth of the minimum concentration for complete inhibition, the resistance became seven times as great as that of the original strain in the seventh generation, although the organisms were selected only in the first generation in the repetitive passage in constant concentrations in contrast to the case of passage through increasing concentrations received in each generation. By mere seven transplants in media of low reagent concentrations as 1 : 600,000 the resistance increased to seven times, and about 700 per 10^8 organisms survived after 24 hr-incubation in media containing reagent at the concentration of 1 : 80,000, in contrast to that of the bacteria of the original strain in which no bacteria out of 10^8 organisms survived.

Thus this experiment gives no new evidence to the choice of two theories, *i. e.*, adaptation or spontaneous mutation.

CONCLUSIONS

The investigation of the development of bacterial resistance of *St. aureus* and *E. coli* to 2-(5-Nitro)-furfural semicarbazone gives the following results.

1. Bacterial growth is inhibited relatively at concentrations as low as one half of the minimum concentration for complete inhibition of bacterial growth.
2. The survival curves for 10^8 organisms show that about 700 *St. aureus* survives at a concentration of 1 : 100,000 and 200 *E. coli* survives at 1 : 80,000 in a 48 hr-culture and that *St. aureus* is much more uniformly sensitive to 2-(5-Nitro)-furfural semi-

carbazon than *E. coli*.

3. *St. aureus* and *E. coli* get resistant as much as twenty times the original strain by the passage through media of increasing test reagent concentrations.

4. By the passage through media in which the test reagent concentration was kept constant *E. coli* became seven times as resistant as the original strain and the survival curve of those which have been transplanted similarly through twenty generations in the media of 1 : 200,000 concentration shows that 50 per 10³ organisms have grown in media of a concentration of 1 : 20,000 in 24 hr.

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REFERENCES

- (1) M. C. Dodd & W. B. Stillman, *J. Pharm. Exp. Therap.*, **82**, 11-18, (1944).
- (2) H. Saikachi, *Jap. J. Pharm. & Chem.*, **20**, 24-27, (1948).
- (3) H. Saikachi *et al.*, *Ibid.*, **21**, 150-159, (1949).
- (4) H. Saikachi *et al.*, *Ibid.*, **22**, 258-260, (1950).
- (5) H. Saikachi *et al.*, *Ibid.*, **23**, 188-190, (1951).
- (6) R. Kimura *et al.*, *Arch. Antibiotics*, **4**, 31-33, (1947).
- (7) R. Kimura *et al.*, *Jap. J. Bact.*, **3**, 132-133, (1948).
- (8) R. Kimura, "The Commemoration Volume for the Silver Jubilee, Inst. for Chem. Research, Kyoto Univ.," 53-62, 1951.
- (9) N. Higashi *et al.*, *Jap. J. Pharm. & Chem.*, **20**, 30-31, (1948).
- (10) D. Mizuno, *Jap. J. Bact.*, **4**, 93-96, (1949).
- (11) Z. Goi, *Arch. Antibiotics*, **2**, 40-42, (1948).
- (12) K. Ikegaki, *Ibid.*, **2**, 442-445, (1949).
- (13) K. Ikegaki, *Ibid.*, **3**, 15-12, (1950).
- (14) K. Ikegaki, *Jap. J. Pharm. & Chem.*, **22**, 148-150, (1950).
- (15) K. Ikegaki, *Ibid.*, **22**, 293-300, (1950).
- (16) K. Ikegaki, *Arch. Antibiotics*, **3**, 179-198, (1951).
- (17) K. Ikegaki, *Ibid.*, **3**, 199-209, (1951).
- (18) K. Ikegaki, *Ibid.*, **3**, 210-214, (1951).
- (19) M. Fujimoto, *Ibid.*, **2**, 42-44, (1948).
- (20) M. Fujimoto, *Ibid.*, **2**, 232-235, (1949).
- (21) H. Iwasaki, *Ibid.*, **3**, 35-50, (1950).
- (22) H. Iwasaki, *Jap. J. Bact.*, **6**, 127-231, (1951).
- (23) H. Iwasaki, *Arch. Antibiotics*, **4**, 53-60, (1951).
- (24) T. Fujino *et al.*, *Med. J. Osaka Univ.*, **4**, 10-12, (1950).
- (25) T. Fujino *et al.*, *Jap. J. Bact.*, **5**, 305-307, (1950).
- (26) K. Shibata, *Arch. Antibiotics*, **4**, 27-31, (1947).
- (27) K. Shibata, *Jap. J. Pharm. & Chem.*, **20**, 32-34, (1948).

Antibacterial Nitrofuran Derivatives

- (28) K. Shibata, *Arch. Antibiotics*, **2**, 152-154, (1948).
- (29) K. Shibata, *Ibid.*, **2**, 155-159, (1948).
- (30) K. Shibata, *Ibid.*, **2**, 240-243, (1949).
- (31) K. Shibata, *Ibid.*, **2**, 300-332, (1949).
- (32) S. Hatta, *Exp. Therapy*, 251, 24-27, (1950).
- (33) S. Hatta, *Tokyo Med. J.*, **67** 16-18, (1950).
- (34) H. Miyamoto, "Nitrofuran derivatives," Igakushoin, Tokyo, 1952.
- (35) S. Kuwabara *et al.*, *Tokyo Med. J.*, **69**, 23-24, (1952).
- (36) Z. Asakura & S. Ogata, *Arch. Antibiotics*, **4**, 252-255, (1951).
- (37) D. L. Cramer & M. C. Dodd, *J. Bact.*, **51**, 293-303, (1946).
- (38) D. L. Cramer, *J. Bact.*, **54**, 119-195, (1947).
- (39) H. Tamiya *et al.*, *Penicillin*, **1**, 257-270, (1947).
- (40) H. Kasuga, *J. Nippon Med. College*, **17**, 534-540, (1950).
- (41) K. Ishigami, *Arch. Antibiotics*, **5**, 1-50, (1952).
- (42) M. N. Green, *Arch. Biochem.*, **19**, 397-406, (1948).
- (43) I. Tachi, *Jap. J. Pharm. & Chem.*, **20**, 38-40, (1948).
- (44) T. Sasaki, *Pharm. Bull.*, **2**, 99-104, (1954).
- (45) T. Sasaki, *Pharm. Bull.*, **2**, 104-108, (1954).
- (46) H. E. Paul *et al.*, *J. Biol. Chem.*, **180**, 345-363, (1949).
- (47) H. E. Paul *et al.*, *Proc. Soc. Exp. Biol. Med.*, **79**, 555-558, (1952).
- (48) R. C. Bender & H. E. Paul, *J. Biol. Chem.*, **191**, 217-222, (1951).
- (49) J. D. Taylor *et al.*, *Ibid.*, **191**, 223-231, (1951).
- (50) I. Yall & M. N. Green, *Proc. Soc. Exp. Biol. Med.*, **79**, 306-308, (1952).
- (51) J. S. Gots *et al.*, *Arch. Biochem.*, **36**, 285-298, (1952).
- (52) S. Toyoshima, *Jap. J. Pharm. & Chem.*, **24**, 616-617, (1952).
- (53) E. Kondo *et al.*, *Ibid.*, **20**, 9-16, (1948).
- (54) R. Kimura *et al.*, *Ibid.*, **21**, 166-167, (1949).
- (55) T. Sasaki, *Ibid.*, **26**, 446-469, (1954).
- (56) T. Sasaki, *Pharm. Bull.*, **2**, 95-98, (1954).
- (57) T. Sasaki, *Ibid.*, **2**, 123-127, (1954).
- (58) K. Suzuki, in press.
- (59) P. Fildes, *Brit. J. Exp. Path.*, **21**, 67-73, (1940).
- (60) S. A. Waksman, "Microbial antagonisms and antibiotics substances," New York, The Commonwealth Fund, 1945.
- (61) A. Goldstein, *New Eng. J. Med.*, **240**, 98-107, 137-147, 180-188, (1949).
- (62) J. W. Foster, *J. Biol. Chem.*, **44**, 285-286, (1942).
- (63) J. W. Foster & B. L. Wilker, *J. Bact.*, **46**, 377-389, (1943).
- (64) S. W. Lee *et al.*, *J. Biol. Chem.*, **152**, 485-486, (1944).
- (65) J. R. McMahn, *Ibid.*, **153**, 246-258, (1944).
- (66) T. Yanagida, *Penicillin*, **1**, 42-44, (1947).
- (67) D. A. Joslyn & M. Galbraith, *J. Bact.*, **54**, 26, (1947).
- (68) D. A. Joslyn & M. Galbraith, *Ibid.*, **59**, 711-716, (1950).
- (69) C. E. Hoffman & O. Rahn, *J. Bact.*, **47**, 177-186, (1944).
- (70) M. Demerec, *Ibid.*, **56**, 63-74, (1948).
- (71) S. E. Luria & M. Delbruck, *Genetics*, **28**, 491-511, (1943).
- (72) C. M. Hinshelwood, "The chemical kinetics of the bacterial cell," New York and London, Oxford Univ. Press, 1946.
- (72) H. E. Paul *et al.*, *Proc. Soc. Exp. Biol. Med.*, **79**, 199-204, (1952).
- (74) Y. Morimura & T. Yanagida, *Jap. J. Pharm. & Pham.*, **24**, 52-62, (1952).