THE MODE OF ACTION OF NITROFURAN COMPOUNDS. I. ACTION VERSUS STAPHYLOCOCCUS AUREUS

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Dodd and Stillman (1944) have reported that the presence of a nitro group in the 5-position of the furan ring confers antibacterial activity on a wide variety of 2-substituted furans. This earlier report demonstrated that the nitro group effect was either bactericidal or bacteriostatic, depending on the concentration present. In the present study the mode of antibacterial action of these compounds was investigated in more detail. The results, as well as comparisons between the action of various compounds and possible effect of structure on the mode of action, are presented by demonstrating graphically the effect of six typical compounds on the population of cultures of *Staphylococcus aureus*. In this instance the method proved especially valuable in demonstrating qualitative as well as previously suggested quantitative differences among the compounds. The results obtained with compounds representing the four chemical types selected by Dodd and Stillman make possible a further differentiation of nitrofuran antibacterial activity on the basis of mode of action. The six compounds used were 2-(5-nitro)-furaldehyde semicarbazone, 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate.

EXPERIMENTAL

The organism chosen for test purposes was *Staphylococcus aureus* Smith, obtained through the courtesy of Dr. H. J. Robinson. This organism was a rapidly growing, coagulase-positive strain. The coagulase-positive characteristic is important, since Spink (1942) has shown that coagulase-negative strains are susceptible to the antibacterial action of normal blood. We have confirmed this observation with a variety of strains of *Staphylococcus aureus*. Since it was our intention to study growth in blood, it was necessary that the test organism be resistant to the normal antibacterial activity of the blood in order to obtain a constant control and to separate the antibacterial effects induced by added nitrofurans from those encountered in the blood samples. A culture was grown by inoculating a loopful of organisms from a blood agar slant of the latest mouse passage culture into 10 ml of brain heart infusion broth containing 10 per cent of defibrinated rabbit's blood; this liquid culture was then incubated for 6 hours at 37.5 C following which it was kept at 4 C overnight. On the day of use the culture thus stored was diluted with physiological saline so as to obtain a concentration of 200,000 to 400,000 organisms per ml. The test material was inoculated with 0.1 ml of this suspension.

The antagonistic action of the nitrofurans upon the growth of this organism was studied in beef infusion broth and citrated rabbit blood. Two members of

the series were subsequently examined in rabbit serum as a test medium. The compounds were dissolved in the sterile test medium in such concentrations as were estimated from the values reported by Dodd and Stillman (1944) to give maximum, intermediate, and minimum antibacterial action within 24 hours. Five-ml portions of such solutions were pipetted into sterile pyrex tubes (100 by 12 mm) and inoculated. After obtaining a sample of 0.1 ml for the initial count of viable organisms, we placed the tubes in the incubator at 37.5 C; tests carried out in whole blood were rotated mechanically at a speed of 6 rpm throughout the test period to insure thorough mixing of cells. A sample of 0.1 ml was withdrawn at 2-hour intervals for a count of viable organisms. Counts of 48-hour samples were usually made as a check on the sterility of tubes containing the maximum concentration of the drug, but for all other concentrations observations made during the first 24 hours only are considered significant. All plates were poured with nutrient agar and incubated 24 hours before the colonies were counted.

RESULTS

Experimental data for the compounds investigated appear in the following tables. Two concentrations for each of the compounds are considered of special significance: first, the minimal concentration to give the maximal antibacterial effect, namely, a logarithmic killing effect resulting ultimately in sterilization; second, the minimal concentration to give any demonstrable effect on the growth of the organism.

The various effects of different nitrofurans upon the growth curve of the test organism, from the data in the tables, may be conveniently diagrammed as in figure 1, showing the types of curves obtained.

The curve of type V is a killing curve, showing that, under the proper conditions, these compounds may effect a sterilization of the medium. The other curves shown in figure 1 represent variations of the curve of the change in population in control cultures, which is shown as type I. We have purposely limited our consideration to the first two phases of bacterial growth, which consist of a short lag period followed by reproduction at a rate such that the population increases logarithmically. Three distinct types of growth curves different from the control, that is, type I, are shown in figure 1: type II, with growth at a reduced rate; type III, with an initial effect of decrease in population, followed by a reduced rate of growth; type IV, showing an abnormally long lag phase, followed by reproduction at a control rate. Table 7 lists the minimal concentration in a given medium at which the typical effect occurs.

The results obtained using broth as a test medium show that four compounds, namely, 2-(5-nitro)-furaldehyde semicarbazone, 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, and 2-(5-nitro)-furfuryl propionate exhibit the property of sterilizing the medium. These data form the simplest case, and the results appear to be a function of concentration only. Propyl-2-(5-nitro)-furoate and ethyl-β-2-(5-nitro)-furacrylate failed to exhibit the sterilizing effect at the limit of solubility. The killing curves of type V (table 7 and figure 1) are of the same

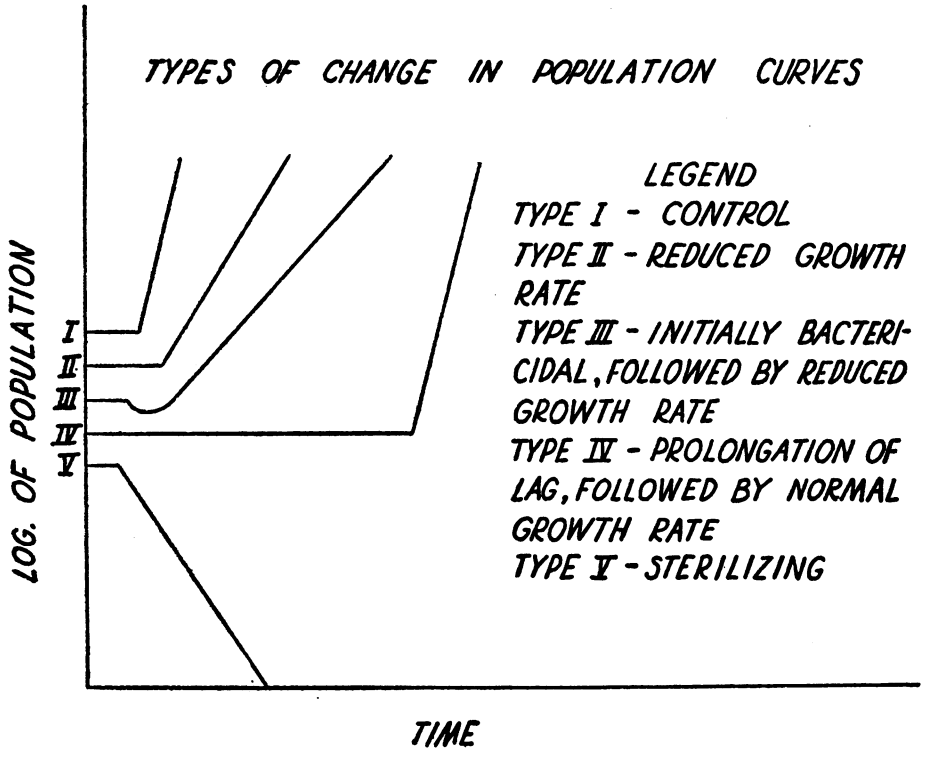
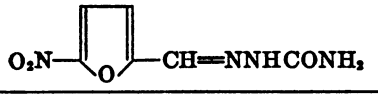


FIG. 1

TABLE 1

2-(5-Nitro)-furaldehyde semicarbazone



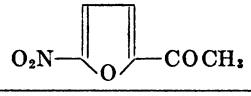
MEDIUM	DRUG CONCENTRATION	PLATE COUNT: Logarithm of the number of organisms per ml at indicated hour									
		0	2	4	6	8	10	12	14	16	24
Broth.....	1:200,000	4.53	4.69	4.75	4.46	4.08	<3.00	<3.00			<1.00
	1:250,000	4.79		5.00	4.70	5.00	4.90	4.75	4.64		4.90
	1:300,000	4.75		5.72	6.45	7.50	8.04	8.51	8.69		
	Control	4.78	5.38	6.88	8.38	9.18		9.55			9.55
Serum....	1:100,000	4.63	4.62	4.48	4.55	4.60	4.52	4.50			<1.00
	1:150,000	4.52	4.58	4.56	4.58	4.75	4.70	4.30			8.69
	1:200,000	4.48	4.56	4.60	4.56	5.98	6.54	6.78			8.11
	Control	4.52	4.63	6.18	6.60	7.34	8.15	8.68			8.65
Blood.....	1:50,000	4.41	4.38	4.40	4.18	3.78	3.74	3.23			<1.00
	1:75,000	4.49	4.48	4.40		4.23		4.30	4.74	5.15	8.44
	1:100,000	4.48	4.30	4.41	4.40	4.60		6.54			8.60
	Control	4.18	4.15	4.18	5.65	7.08					8.44

general type regardless of difference in the chemical structure of the compounds. Hence, we may consider the bactericidal properties conferred on the furan ring by the 5-nitro group as a common function where the effect of the 2-substitution has not reduced the solubility in the medium to a limiting value.

Table 7 also shows that a more complex case, in which both concentration and medium are involved, must be considered. The sterilizing properties of 2-(5-nitro)-furaldehyde semicarbazone and 2-(5-nitro)-furfuryl propionate are

TABLE 2

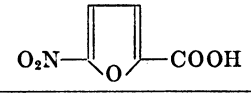
2-(5-Nitro)-furyl methyl ketone



MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:50,000	4.70	4.61	4.34	4.15	3.86	2.30	<1.00
	1:300,000	4.63	4.60	4.34	4.78	5.00	7.30	
	Control	4.66	5.15	6.62	7.62	8.57	9.64	8.73
Blood.....	1:50,000	4.60	4.63	4.53	5.25	5.50	7.76	
	Control	4.54	4.52	5.30	6.40	7.00	7.91	

TABLE 3

2-(5-Nitro)-furoic acid



MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:5,000	4.61	4.61	4.53	4.57	4.76	2.78	<1.00
	1:10,000	4.60	4.82	5.08	5.48	5.30	6.53	8.00
	Control	4.69	4.77	4.91	5.81	6.15	9.20	6.00
Blood.....	1:5,000	4.62	4.62	4.87	5.76	6.48	6.48	
	Control	4.66	5.52	5.52	6.15	7.45	8.75	

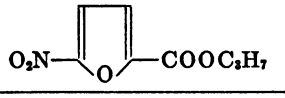
maintained in both serum and blood. With these compounds the medium used for testing has modified the concentration necessary for bactericidal activity. In the case of semicarbazone, the activity is depressed by serum, more noticeably depressed by whole blood. The order of activity for 2-(5-nitro)-furfuryl propionate is the reverse of the semicarbazone. The use of blood as a medium for testing 2-(5-nitro)-furyl methyl ketone and 2-(5-nitro)-furoic acid abolished the bactericidal properties found in broth. This suggests that if nitrofurans were

examined for bactericidal properties in a simple, chemically defined medium, it might be found that the function is common to all members when sufficient solubility is maintained.

The simplest bacteriostatic activity is exhibited by 2-(5-nitro)-furoic acid, which shows only type II activity (table 7), that is, a reduction in the rate of growth. This effect depends upon both concentration and medium, since it was observed that the activity was considerably less in blood than in broth, and this

TABLE 4

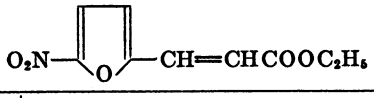
Propyl-2-(5-nitro)-furoate



MEDIUM	DRUG CONCENTRATION	PLATE COUNT: Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:40,000	4.60	4.62	4.64	4.74	4.77	3.70	6.45
	1:100,000	4.56	4.90	5.15	5.57	5.78	8.30	
	Control	4.62	5.04	6.72		8.81	8.30	8.00
Blood.....	1:10,000	4.79	4.78	6.04	6.84	7.23	8.54	
	Control	4.82	4.82	6.20	7.11	7.94	8.87	

TABLE 5

Ethyl-β-2-(5-nitro)-furacrylate



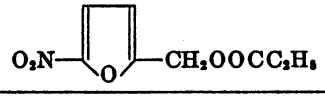
MEDIUM	DRUG CONCENTRATION	PLATE COUNT: Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:30,000	4.74	4.62	4.57	4.58	4.69	4.15	9.08
	1:100,000	4.59	4.74	5.15	5.71	6.14	9.26	
	Control	4.62	5.04	6.72	8.00	9.35	9.53	8.66
Blood.....	1:20,000	4.83	4.91	5.55	6.15	6.75	7.34	
	Control	4.76	4.89	5.60	6.63	7.70	8.68	

growth reduction is the only type of bacteriostasis shown by the acid. In low concentrations 2-(5-nitro)-furyl methyl ketone, propyl-2-(5-nitro)-furoate, and ethyl-β-2-(5-nitro)-furacrylate also exhibit this simple bacteriostatic action of reducing growth rate. In addition to concentration, the medium used is a factor.

A combination of an initial killing effect followed by a decreased growth rate, resulting in an ultimate bacteriostatic effect, shown in figure 1 as type III, is achieved in broth with 2-(5-nitro)-furyl methyl ketone, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate.

TABLE 6

2-(5-Nitro)-furfuryl propionate

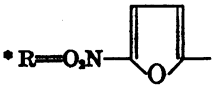


MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:5,000	4.60	3.34	3.00	2.68	2.30	<1.00	<1.00
	1:10,000	4.58	3.92	3.99	3.92	3.91	4.69	8.93
	1:20,000	4.61	4.57	4.48	4.90	5.08	7.30	8.90
	Control	4.66	5.15	6.62	7.62	8.57	9.64	8.73
Serum.....	1:10,000	3.78	3.00	3.30	3.00	2.78	1.60	<1.00
	1:40,000	4.37	4.46	3.08	2.80	2.20	5.78	8.50
	1:80,000	4.35	4.04	5.49	6.78	7.65	8.15	8.00
	Control	4.51	4.36	6.04	7.43	8.42	9.28	9.28
Blood.....	1:40,000	4.38	3.63	2.60	2.20	1.48	<1.00	
	1:80,000	4.36	4.38	4.43	5.08	5.68	8.30	
	1:100,000	4.36	4.25	5.18	5.78	6.78	8.18	
	Control	4.38	4.25	5.23	5.90	6.80	8.38	

TABLE 7

The effect of varying concentration and medium upon the population curves

COMPOUNDS*	TYPE OF EFFECT			
	II	III	IV	V
R—CH=NNH-CONH$_2$.....			Broth 1:300,000 Serum 1:200,000 Blood 1:100,000	Broth 1:200,000 Serum 1:100,000 Blood 1:50,000
R—COCH$_3$.....	Blood 1:50,000	Broth 1:300,000		Broth 1:50,000
R—COOH.....	Broth 1:10,000 Blood 1:5,000			Broth 1:5,000
R—COOC$_2$H$_7$....	Broth 1:100,000 Blood 1:10,000	Broth 1:40,000		
RCH=CHCO-OC$_2$H$_5$.....	Broth 1:100,000 Blood 1:20,000	Broth 1:30,000		
RCH$_2$OOCC$_2$H$_5$..		Broth 1:20,000 Serum 1:80,000 Blood 1:100,000		Broth 1:5,000 Serum 1:10,000 Blood 1:40,000

* R= 

This may be a concentration effect and represent a transition from type II to type V activity for the ketone and the propionate, since at higher concentrations

these compounds exhibit a sterilizing effect. On the other hand, if both reproduction and killing were being effected simultaneously, with only a small proportion of each generation being killed, then the population curve would show an initial drop, followed by an increase resembling growth at a reduced rate. Similar factors in the mode of action of penicillin were discussed by Lee, Foley, and Epstein (1944). This type III effect is also influenced by the medium. None of the compounds exhibited the phenomenon in blood, with the exception

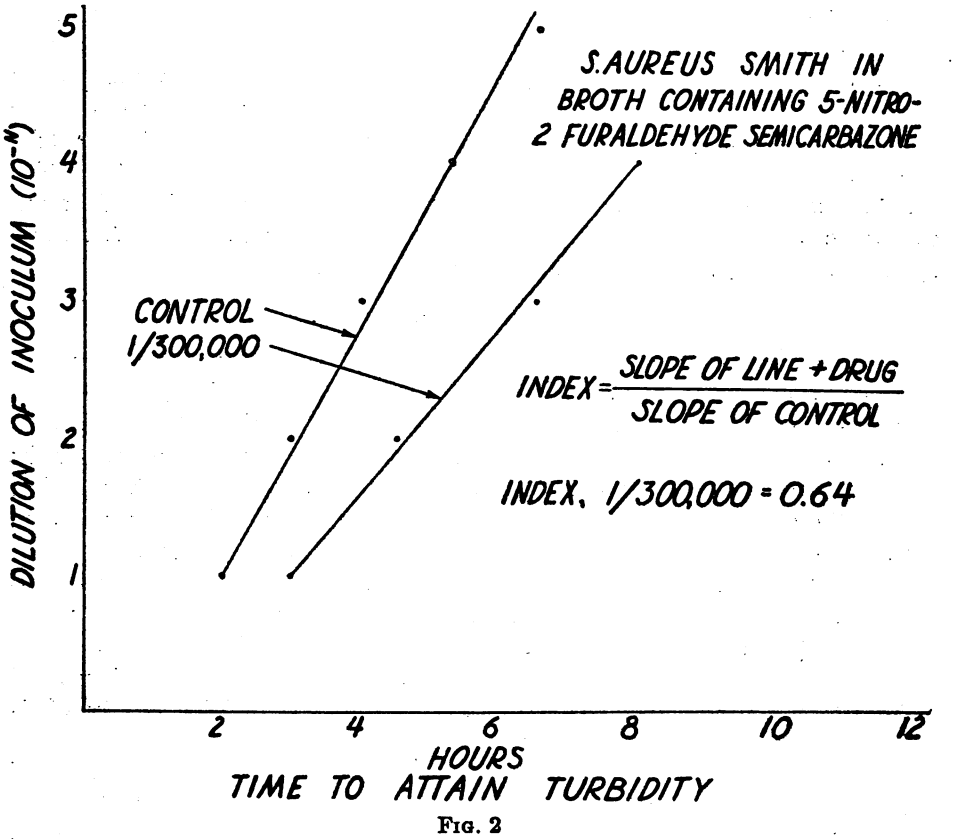


FIG. 2

of 2-(5-nitro)-furfuryl propionate. With this compound the activity is greater in serum and in blood, in the order named.

The compound 2-(5-nitro)-furaldehyde semicarbazone exerts an entirely different mechanism of bacteriostatic action. With bacteriostatic concentrations of this drug, the growth curve (figure 1, type IV) shows an abnormal prolongation of the lag phase followed by reproduction at the control rate. We have tested this effect in three separate media: broth, serum, and blood. The same mechanism holds, regardless of the medium. However, the effectiveness of this semicarbazone is reduced by both serum and blood, the latter exhibiting the greater antagonistic effect.

We have also determined the type of growth curve obtained with bacteriostatic concentrations of this compound greater than the minimal bacteriostatic concentration but less than the amount necessary to kill, in any given medium. These increased concentrations do not affect the mode of action other than to prolong the time that the organism will remain in the lag phase.

A similar phenomenon in the bacteriostatic action of crystal violet has previously been shown by Dubos (1929), Ingraham (1933), and Hoffman and Rahn (1944). We have applied the test described by Ingraham (1933). A series of tubes each containing the same given concentration of the compound in nutrient broth is inoculated with a tenfold dilution series of the test organism. If the rate of growth is undisturbed, but the lag phase prolonged, the time for each successive tube to reach just visible turbidity when plotted against the dilution number of that tube, and hence the dilution of the inoculum, should give a straight line. In this manner the length of the lag phase is made the controlling factor. The results from a representative experiment using 2-(5-nitro)-furaldehyde semicarbazone at a concentration of 1:300,000 in broth, inoculated with *S. aureus*, appear as figure 2; the straight-line relationship is obvious.

DISCUSSION

Within a group of six widely different chemical compounds of the nitrofuran class, it has been shown that the 5-nitro group may confer killing properties to four of the compounds. In broth the chemical nature of the 2-position substitution in the compounds seemed to bear only a quantitative relationship to the activity. The property is therefore a function of the concentration and is apparently absent in the other two compounds because the concentration necessary to reach the sterilizing effect exceeds the maximum solubility in the medium. Moreover, the test medium employed may affect the potency of this killing action. The ability of blood to produce these changes is greater than that of serum. The effect is depressant with 2-(5-nitro)-furaldehyde semicarbazone and completely abolishes the sterilizing activity with 2-(5-nitro)-furfuryl methyl ketone and 2-(5-nitro)-furoic acid. The bactericidal property of 2-(5-nitro)-furfuryl propionate is exalted both in serum and in blood.

The bacteriostatic activity of these compounds may be divided on the basis of the effect on bacterial growth curves. One group, consisting of 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate, acts as a growth rate depressant. In this group the nature of the side chain in the 2-position on the furan ring exerts only a quantitative effect. With a given medium, the compounds exhibiting this type of activity differ only in the concentration necessary to produce a bacteriostatic effect. The activity of all except one may be reduced by employing serum or whole blood as a test medium, the latter exerting a more antagonistic effect. Dodd and Stillman (1944) have shown that in a simple, chemically defined medium the antibacterial activity of the nitrofurans against *Escherichia coli* is even greater than the activity in broth. These facts suggest that nitrofurans affect adversely a metabolic function of the organism; the nature

of this effect, or the substrate involved, is as yet unknown. The exception to the depressant action of blood and serum is 2-(5-nitro)-furfuryl propionate. The activity of this compound appears to be enhanced by both serum and blood. No explanation can be given for this unusual effect, unless we assume conversion to a more active compound. Obviously, this explanation involves an effect of the 2-position substituent of the furan ring on the activity and would remove this compound from the group as defined.

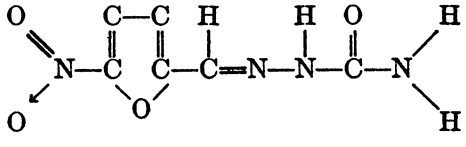
A variation of this depressed growth rate type of bacteriostatic activity was exhibited in broth by 2-(5-nitro)-furyl ketone, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate. Upon examination of the population at intervals after the inoculation of the drug-containing medium, a slight decrease, followed by an increase at a reduced growth rate, is noted. Obviously, the concentration necessary to demonstrate this is an important factor. It may be that this represents a transition point from bactericidal to simple bacteriostatic activity. If so, then structure is not involved. However, a partial or proportional killing process would yield the same type of curve if only a small percentage of each succeeding generation were killed. Since all of the compounds tested do not demonstrate this type of activity, then the nature of furan ring substitutions other than the nitro group modifies the effect, if we assume the second explanation. The medium used in testing also contributes an effect; only the propionate gives this type of curve in serum and blood.

A distinctly different mode of bacteriostatic action was demonstrated with 2-(5-nitro)-furaldehyde semicarbazone. This compound apparently has no effect on the rate of growth, once growth is initated; it does, however, prolong the time of the lag phase, in which the population remains stationary. This phase of growth, distinguished by the lack of reproduction, is generally conceded to be a period of intense vital activity of the organism. Whether the semicarbazone affects some vital process other than reproduction in such a fashion as to prolong the time to attain maturity, or merely interferes with cell division, cannot at this time be stated definitely. However, erratic results observed with coagulase-negative staphylococci suggest that metabolic processes during the lag phase are affected. Details of this activity are being studied and will be published later. We have found that the only effect of increasing the bacteriostatic concentration of the semicarbazone in a given medium is to increase the length of the lag period. The basic features of the mode of action remain the same. This favors the explanation that the increase in lag period is merely a poising of the organism. The point is an important one, for it is theoretically possible that in this case we have a drug which for at least a period maintains a rate of kill equal to the rate of growth. If this be true, however, then it would be expected that even slight increases in concentration would depress the population as an increasing linear function of the concentration. This does not agree with the observed facts.

Dubos (1929), in postulating a mechanism for a similar lag prolongation with crystal violet, ascribed the effect to the ability of the dye to poise the oxidation-reduction potential at an unfavorable point; the idea has been elaborated by

Ingraham (1933) and Hoffman and Rahn (1944). In the present case it is possible that oxidation-reduction enzymes and processes are involved. Certainly one point stands out, namely, that the mode of action of this semicarbazone is different from that of the other nitrofurans examined. However, although the degree of activity is depressed by both blood and serum, the mode remains the same regardless of the medium.

Such unique properties must be due, at least in part, to the qualitative contribution of the structure of the semicarbazone chain in the 2-position of the furan ring to the antibacterial properties of the 5-nitro furan radical. Possibly the semicarbazone chain has conferred unusual electrobiological properties to the nitrofuran ring. A poising of potential would result if the nitrofuran semicarbazone were preferentially reduced at any point in the hydrogen transport system of bacterial respiration. Moreover, this interference with a normal metabolic system could be manifest as an antibacterial property of the compound. The structure of this semicarbazone



contains a highly conjugated system of double bonds, as well as nitrogen at its maximum state of oxidation, in the nitro group. These circumstances favor the role of hydrogen acceptor in biological oxidation mechanisms.

As a chemical compound, this semicarbazone in aqueous solution can be reduced by a variety of methods, including the action of sodium hydrosulfite. Preliminary results indicate the nitro group as the first point of attack. The extent of reduction and the characteristics of reduction products are at present being studied.

SUMMARY

The mode of antibacterial action of 2-(5-nitro)-furaldehyde semicarbazone, 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate was studied in broth, rabbit blood, and rabbit serum. The activity of these compounds was examined qualitatively and quantitatively by comparisons of population changes in test samples with population changes in control cultures of *Staphylococcus aureus*.

Four compounds were capable of sterilizing the inoculum in broth. This effect was modified by serum and blood as the test medium.

Five compounds are bacteriostatic by reducing the rate of growth; in four cases this may, at the proper concentration, be preceded by a slight bactericidal effect. The influence of concentration and medium was discussed. The possible relations between structure and mode of action were pointed out.

One compound, 2-nitro-(5-nitro)-furaldehyde semicarbazone, was unique in its mode of bacteriostatic action. Activity was shown in the lag phase of growth.

The rate of reproduction, once initiated, was unaffected by the compound. Both concentration and medium affect the activity. The possible contribution of structure to this mode of action was discussed.

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