THE MODE OF ACTION OF NITROFURAN COMPOUNDS

II. Application of Physicochemical Methods to the Study of Action Against Staphylococcus aureus

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The first report of this series called attention to the fact that, of a group of varied nitrofurans, the 2-(5-nitro)-furaldehyde semicarbazone, named "furacin," was distinctly different from the others in its mode of antibacterial activity when tested with a coagulase-positive strain of *Staphylococcus aureus* (Cramer and Dodd, 1945). We have further examined the effect of furacin upon the oxidation-reduction potential of growing cultures of a coagulase-positive staphylococcus, and also the effect of eventual growth upon the concentration of furacin. We have found that a poising of E_h does occur, and that subsequently if growth takes place the chemical compound is reduced, undoubtedly at the 5-nitro group. We wish to present data related to these events, and to discuss the implications with respect to the vital processes of the bacteria.

These data have been obtained by the application of purely physicochemical methods, and at least in the instance of the polarographic method of analysis of bacterial cultures, represent an unusual and simple approach to the determination of a single constituent in the complex mixture that results from bacterial growth, without the necessity of detailed separation procedures.

EXPERIMENTAL

The changes of potential occurring in growing cultures were measured by means of a simple potentiometer, a Leeds and Northrup type K_1 (Hewitt, 1936). Electrodes were made by sealing 22-gauge platinum wire into 2-mm soft glass tubing; mercury was used to connect the Pt electrode to the potentiometer circuit. The culture vessels were 25-mm pyrex test tubes. The electrode and an inverted U-tube, the latter to serve as an electrical bridge, were rolled into a cotton plug and sterilized by autoclaving, following which the bridge was filled aseptically with a sterile, saturated KCl solution containing 3 per cent agar. For experimental observations exactly 25 ml of the medium to be examined were placed in a sterile culture vessel and inoculated with 50,000 to 100,000 organisms per ml, and the electrode- and bridge-containing plug was inserted. The vessel was then placed in a water bath at 37 C, the outer tip of the KCl-agar bridge being placed in a reservoir of saturated KCl solution. Into this reservoir also was placed the side arm of a saturated calomel electrode, and the cell thus formed was connected to the potentiometer. It was determined that the calomel half-cell under the

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conditions used would produce an emf of +250 millivolts. Thus, experimental readings in millivolts, obtained during growth, were referred to the hydrogen electrode, i.e., converted to E_h, by adding +250 to the observed reading.

The furacin present in culture media may be quantitatively estimated by the polarographic technique (Kolthoff, 1941). The 5-nitro group of the furacin molecule is reducible at the dropping mercury electrode. The half-wave potential observed for aqueous solutions buffered at pH 7.0 is −0.4 volt, against a saturated calomel external anode, and the current-voltage curve for the compound is flat in the range −0.6 to −1.0 volt. If, therefore, one makes a polarogram of a culture medium, e.g., broth, both with and without a known quantity of furacin present, the difference in diffusion current at −0.8 volt, or $\Delta\ i_d^{0.8}$, should be proportional to the amount of furacin present. The technique is particularly suitable for our purposes; it is sufficiently sensitive, accurate, and is relatively unaffected by turbidity, particulate material, or compounds present in addition to furacin.

For any particular sample of culture material the procedure of analysis consists in heating the sample to 56 C for a period of 35 minutes to 1 hour, to kill any organisms present and to stop enzyme reactions, then making a polarogram in the range 0 to −1.0 volt. The sample must be deaerated by bubbling pure nitrogen through the medium for 15 minutes. The difference in the sample and suitable controls permits one to observe the concentration of the nitro compound. We have expressed the concentration of furacin remaining in solutions as the percentage of residual furacin, or the ratio: $\frac{\Delta i_d^{0.8}\ \text{final}}{\Delta i_d^{0.8}\ \text{initial}}$.

This seemed advisable in comparative data such as we present, since it obviates correction of diffusion current values which may change slightly during the course of several experiments because of changes in temperature, fluctuations in drop rate, and similar variables. An Aminco polarometric analyzer was used in these determinations.

The medium used in this investigation was Difco brain heart infusion broth. The organism was a recently isolated, coagulase-positive strain of *S. aureus*. Inoculations were made from 24-hour broth cultures of the organism, using 50,000 to 100,000 organisms per ml of the medium to be examined.

RESULTS

E_h measurements. The data from a representative experiment showing the change of E_h during growth in the control, in two samples containing different bacteriostatic concentrations, and in a sample containing bactericidal concentration of furacin are presented in table 1. The concomitant change of pH in the control was found to be 0.4 to 0.5 units, e.g., from 7.20 to 6.75. It would be desirable, in similar future experiments to measure pH simultaneously with E_h. Such refinement, with subsequent corrections, has not been done in this work. An idealized diagram for comparison of the concentration effect appears as figure 1.

From consideration of figure 1 it is evident that the principal effect of increasing the concentration of furacin is to prolong the time before the initial E_h will drop.

TABLE 1

Change of E_h during growth of S. aureus in broth containing furacin

HOURS AFTER INOC.	CONTROL		1/200,000		1/100,000		1/50,000	
	E_h, mv	Growth	E_h, mv	Growth	E_h, mv	Growth	E_h, mv	Growth
0	+157		+170		+166		+100	
1	164		190		176		96	
2	164		202		175		98	
3	156		190		179		102	
4	111	+	168		176		109	
5	107		168		170		103	
6	7	++	168	trace	167		102	
7	20		111		163		95	
8	10	+++	88	++	147		91	
9	15		6		125		77	
10	10		5		117			
11	+2		30		108			
12	−2	++++	+20			trace		
15	−30		−10	++++	45	++	80	
18	−63		−40		+7		80	
24	−88		−65		−28	++++	85	
48	−92		−110		−95		80	
96	−85		−105		−100		70	−

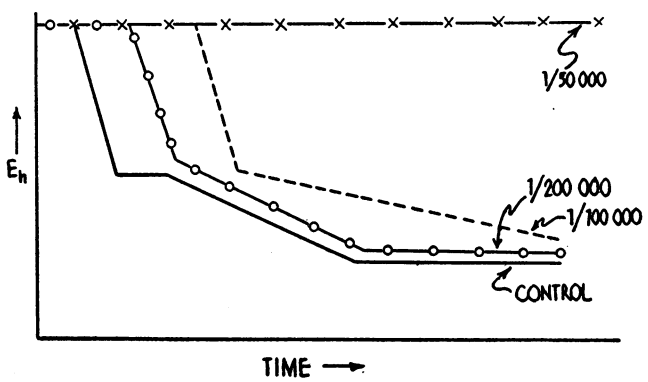

FIG. 1. CONCENTRATION EFFECT OF 2-(5-NITRO)-FURALDEHYDE SEMICARBAZONE UPON THE E_h OF GROWING STAPHYLOCOCCUS AUREUS

——— control.
-X—X- 2-(5-nitro)-furaldehyde semicarbazone 1:50,000.
——— 2-(5-nitro)-furaldehyde semicarbazone 1:100,000.
-O—O- 2-(5-nitro)-furaldehyde semicarbazone 1:200,000.

With a bactericidal concentration, this initial value does not significantly change. With simply bacteriostatic concentrations the initial level is maintained for a time period in excess of that in the control.

Change in concentration of furacin during growth. Table 2 and figure 2 show current-voltage data for broth—broth in which *S. aureus* has grown fully and broth containing furacin at a concentration of 1:100,000.

TABLE 2

Current-voltage relations for furacin in broth

EMF., -VOLT	BROTH	BROTH AFTER FULL *S. aureus* GROWTH	FURACIN 1/100,000 IN BROTH
0.0	−3.3	−3.1	−3.3
0.2	−1.5	−1.5	−1.6
0.3	−1.4	−1.4	−1.4
0.4	−1.3	−1.35	−1.3
0.5	−1.3	−1.3	−0.7
0.6	−1.3	−1.3	−0.6
0.8	−1.05	−1.05	−0.35
1.0	−0.75	−0.65	0.0

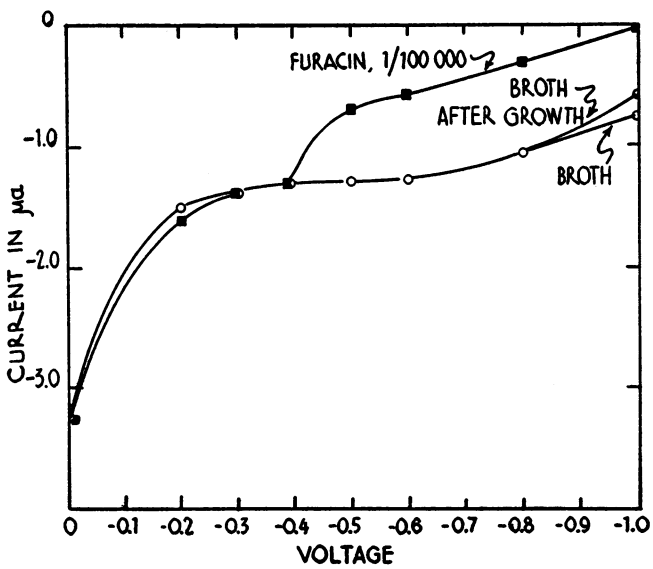

FIG. 2. POLAROGRAMS FOR BROTH AND 2-(5-NITRO)-FURALDEHYDE SEMICARBAZONE IN BROTH

-O—O- broth.

-■—■- 2-(5-nitro)-furaldehyde semicarbazone in broth, concentration, 1:100,000.

The effect of added furacin is obvious. The average difference in the diffusion current at −0.8 volts due to the presence of 1:100,000 furacin is 0.7 μa, i.e., $\triangle i_d^{0.8} = 0.7\ \mu a = 1:100{,}000$. Similarly, we have determined that for a concentration of 1:50,000 $\triangle i_d^{0.8} = 1.4\ \mu a$, and for 1:25,000 $\triangle i_d^{0.8} = 2.75\ \mu a$. Thus, for this range of concentration, the amount of furacin is a linear function of $\triangle i_d^{0.8}$. The average value for the dilution of furacin producing $\triangle i_d^{0.8} = 1\ \mu a$

under these conditions is 1:70,000. This is the quantitative analytical basis for estimating furacin in bacterial culture media.

Table 3 shows representative data for the amount of furacin remaining in a culture of *S. aureus* growing in the presence of 1:100,000 furacin; visible growth indication is also included.

TABLE 3

Changes in concentration of furacin during growth

(Initial concentration furacin = 1:100,000)

HOURS AFTER INOC.	$\Delta i_d^{0.6}$, μa	RESIDUAL FURACIN	VISIBLE GROWTH
		%	
0	0.7	100	−−
2	0.7	100	−−
4	0.7	100	−−
6	0.65	93	−−
8	0.55	79	−−
10	0.50	71	−−
12	0.45	65	trace
15	0.25	36	++
18	0.15	21	++++
24	0.10	14	++++

DISCUSSION

It has long been known that in broth *S. aureus* has a lag period of 1 to 2 hours, followed by a rapid growth that is complete in 8 to 9 hours. The effect of furacin in a concentration of 1:100,000 is to prolong the lag period, in this case the prolongation being at least 6 to 7 hours. We have now demonstrated that during this prolonged lag the E_h of the culture remains poised. Moreover, during subsequent growth, furacin disappears.

The poising of potential, followed by what seems to be a normal drop in E_h, confirms our previous conclusion (Cramer and Dodd, 1945) that the only effect observable in population curves of this particular drug-organism combination occurs during the lag phase. It may well be that the poising of the E_h at a point unfavorable to growth is the primary cause for bacteriostasis, in line with the suggestion of Dubos (1929) concerning crystal violet.

It is also apparent that an induction period is necessary before the reduction of the 5-nitro group of the furacin molecule occurs (table 3). With furacin at a concentration of 1:100,000 the organism under these conditions has a lag period of 8 to 9 hours. Reduction, however, is initiated 5 to 7 hours after inoculation, and at 9 hours is approximately 25 per cent complete. This suggests that reduction is initiated shortly before the lag period is ended. This would appear to indicate that at least the initiation of reduction may be necessary before growth can take place, and may signify that furacin must be reduced below a critical level before growth starts.

It is only with difficulty that the time relations in experiments designed to show the change in E_h with growth, or the rate of reduction of furacin, may be duplicated within a narrow range. Successive cultures of the same organism, and also successive lots of the same nutrient material, show a considerable effect in varying the hour at which an expected change occurs. However, the over-all trend of these changes with time remained the same in successive repetitions of our experiments.

We propose then this sequence of events for a culture growing in a bacteriostatic concentration (1:100,000) of furacin: (a) growth is delayed, as manifested by a prolonged lag and a poised E_h; (b) reduction of furacin begins; and (c) growth is initiated and proceeds at a normal rate. The latter event is accompanied by a drop in E_h, and the reduction of furacin in an apparent zero order reaction.

Hinshelwood (1944) has recently reviewed various factors influencing events of the bacterial growth cycle. He has pointed out the necessity, early in the lag phase, of a regeneration of inactivated enzymes and a fresh accumulation of labile or diffusible intermediates responsible for the extension of enzyme patterns prerequisite to logarithmic growth. Certain correlations of the phenomena we observe with these vital events can be made. The induction period before the reduction of furacin is probably explained at least in part by the reactivation of an enzyme. However, it is probable that the effective concentration of reactivated enzyme is in the beginning so small that the reduction in the amount of furacin, although it may start soon after the introduction of furacin into the system, is so slow that by our present methods differences in furacin concentration are not measured. When the enzyme is fully reactivated, as for example, 5 hours after inoculation of broth containing 1:100,000 furacin (table 3), which is roughly midway through the extended lag period, then the reduction is proceeding at its maximum velocity, having probably changed, in our opinion, from a first order to a zero order reaction. Under these conditions the change in the concentration of furacin becomes measurable, as the data show. The diversion of at least a part of this enzyme activity from normal metabolic processes into the reduction of furacin must result in an interference with the production by one enzyme of a sequence of an intermediate utilized by a later member of the sequence. This would be an uncomplicated explanation. This interference is manifested as a temporary delay in the completion of the necessary extension of enzyme patterns that must occur before growth becomes logarithmic. When either the velocity of such an extension becomes great enough that the reaction rate of furacin reduction is no longer critical, or when the concentration of furacin itself is lowered beyond a critical level, then growth proceeds simultaneously with a continued reduction, and appears unaffected by that reduction. It must be pointed out again that, under such conditions as we have defined, the effective events in the antibacterial action of furacin have all occurred by the time that the lag phase has ended.

Thus, as previously suggested (Cramer and Dodd, 1945), furacin does indeed

interfere with a normal metabolic process of the organism, and does so by virtue of its capabilities of being reduced. These facts emphasize the importance of a better knowledge of the reducing system present in the organism, which for convenience may be called "furacin reductase," since susceptibility may be intimately linked to this factor. Such information we hope to obtain.

We wish to emphasize the utility of physiochemical methods in this study, particularly the polarographic method. The only technique previously available for quantitative determination of nitrofurans utilized spectral absorption in the near ultraviolet. This proved quite unsatisfactory for use in turbid, protein-containing culture liquors. On the other hand, the polarographic technique, when applicable to the compound in question, does not suffer unduly from such interfering influences. Moreover, in practice it proved remarkably simple in regard to operations necessary to prepare a viable culture for analysis.

SUMMARY

Examination of the effect of 2-(5-nitro)-furaldehyde ("furacin") upon changes in the oxidation-reduction potential of a growing culture of *Staphylococcus aureus* has revealed that a poising occurs.

By means of a polarographic method of analysis for furacin in bacterial culture liquors it has been demonstrated that it is destroyed by bacteria. A mechanism for such destruction is suggested.

The implications of the time relationships between such changes upon the bacterial growth process were discussed.

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