THE INFLUENCE OF GERMICIDES ON THE DEHYDROGENASES OF *BACT. COLI*

PART I. THE SUCCINIC ACID DEHYDROGENASE OF *BACT. COLI*

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It is well known that many bacteria, including *Bact. coli*, carry an enzyme, or enzyme system, which is capable of removing hydrogen from a variety of substrates. This dehydrogenase has been found to be active upon many organic acids (including succinic, lactic and formic acids), sugars, certain aldehydes and alcohols in low concentrations. Quastel (1926) has shown that the enzyme cannot be separated from the organisms by the usual methods of extraction, and further, that it cannot be within the bacterial cell, since the rate of reaction is too high to allow of diffusion of the molecules through the cell wall. Hence it must be an integral part of the organism, situated in the surface of the cell. This conclusion is supported by Yudkin (1937) who, working on the effect of freezing on the glucose and lactic dehydrogenases, stated that "the enzymes appear to be linked in some way with the structure of the cell". Only the lactic dehydrogenase has been separated from the bacteria (Stephenson, 1928) by autolysis of the cells.

The addition of certain compounds to bacterial suspensions has been shown to inhibit, partially or completely, the dehydrogenation of other compounds. Thus, the dehydrogenation of succinic acid by *Bact. coli* was inhibited partially by benzene, acetone, toluene and phenol, and completely by cyclohexanol and monohydric alcohols above certain concentrations (Quastel & Whetham, 1925; Quastel & Wooldridge, 1927). The succinic dehydrogenase of *S. aureus*, according to Bach & Lambert (1937), was completely inhibited by benzene, toluene and phenol, and the lactic dehydrogenase (known to be more resistant than the succinic) was also inhibited, partially or completely, by these compounds and by cyclohexanol, resorcinol, and some alcohols.

Changes in temperature are known to affect considerably the viability of bacteria, and the effect of these changes on the dehydrogenases of *Bact. coli* has been studied by several workers. Quastel & Whetham (1924) found that at 70° C. in the presence of substrate the bacteria were rapidly killed and coagulated and the enzyme was completely destroyed. They showed that the enzyme activity increased with temperature up to a maximum at 60° C. but was considerably retarded if the bacteria were heated for a short period at this temperature before adding the substrate.

In the papers quoted above it has been shown that the succinic dehydrogenase of *Bact. coli*: (1) forms an integral part of the cell surface, and (2) is affected by treatments known to be bactericidal. It was decided to investigate further the effect on the succinic dehydrogenase of *Bact. coli* of: (*a*) variation in concentration of living bacteria; (*b*) heat (up to the lethal temperature); (*c*) various germicides. Investigations (*a*) and (*b*) were undertaken in order to establish the conditions for (*c*) which was the principal object of research.

Experiments

Suspensions of *Bact. coli* were prepared by the technique of Harden & Zilva (1915). Cultures were grown for 18–24 hr. at 37° C. on a nutrient agar made with a broth prepared from the tryptic digestion of lean meat. The growth was washed off with sterile Ringer solution and centrifuged. Quastel & Whetham (1924) emphasize the necessity of removing all traces of culture medium, which may contain powerfully reducing substances, so the cells were washed three times more, and finally suspended in Ringer solution, aerated, and kept under nitrogen. The concentration of these "washed bacterial cells" was always adjusted to about $4{\cdot}5 \times 10^9$ per c.c. (as estimated on an opacity meter), since 1 c.c. of this suspension reduced 90% of the methylene blue under standard conditions in about 10 min. (Quastel & Whetham (1924) and Quastel (1926) indicated that the rate of reduction was approximately constant up to 90%; beyond this it decreased appreciably.) Stock suspensions were stored in the cold at 4° C. and were always used within 10 days of preparation. Plate counts showed that practically all of the bacteria present were viable, the product of the dilution of suspension times the number of growing colonies being $5{\cdot}0 \times 10^9$, $4{\cdot}55 \times 10^9$ and $4{\cdot}7 \times 10^9$ per c.c. in typical experiments. The numbers remained constant for at least 14 days. Four different strains of *Bact. coli*, obtained from the National Collection of Type Cultures, all showed the same order of enzyme activity at the same concentration of bacteria, and for the work reported in this paper the strain *Bact. coli* Escherich (N.C.T.C. No. 86) was chosen.

The methylene-blue technique, as given by Thunberg (1920) and by Hopkins & Dixon (1922), was used in principle for estimating enzyme activity: 1 c.c. of the washed bacterial cells was treated at room temperature with a known concentration of the germicide, the volume being made up to 2 c.c. with water. After the expiry of a given time, 3·5 c.c. of phosphate buffer (*p*H 7·2), 1 c.c. of 1 in 5000 methylene blue and 0·5 c.c. of ammonium succinate solution ($\equiv 1\%$ succinic acid) were added immediately. The tube was evacuated and then filled with oxygen-free nitrogen. This process was repeated three times to ensure complete removal of oxygen from the solution. The tube was then placed in a water bath at 37° C. and the time to reduce 90% of the methylene blue was observed. Reduction was estimated by comparison with a standard tube containing only 10% of the original amount of methylene blue. The temperature of 37° C. was selected as being the optimum for bacterial growth;

many workers have used 45° C., but, as shown below, this would probably have a retarding effect on the longer times of reduction. Experiments were carried out at least in duplicate, and reduction times agreed closely when they were short, but when they were of the order of 50 min. or more, greater variations were frequently observed.

In the experiments with phenol, viable counts were made on the suspensions after treatment with the germicide, and in tests with other germicides, cultures were made in order to confirm the presence or absence of living bacteria.

I. *The effect on reducing power of varying the concentration of living bacteria*

The dehydrogenating activity of a bacterial suspension is considered by Quastel & Whetham (1924) and by Sandiford & Wooldridge (1931) to be proportional to the total number of bacteria present, or, alternatively, to the amount of enzyme present, but Quastel & Wooldridge (1927) showed that the proportionality exists only over a range where the bacterial concentration is relatively high. The results recorded in Table I are in agreement with the last authors.

Table I. *The effect of varying the concentration of living bacteria*

Volume of viable bacteria c.c.	Reduction time min.
1·0	10
0·8	11½
0·7	14
0·6	17
0·5	28½
0·4	38
0·3	>90

They show that reduction times remain proportional to the dilution of the bacterial suspensions under consideration over the range 1·0–0·6 c.c., and that from 0·6 c.c. downwards reduction times tend to become proportional to the square of the dilution.

In all of the following experiments, the amount of bacterial suspension was the same throughout any one series. Therefore increases in reduction times may be considered due to progressive destruction of the enzyme, or of a constituent of the enzyme system.

II. *The effect of temperature*

The temperature required for carrying out the reduction tests was that at which the reaction proceeded at a reasonable velocity, but which in itself was not injurious to bacteria or enzyme. Suspensions were heated in a water bath for varying times at different temperatures, then substrate, etc. was added and reduction times at 37° C. were measured.

Table II. *The effect of heating bacterial suspensions*

Temperature ° C.	Reduction times (in min.) after heating for: 5 min.	10 min.	15 min.	20 min.	30 min.
60	>60	∞	—	—	—
55	36	55	∞	—	—
50	14	20	22	25	40
45	13	13	14	14	—
Control	13	—	—	—	—

(∞ = Reduction time greater than 100 min.)

The results given in Table II show a decided inhibition of reducing power at 50° C., and possibly a slight effect at 45° C. Quastel & Wooldridge (1927) had already observed that a temperature of 45° C. might have some effect on the dehydrogenase if maintained for a long period. Thus the choice of 37° C. for carrying out the tests reported in this paper is justified.

III. *The relation between reduction times and numbers of living bacteria after treatment with germicides*

(*a*) *Phenol.*

Phenol was used as the standard germicide. The reduction times of two suspensions of *Bact. coli* were found after they had been treated with varying concentrations of phenol for 10 min. in the standard way. The numbers of viable organisms were also found by plating suitable volumes of the treated suspension and estimating the approximate number per unit small volume.

Table III. *The effect of phenol on reduction times*

Concentration of phenol, 1 in:	Test I: Reduction time (min.)	Test I: No. of surviving bacteria per unit volume	Test II*: Reduction time (min.)	Test II*: No. of surviving bacteria per unit volume
80	∞	0	∞	0
90	>60	20	>60	10
100	39½	300,000	29	+ +
110	24½	500,000	23	+ + +
120	20	800,000	22½	+ + + +
140	18	—	18	+ + + +
200	14	—	—	—
Control	9	1,500,000	8½	+ + + + +

* In Test II, actual viable counts were not made. The + signs indicate the order of the numbers of surviving bacteria. ∞ = reduction time greater than 100 min.

It will be seen that there is an increase in the time of reduction of about 400% at a concentration of 1 in 100 of phenol and that at the next concentration (1 in 90) there is a much greater increase in reduction time (tending to infinity), practically all the bacteria being killed.

A concentration of 1 in 100 of pure phenol (range 1 in 110 to 1 in 95) will kill a culture of *Bact. typhosum* under given conditions in 7½ min. but not in

5 min., and this is used as the Standard in the Rideal-Walker test for germicides. The quotient (concentration of germicide which kills in the standard time divided by the concentration of phenol which kills in the same time) gives the Rideal-Walker coefficient of the germicide. Thus, with this comparison at hand, several other germicides of known Rideal-Walker coefficient were examined for their inhibiting power on the succinic dehydrogenase of *Bact. coli* over ranges of concentrations which might be used in the Rideal-Walker test.

(*b*) *Aliphatic monohydric alcohols.*

1 c.c. of the standard suspension was treated with suitable concentrations of the alcohols, the volumes in each case being made up to 2 c.c. After the expiry of 10 min., substrate, methylene blue and buffer solution were added and the reduction times measured. These times are given in Table IV along with the concentrations from which the Rideal-Walker coefficients (modified tests) of the alcohols (Tilley & Schaffer, 1926) are derived. Reduction times at these concentrations were very considerably increased and at slightly greater concentrations of alcohol they tended to infinity, indicating complete destruction of the enzyme.

Table IV. *Times of reduction (in min.) after treatment with alcohols*

Concentration of alcohols, 1 in:	Ethyl (R.W. = 0·04)	*iso*-Propyl (R.W. = 0·064)	*n*-Butyl (R.W. = 0·273)	*n*-Amyl (R.W. = 0·38)
2·5	∞	—	—	—
3·3	65	∞	—	—
4·0	34*	∞	—	—
5·0	11½	∞	—	—
6·6	8½	21*	—	—
10·0	—	17½	—	—
12·5	—	—	∞	—
16·6	—	—	∞	—
20·0	—	12½	∞	—
25·0	—	—	22½*	—
33·3	—	—	14½	—
40	—	—	14	∞*
50	—	—	14	28½
60	—	—	—	26½
70	—	—	—	17
80	—	—	—	11½
Control	9	10	10	10

∞ = reduction time greater than 100 min.

* Results using approximate concentrations from which the respective Rideal-Walker coefficients are derived.

(*c*) *Phenol derivatives.*

Tests similar to those on the alcohols were carried out on *p*-chloro-*m*-cresol, *p*-butyl phenol, 4-*n*-amyl-*m*-cresol and hexyl resorcinol. These compounds are typical germicides of the group, with Rideal-Walker coefficients ranging from 13·3 to 250. Owing to very low solubilities in water, the butyl phenol and hexyl resorcinol solutions were prepared from mother dilutions of 1 in 1000

in 20% alcohol, and the amyl-*m*-cresol solutions from one of 1 in 5000 in *N*/100 NaOH: *p*-chloro-*m*-cresol is easily soluble at the dilutions tested.

Cultures, made from the suspension-germicide mixture immediately after the 10 min. period (that is, just before adding the substrate-methylene blue solution), showed that treatment with 1 in 1000 *p*-chloro-*m*-cresol killed all of the bacteria, and with 1 in 1200 only a few viable organisms remained; similarly the lethal concentration of butyl phenol was between 1 in 5000 and 1 in 6000, and of amyl-*m*-cresol was about 1 in 20,000. At weaker concentrations the numbers of surviving bacteria were progressively greater. In the case of hexyl resorcinol there were a few viable organisms remaining in the 1 in 5000 concentration but not in the 1 in 4000, and the number even in the 1 in 10,000 concentration was not large. Thus neither in the count test nor in the reduction test was the "end-point" well defined.

The concentrations from which the Rideal-Walker coefficients of the phenols are derived are indicated in Table V, and it will be seen that, as in the case of the alcohols, the enzyme activity is completely destroyed at slightly stronger concentrations of germicide.

Table V. *Times of reduction (in min.) after treatment with phenols*

Concentration of germicide, 1 in:	*p*-Chloro-*m*-cresol R.W. = 13·3 (*a*)	Hexyl resorcinol R.W. = 50 (approx.)	*p*-Butyl phenol R.W. = 70–75 (*b*)	Amyl-*m*-cresol R.W. = 250 (*b*)
1,000	60	—	—	—
1,200	25	—	—	—
1,300	—*	—	—	—
1,400	17	—	—	—
1,600	16½	—	—	—
2,000	14½	—	—	—
2,400	14	—	—	—
4,000	—	∞	—	—
5,000	—	>60*	51	—
6,000	—	51	36	—
7,000	—	50	21	—
8,000	—	52	21*	—
10,000	—	47	16	∞
12,000	—	30	—	∞
15,000	—	20	—	∞
20,000	—	—	—	∞
25,000	—	—	—	38*
30,000	—	—	—	23
40,000	—	—	—	18
50,000	—	—	—	15
Control	8½	9	9	10

∞ = Reduction time greater than 100 min.

* Results using approximate concentrations from which respective Rideal-Walker coefficients are derived. † Rapps (1933). ‡ Coulthard, Marshall & Pyman (1930).

Discussion

From the results given in the foregoing tables it is evident that when a suspension of *Bact. coli* has been treated with certain phenols or aliphatic alcohols a relationship exists between the dehydrogenating capacity of the suspension towards succinic acid and the number of living organisms present.

This relationship is not one of direct proportionality since there is a lag in the effect of a germicide on the enzyme system as compared with its effect on the viability of the organisms. Nevertheless, at concentrations of germicide equal to those which kill the whole of a suspension of *Bact. coli*, the activity of the enzyme is considerably, if not completely, inhibited, and at slightly stronger concentrations the enzyme is in all cases completely destroyed.

In view of the close correlation between the effect of a germicide on bacteria and its effect on the succinic dehydrogenase, it may be possible to use this type of test in conjunction with, or as a substitute for, the Rideal-Walker test. One feature of the reduction test is that results can be obtained within 2 hr.

It is hoped to continue work in this direction by including other types of germicide and by investigating their effects on the lactic and other dehydrogenases of *Bact. coli*.

SUMMARY

1. The effect of alcohols and of phenol on the succinic acid dehydrogenase of *Bact. coli*, hitherto recorded qualitatively in the literature, has been studied in greater detail.

2. The effect of heat and of *p*-chloro-*m*-cresol, hexyl resorcinol, *p*-butyl phenol and amyl-*m*-cresol, has also been studied.

3. The concentrations of these germicides required to inhibit completely the succinic dehydrogenase is such as to allow for a slight excess over the concentrations which are lethal to *Bact. coli*.

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