CCVIII. HYDROGENLYASES.

III. FURTHER EXPERIMENTS ON THE FORMATION OF FORMIC HYDROGENLYASE BY *BACT. COLI.*

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IN a recent paper [Stephenson and Stickland, 1932] we showed that a culture of *Bact. coli* grown in Roux bottles on tryptic broth was devoid of the enzyme formic hydrogenlyase, while a culture grown in similar circumstances with addition of formate to the medium possessed this enzyme. Yudkin [1932] pointed out that there are two possible explanations of this phenomenon. (1) The difference might be due to a process of "natural selection," or (2) it might be due to a direct chemical action of the formate and broth, stimulating the formation of a new enzyme in the cells. He decided against the former theory, his evidence being (1) the probable uselessness to *Bact. coli* of being able to decompose formic acid anaerobically, and (2) the fact that when a culture (in this case of *Bact. freundii*) grown for many generations on broth containing formic acid was resown on to plain broth, it completely lost its formic hydrogenlyase during sixteen hours' growth (about ten generations). We propose now to offer some further evidence on this point.

For "natural selection" to operate, the formate must have either a beneficial or a harmful effect on the bacteria; in the former case those cells able to profit by the decomposition of formic acid, and in the latter those able to rid themselves of it by decomposition, would have an advantage. In either case, the addition of formate to a young, rapidly growing culture of *Bact. coli* would be expected to have some effect on the subsequent growth rate, and this point was tested experimentally.

Two one-litre flasks containing 500 cc. of tryptic broth were sterilised and brought to 37° by incubation overnight. Each flask was then sown with one drop of a young broth culture of *Bact. coli*, and a sample of each taken for counting. At intervals of 1, 2, 2½, 3, 3½ and 4 hours further samples were withdrawn for counting, to establish the growth rate, and at 4 hours 10 cc. of sterile 25 % formic acid as sodium salt were added to one flask (giving a concentration of 0·5 %) and 10 cc. of sterile water to the other, both solutions having been previously warmed to 37°. After the additions samples were again taken at intervals of 5 minutes, ½, 1, 1½, 2, 2½ and 3½ hours; the counting was carried out by Wilson's viable count method [1922]. The results, shown graphically in Fig. 1, show that the addition of sodium formate had no effect on the growth rate of the culture, so we must conclude that the mode of action of the formate is not "natural selection."

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The second alternative, *viz.*, a direct chemical action of the formate and broth on the cells, was therefore provisionally accepted, and we proceeded to try to find further experimental support of this view.

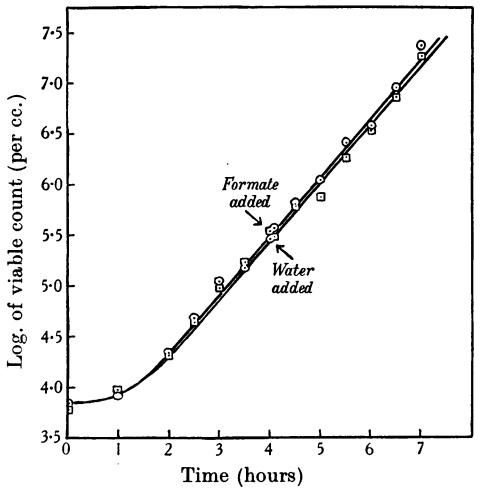

Fig. 1. ⊡ Exp. (formate added); ⊙ Control (water added).

Growth and enzyme formation.

Our next step was to determine how many generations and how much time were required for the appearance of the enzyme after addition of the formate. This could not be done while growth was still in the logarithmic phase, as at that stage the concentration of bacteria was insufficient for enzyme determinations; the youngest culture from which sufficient bacteria for testing could be obtained by centrifuging was ten hours old, and a culture of this age was consequently used.

A four-litre flask containing three litres of medium, fitted with a rubber stopper carrying a wide glass inlet tube and a siphon, was sterilised and sown with two drops of an eight-hour old culture of *Bact. coli*. Ten hours later, when growth was just visible, a sample of 500 cc. was siphoned off into sterile centrifuge-tubes, and 50 cc. of sterile 12·5 % formic acid (as sodium salt) were added to the remaining culture through the wide tube in the stopper, giving a final concentration of 0·5 % formic acid. Further samples of 500 cc. were run off into sterile centrifuge-tubes at intervals of 1, 2, 3 and 4 hours after the addition of the formate. Each sample as it was obtained was treated as follows. The cups were cooled to roughly 10° by immersion in ice, and a small sample (about 1 cc.) was taken into a sterile dry tube and diluted immediately for viable counting. The remainder was centrifuged and washed once with sterile Ringer's solution. The washed suspensions thus obtained were tested for formic hydrogenlyase (as described by Stephenson and Stickland [1932], and with the help of nitrogen estimations on the bacteria values for Q_{H_2} were calculated. The results of one experiment are given in Table I.

It will be seen that the formic hydrogenlyase had appeared in less than one hour after the addition of the formate, while the viable count had increased by only 18 %, and the Q_{H_2} had reached its maximum after two hours with an increase of only 34 % in the viable count. It appears, therefore, that the enzyme formation

Table I.

Time after addition of formate (hours)	Viable count (10^7 per cc.)	Q_{H_2}
0	5·0	0
1	5·9	450
2	6·7	710
3	8·0	650
4	7·7	650

reaches its maximum during considerably less than one generation time, which fact makes it impossible for the phenomenon to be one of "natural selection."

It might be objected that viable counts in a relatively old culture do not give a true indication of the amount of cell division taking place, as a rapid cell division might be balanced by a high death rate. This objection could be met by using the method of total counting, which would detect any increase of cell numbers with an error of 5 %. This method of counting was used in all subsequent experiments, and at the same time a simpler method was devised for following the course of the production of the enzyme.

The course of the production of formic hydrogenlyase.

A pure suspension of *Bact. coli* containing no formic hydrogenlyase was prepared by growing a culture on plain broth in Roux bottles, centrifuging and washing aseptically. Barcroft cups were sterilised by standing them in chromic acid overnight, washing them six times with sterile distilled water and plugging them with sterile cotton wool plugs from test-tubes; they were dried by leaving them in an incubator for a few hours. The apparatus was set up with 3 cc. of water in the left-hand cup and the following sterile solutions in the right-hand cup:

Tryptic broth (three times normal strength) p_H 7·0 ...	1·0 cc.
Sodium formate 1·0 *M*	0·3 cc.
Phosphate buffer 0·2 *N*, p_H 7·0	0·5 cc.
Water	0·2 cc.

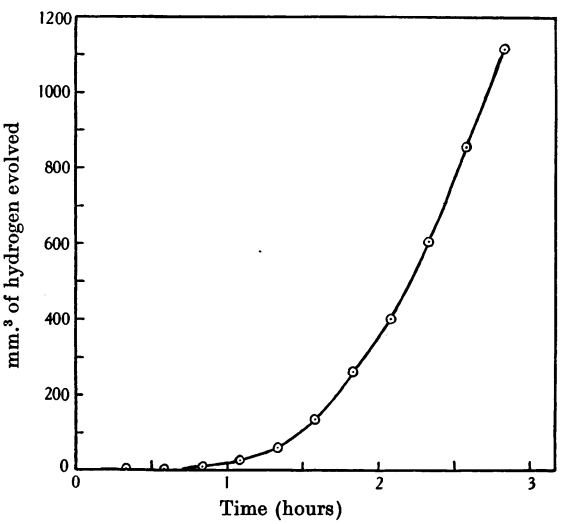

Fig. 2.

1 cc. of a suitably diluted bacterial suspension was then added and the apparatus filled with nitrogen as usual and placed in the bath at 40°.

In Fig. 2 the result of a typical experiment is given, the volume of hydrogen evolved being plotted against time in the usual way, while in Fig. 3 the same experiment is plotted with velocities of hydrogen evolution as ordinates.

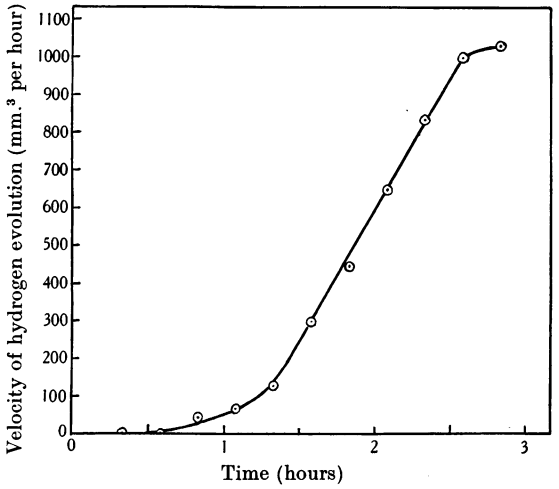

Fig. 3.

Table II.

No.	Duration of exp. (hours)	Total count 10^8/cc. Initial	Total count 10^8/cc. Final	Increase (%)	Q_{H_2} Initial	Q_{H_2} Final
1	2½	1·14	1·19	0	0	3950
2	2	5·4	4·9	0	0	3900
3	3½	1·17	1·12	0	0	3500
4	1½	2·6	2·6	0	0	2160
5	3½	1·6	1·5	0	0	1900
6	5½	1·5	1·5	0	0	1670
7	4½	2·1	2·1	0	0	1600
8	3	4·3	4·45	0	0	1400
9	3	2·5	2·5	0	0	1400
10	3	5·4	5·2	0	0	1200
11	2½	2·6	2·5	0	0	1120
12	3½	7·6	7·9	0	0	870
13	3	7·6	7·6	0	0	770
14	3	9·3	9·2	0	0	760
15	2	1·7	1·7	0	0	750
16	2½	3·2	3·2	0	0	720
17	3	1·14	1·24	9	0	4850
18	3	3·2	3·6	12	0	900
19	3	1·14	1·39	20	0	3600
20	4½	2·3	3·15	40	0	1200
21	3	3·2	4·8	50	0	560
22	3½	1·7	3·0	95	0	1240
23	4	1·7	4·0	120	0	1210

It will be observed that, as would be expected, the initial velocity is zero, and after a latent period the velocity begins to increase. The rate of increase of velocity becomes linear and remains so until the maximum is reached; this may

be interpreted as showing that the synthesis of formic hydrogenlyase is a linear reaction.

In a large number of such experiments a total count was carried out, both on the initial suspension of *Bact. coli* and on the contents of the Barcroft cup after the maximum velocity had been reached; the results are given in Table II. It will be seen that in the majority of the experiments no growth (*i.e.*, less than 5 % increase in total count) took place, while the value of Q_{H_2} increased from 0 to 720–3950, and in the remaining cases (7 out of 23) slight growth (never more than 120 % increase) was found with a similar increase in Q_{H_2}.

Discussion.

The experiments on the growth rate of *Bact. coli* after addition of formate to a young culture, and on the appearance of formic hydrogenlyase after such an addition, are sufficient to show that "natural selection" plays no part in the formation of the enzyme. From the experiments in the Barcroft apparatus just described further conclusions can be drawn. In presence of formate alone no formic hydrogenlyase is developed, but in presence of formate and tryptic broth, after a latent period, the appearance of formic hydrogenlyase proceeds proportionally to the time, while no cell division is taking place. It seems reasonable to deduce from this that enzyme formation is simply a chemical reaction between the medium and the cells. Further work is being carried out to try to elucidate the nature of the reaction.

Summary.

1. This work aims at deciding whether the enzyme formic hydrogenlyase appears in *Bact. coli* by a process of "natural selection" or in some other way.

2. Addition of sodium formate to a young culture of *Bact. coli* has no effect on the subsequent growth rate.

3. The enzyme can be produced by the bacteria while no cell division is taking place, and this production proceeds linearly.

4. The deductions made are that (1) the operation of "natural selection" is impossible, and (2) the formation of formic hydrogenlyase is probably a chemical reaction between the medium and the cells.

REFERENCES.

Stephenson and Stickland (1932). *Biochem. J.* **26**, 712.
Wilson (1922). *J. Bact.* **7**, 405.
Yudkin (1932). *Biochem. J.* **26**, 1859.