CCXXXIV. "RESTING" BACTERIA.

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DURING recent years much work has been reported upon the existence and properties of various bacterial oxidising enzymes. For most of these researches thick suspensions of well-washed bacterial cells in physiological saline or Ringer's solution have been used. These bacterial preparations, when used under conditions such that growth cannot occur [Quastel and Whetham, 1924], have been called "resting" bacteria, a term suggested by analogy with that of "resting" muscle, a similar preparation of well-washed muscle tissue. Although the term "resting" bacteria appears to be justified in the biochemical sense it has been a somewhat unfortunate choice for the bacteriologist if it be taken to imply that "resting" bacteria are normal viable cells which are only unable to proliferate because they are used under conditions unsuitable for their growth; *e.g.* in the absence of necessary food materials or at unfavourable temperatures. It has seemed to him unjustifiable to describe bacterial suspensions as "resting" when no effort was made in the early work to demonstrate any connection between the different stages of the experiments and the total and viable counts of the organisms present. At that time the main concern was to simplify the complexity of bacterial processes as much as possible so that certain of their systems could be more rigorously examined, and hence the actual relationship between the enzymic mechanisms and the life or death of the cells present was not emphasised. In 1927 Quastel and Wooldridge [1927, 1, 2] showed that bacteria previously submitted to various treatments, *e.g.* toluene, chloroform, permanganate *etc.*, so that they would no longer proliferate when inoculated on to agar slopes, nevertheless exhibited little variation in the enzymic activity of many of the mechanisms studied. In these experiments no actual counts were made. After discussing at some length the question of whether the activations of the cell are due to the latter acting as an organised whole, the authors conclude that "we have indeed no evidence to support a contention that the reactions of the organism are dependent upon its survival as a living unit...." Later Cook and Stephenson [1928], who were examining the behaviour of bacterial suspensions in Ringer's solution in a Barcroft differential manometer, *i.e.* in the presence of oxygen,

carried out a series of bacterial counts on their preparations both before and after treatment with ultra-violet light and compared these counts with the oxygen uptake. They conclude "that the oxidation phenomena studied are the work of dead equally with living organisms."

The bulk of the work with "resting" bacteria has been effected under anaerobic conditions, usually at 45°, and it was thought advisable to make total and viable counts of organisms used under these conditions, not only at the beginning and end of the experiments, but at various intervals over a period of several hours. The importance of these Thunberg tube-methylene blue experiments to the bacteriologist was emphasised as early as 1925 [Quastel and Wooldridge, 1925; Quastel and Stephenson, 1925] by the correlation of the results obtained from a study of the enzymic powers of various organisms with their ability to grow upon synthetic media. Since then the term "resting" bacteria has invaded bacteriological literature to such an extent that it has become more than ever necessary to demonstrate the true nature of these suspensions from the angle of the strict bacteriologist. Accordingly the work described in this paper was undertaken.

EXPERIMENTAL.

An organism is said to activate a substrate, examined by the anaerobic methylene blue technique, when the methylene blue is decolorised within a period of 2 hrs., whilst a control experiment with the organism in the absence of the substrate does not reduce the methylene blue at all. Frequently the reduction time with an activated substrate is only a few minutes, but as occasionally experiments have been carried out, and the results reported, over a period of 22 hrs. it was decided to examine the organism over this long period. Accordingly series of Thunberg tube experiments have been put up with *B. coli* suspensions in the presence of various substrates and these have been incubated anaerobically at 45° for different periods of time ranging up to 22 hrs. At the end of the incubation periods total and viable counts of the organisms present in the suspensions have been made. In parallel with these experiments other series of tubes have been incubated so that the effect of the incubation period upon the enzymic activity of the organism could be ascertained. In this way it has been possible to obtain curves showing the variation of total and viable counts with the period of incubation and also to compare these variations with any changes discovered in the enzymic activity.

In the preparation of the organism an 18-hr. broth culture of *B. coli* was inoculated into Roux bottles containing caseinogen digest broth. After 36 hrs. incubation at 37° the organism was centrifuged free of broth, washed twice by centrifugation with sterile saline, made up to a suitable suspension with sterile saline, aerated for half-an-hour, and the organism then stored at 0° until required for use. All experiments were made with an organism that had not been prepared for more than 5 days. For actual use

the suspension was adjusted, by comparison with turbidity controls of *B. coli*, so that its final dilution in the Thunberg tube would contain between 500 and 1000 million cells per cc.

For the Thunberg tube experiments each tube contained 2 cc. of sterile phosphate buffer, p_H 7.4, 1 cc. of sterile substrate, $M/20$ glucose, lactate, formate or succinate—all adjusted to p_H 7.2 and 1 cc. of the *B. coli* suspension. Some of the tubes were incubated with 1 cc. of methylene blue (1/5000 solution in water) whilst to the others 1 cc. of sterile physiological saline was added instead. The organism was added last, the tube closed, immediately evacuated, the side-arm filled with sterile water and placed in the water-bath at 45°. The first of each series of experiments was only left in the bath for 30 secs. when it was removed, cooled under running water, the water removed from the side-arm and the tube carefully opened so that no grease was allowed to descend into the fluid. Total (*i.e.* living and dead cells) and viable counts were begun on this suspension almost immediately. Viable counts were made similarly after incubation for the following times: 20 mins., 40 mins., 1 hr., 2 hrs., 3 hrs., 4 hrs., 5 hrs., 6 hrs. and 22 hrs. Total counts were estimated after 1-hr., 6-hr. and 22-hr. incubation periods. At times various tubes of a series were examined for purity by inoculating nutrient media and incubating both at 37° and at 45° and sometimes at 53° in order to ascertain whether any thermophilic or other contaminants were present. In all these experiments the suspensions were found to consist solely of *B. coli*.

For assessing the total number of organisms present the technique followed was that described by Wilson [1922]. In this the Helber chamber is used and the unstained organisms are counted against a dark background.

The viable counts were made by the agar roll-tube method discussed in the same paper.

All dilutions were effected by the use of dropping pipettes [Donald, 1915]. The margin of error of the results given in the present paper may be slightly greater than usual owing to the fact that at times the dropping pipettes became contaminated with grease, which would affect somewhat the size of the drops.

The experiments for examining the effect of the anaerobic incubation at 45° upon the activity of the enzymes were carried out in Thunberg tubes just as for the bacterial counts. At the end of the required incubation the tube was cooled, the water in the side-arm withdrawn and the stopper carefully opened. The stopper and neck of the tube were cleaned with a dry rag and the former re-greased. If the incubation had taken place in the absence of methylene blue this was now added, the tube closed and re-evacuated and the time required for the reduction of the dye on replacing in the bath at 45° noted. If the first incubation had taken place in the presence of methylene blue, then before the second evacuation the contents of the tube were well shaken to oxidise the dye, and just before immersion in the bath the proportion of oxidised dye present was ascertained by comparison with a series

of controls. Any necessary correction was then made in the second reduction time before comparing this with that previously obtained.

Results representative of the many obtained are set forth in the following tables or shown graphically in the figures.

DISCUSSION OF RESULTS.

Viable counts. The curves of Fig. 1 show that the number of viable organisms present in bacterial suspensions incubated anaerobically at 45° in the presence of various substrates, but in the absence of methylene blue, decreases with increase in the period of incubation.

If, in addition to the substrate, methylene blue be added, there is a fairly rapid diminution in the number of viable organisms within the first hour, but this is followed by an increase in numbers in all instances except with

Fig. 1. Showing the relationship between the number of viable organisms and the previous period of incubation at 45° in the presence of the named substrate but in the absence of methylene blue. Glucose=curve ABC. Formate=curve DEF. Lactate=curve DGH. Succinate=curve AKL. Saline=curve PMN. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

the saline series (see Fig. 2). This increase tends to balance the previous decrease but subsequently the viable count diminishes again. With the saline-methylene blue series no growth at all was obtained from the dilutions planted from the 2-hr. to 6-hr. tubes, but this was probably the result of diluting the suspensions too much, for with the 22-hr. tube, where the suspension was inoculated undiluted, some growth, although less thick than that obtained in the earlier tubes, was noted. It is therefore evident that the increase in the number of viable organisms observed with the other series is absent from that with saline and methylene blue.

It may be possible to explain these results from a consideration of the number of viable organisms involved. There is evidence to suggest [see Bail, 1929] that many organisms will only grow upon media, provided that the

initial concentration of viable organisms is below a certain critical value. This fact might explain the results obtained with the lactate and formate series, for if the initial concentration of viable organisms was already at the critical value in the experiments without the dye then no growth would be expected but in the methylene blue tubes the initial viable count is much less, possibly below this critical "saturation" value, and growth might take place. It is more difficult to explain the glucose results in this way as with these the initial concentrations of viable organisms differ little.

An alternative biochemical explanation may be suggested, depending upon the presence of methylene blue in those tubes where an increase in number of viable organisms is noticed. Methylene blue, in addition to its known inhibitory effect on many enzymes, will also act as a hydrogen acceptor and so will facilitate the oxidation of such hydrogen donors as glucose, lactate and formate in the presence of the organism. These oxidation changes may

Fig. 2. Showing the relationship between the number of viable organisms and the previous period of incubation at 45°, in the presence of a substrate and of methylene blue. Glucose = curve ABC. Formate = curve DEF. Lactate = curve GHK. Saline = curve PMN. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

result in the formation of some substance essential to or definitely stimulatory of growth and would, in addition, yield a supply of energy and, as a result of one or both of these factors, growth might ensue. This growth would be facilitated by the presence of some nitrogenous materials probably made available from the dead cells. Growth will only occur when in addition to the hydrogen acceptor, methylene blue, a hydrogen donor is present and hence no increase in the viable count would be expected in the saline-methylene blue series of experiments—an expectation borne out by experiment. But even in the presence of a hydrogen donor growth will not continue for long under the conditions of these experiments for two reasons; firstly the methylene blue will soon be entirely reduced to the leuco-compound and, although this might be re-oxidised by protein products, the action would continue for but a short time; secondly the inhibitory action of the dye would become increasingly

important. Hence after a short period of growth the number of viable organisms would again decrease. This early cessation of growth might, of course, be due to other factors, such as the lack of adequate nitrogenous material. The above explanation, however, is tentative and needs further work for corroboration. A further fact that should be noticed is that the delay in growth corresponds to the ordinary lag phase of bacterial growth on nutrient media and so the changes noticed may be due to the same causes as are responsible for this lag phase. What relation the phenomena described in the above explanation of the results obtained may have to the lag phase, if any, still remains to be determined.

An examination of the figures given in Table I shows that the total number of cells remains practically stationary throughout the period of incubation. At the beginning the ratio of viable organisms to total cells varies between 1 : 2 and 1 : 7 in the different experiments, except with the lactate-methylene blue series, but at the end of the period of incubation this ratio is always greatly altered—varying between 1 : 200 and 1 : 6,000,000. It follows therefore that if the enzymic activity of these suspensions were examined in parallel with the growth series, then the enzymic activity should either be little changed, if it is independent of whether the cell is alive or dead, or it should fall off greatly and become immeasurable by our technique if the activity is due solely to the presence of living organisms. This holds only if the velocity of the enzyme reaction as measured by the Thunberg technique is proportional to the concentration of the enzyme present. That this is so is shown by the curves given in Fig. 3. The results given in Table II show that for practical purposes the enzymic activity remains constant, the greatest change recorded being a sixfold increase in the reduction time. These results therefore conclusively demonstrate that the enzymic activity is not solely dependent upon the living cell but appears to be a property both of cells that are unable to grow, even given suitable conditions, and of cells that are still alive and only unable to grow during the experiment because of the conditions of this experiment. The implication that preparations described as "resting" bacteria consist mainly of cells which are in a state of suspended animation as regards growth is incorrect, although the claim that the synthetic reactions associated with growth are largely inhibited by their use under specified conditions remains valid. The term "resting" bacteria therefore should signify a suspension of washed bacterial cells, some alive and some dead, used under conditions such that proliferation, that is increase in the number of living cells, is inhibited during the usual period of observation. Then the reactions studied will generally be those associated with both the living and the dead cells.

As the cardinal point in this definition is that multiplication of the bacteria is inhibited during the use of "resting" bacteria it is obviously essential, if the term be applied at all generally to the use of suspensions of washed bacterial cells, that control experiments on bacterial counts be conducted in parallel with the biochemical reactions being studied. Under conditions such

Table I. Showing the effect of varying the substrate upon the total and viable counts of organisms present in suspensions of *B. coli* incubated anaerobically at 45°. Except for the series marked with an asterisk, the results given (no. per cc.) with one substrate were obtained with the same bacterial suspension.

Substrate	Count	0 min.	20 mins.	40 mins.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	22 hrs.
1. Saline	Total	363,000,000	—	—	323,000,000	—	—	—	—	327,000,000	330,000,000
2. " + M.B.	Total	363,000,000	—	—	330,000,000	—	—	—	—	357,000,000	367,000,000
3. " + M.B.	Viable	73,720,000	30,510,000	3,390,000	8,052,000	0	0	0	0	0	133,900
4. " *	Viable	66,900,000	83,030,000	42,450,000	23,030,000	16,940,000	7,340,000	191,220	205,700	125,700	983,700
5. Lactate	Total	1,000,000,000	—	—	1,220,000,000	—	—	—	—	1,212,000,000	894,000,000
6. " + M.B.	Viable	171,000,000	123,700,000	63,700,000	44,910,000	11,870,000	5,932,000	308,600	843,000	291,000	25,710
7. " + M.B.	Total	1,018,000,000	—	—	1,034,000,000	—	—	—	—	876,000,000	1,040,000,000
8. " + M.B.	Viable	5,453,000	471,500	120,000	222,900	4,286,000	4,629,000	4,887,000	3,517,000	4,286,000	214,500
9. " *	Viable	210,000,000	90,000,000	33,000,000	14,800,000	4,661,000	3,000,000	271,500	225,000	132,000	7,310
10. Formate	Total	616,000,000	—	—	654,000,000	—	—	—	—	570,000,000	566,000,000
11. " + M.B.	Viable	194,800,000	46,610,000	67,790,000	10,590,000	9,745,000	4,661,000	34,300	154,300	56,010	8,570
12. " + M.B.	Total	694,000,000	—	—	724,000,000	—	—	—	—	674,000,000	450,000,000
13. " + M.B.	Viable	9,322,000	1,611,000	124,300	185,700	638,300	1,277,000	165,000	85,270	178,400	9,840
14. " *	Viable	152,400,000	119,900,000	93,240,000	74,730,000	8,664,000	5,484,000	3,368,000	2,887,000	1,987,000	3,273,000
15. Glucose	Total	832,000,000	—	—	1,002,000,000	—	—	—	—	762,000,000	859,000,000
16. " + M.B.	Viable	169,400,000	135,500,000	77,900,000	266,400,000 ¹	103,400,000	96,580,000	64,390,000	50,860,000	45,750,000	13,560,000
17. " + M.B.	Total	830,000,000	—	—	878,000,000	—	—	—	—	747,000,000	628,000,000
18. " + M.B.	Viable	119,600,000	110,100,000	18,880,000	4,237,000	2,966,000	10,590,000	7,628,000	38,140,000	43,220,000	118,870,000
19. " *	Viable	13,920,000	10,490,000	10,670,000	10,250,000	7,509,000	7,750,000	6,991,000	2,030,000	140,700	—
20. Succinate	Viable	165,900,000	111,100,000	84,330,000	43,190,000	12,850,000	4,230,000	4,268,000	3,210,000	2,762,000	1,663,000

¹ The suspension in this tube was badly affected with grease.

as those used by Quastel and Wooldridge [1927, 1, 2; 1928] in which the organisms are initially exposed to high concentrations of inhibitors such as toluene, KMnO_4 etc., this precaution is not so necessary. But the fact that growth has been observed to take place under conditions thought to be inhibitory of any increase in numbers, as described in the present paper, makes such controls generally advisable. It is true that this increase in numbers

Fig. 3. Showing the relationship between the concentration of "resting" *B. coli* and the velocity of reduction of methylene blue ($v=100/T$, where T =time of reduction in mins.) in the presence of various substrates. Concentration 16 of *B. coli* = approx. 5×10^8 cells per cc.

Table II. Showing the effect of an initial anaerobic incubation of variable duration at 45° and p_H 7.4 upon the times (in mins.) taken by "resting" *B. coli* to reduce M/25,000 methylene blue solution, the preliminary incubation taking place in the presence or the absence of the dye, as noted at the head of each column.

Period of first incubation at 45°	Lactate	Lactate + M.B.	Formate	Formate + M.B.	Glucose	Glucose + M.B.	Succinate	Succinate + M.B.
0 min.	10	8	8	9	13	12	8	8
5 mins.	10	12	12	17	15	15	6	8.5
10 "	8.5	9	7	7	13	14.3	7	8
15 "	12	7	7	8	11	19	8	8
20 "	9	11	8	8.5	11	21	10	8
30 "	12	10	11	7	14	13	10	11
45 "	13	10	8	6.5	11.3	12	14	12
1 hr.	11	13	6.5	8	11	12	14	15
2 hrs.	8	14	7	9	10	14.3	20	21
3 "	14	10.5	6.5	8	12.5	11.3	18	20
4 "	11	16	6.5	11	12.5	10.7	18	22
5 "	10	16	7	6.5	12	9.5	14	20
6 "	11	18	7	8	10	12.7	19	21
22 "	20	49	9	18	12.3	17.7	51	70

was small and transient and that it only occurred after the usual period of observation in these enzyme studies. (In the great majority of dehydrogenase experiments reported in the previous work upon these bacterial enzymes the period of observation did not exceed 30 mins. and so the conclusions of, and deductions drawn from, this work are in no way invalidated.) Nevertheless, owing to the recent extension of the use of so-called "resting" bacteria,

especially in America, it seems advisable to discontinue the use of this term before absolute confusion results. The only justification for the use of the term is if bacterial growth definitely does not take place, and as this is generally doubtful and rarely proven it is preferable that future workers along these lines should simply allude to the use of washed bacterial cells and describe the conditions under which they are employed.

SUMMARY.

1. It is shown that preparations described as "resting" bacteria consist both of cells that will proliferate when inoculated upon suitable media and of cells that are unable to grow under such conditions.

2. The total number of cells remains practically constant throughout long periods of anaerobic incubation at 45° in the presence of various substrates with or without methylene blue.

3. In the absence of the dye the number of viable organisms steadily decreases, whereas in the presence of methylene blue the viable count first shows a fall followed by a temporary rise, subsequently falling more slowly. An explanation of this phenomenon is suggested.

4. The enzymic activity of these preparations towards the various substrates used is shown to remain practically constant throughout the period of incubation.

5. The enzymic activities examined in this work are not solely dependent upon the presence of living cells; both viable and dead cells are active.

6. As the number of living cells may sometimes increase under conditions thought to be inhibitory of growth it is essential that control experiments assessing the number of viable organisms be conducted whenever the term "resting" bacteria is used.

7. It is preferable to discontinue the use of this term entirely and to refer simply to washed bacterial cells used under certain specified conditions.

REFERENCES.

- Bail (1929). *Z. Immunität*. **60**, 1.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Donald (1915). *Lancet*, ii, 1243.
Quastel and Stephenson (1925). *Biochem. J.* **19**, 660.
— and Whetham (1924). *Biochem. J.* **18**, 519.
— and Wooldridge (1925). *Biochem. J.* **19**, 652.
— — (1927, 1). *Biochem. J.* **21**, 148.
— — (1927, 2). *Biochem. J.* **21**, 1224.
— — (1928). *Biochem. J.* **22**, 689.
Wilson (1922). *J. Bact.* **7**, 405.