LXXIII. VARIABILITY IN THE ACTIVITY OF BACTERIAL ENZYMES

II. FACTORS ASSOCIATED WITH VIABILITY AND GROWTH

By WALTER REGINALD WOOLDRIDGE AND VLADIMIR GLASS

From the London School of Hygiene and Tropical Medicine

(Received 26 January 1937)

IN a previous paper [Wooldridge *et al.* 1936] it was shown that the activities of most bacterial dehydrogenases varied with the culture time of the organism. In the present paper this variation is studied in relation to the viability of the cells responsible for the enzymic activity.

EXPERIMENTAL

The bacterial suspensions used in this work were prepared and the activities of their enzymes examined by the methods described in the previous paper. The suspensions, which were adjusted to a strength equivalent to that of a suspension of *Bact. coli* containing 5×10^9 cells per ml., were examined by the Thunberg technique within an hour or two of their preparation: total and viable counts were made at the same time as the enzymic tests. The number of viable organisms present in the culture media at the time of reaping was also estimated. The total number of organisms present in the suspensions of washed cells was assessed by the technique described by Wilson [1922]. In this the Helber chamber is used and the unstained organisms are counted against a dark background. Viable counts were made by the agar-roll tube method described in the same paper. All dilutions were effected by the use of dropping pipettes [Donald, 1915].

Viability and the enzymic activity of bacterial suspensions

Bact. coli was inoculated into a series of Roux bottles containing 120 ml. of liquid medium, the flasks incubated at 37° and batches of them reaped after different periods of growth. Each harvest of bacteria was centrifuged from the medium, washed thrice with saline, aerated for 20 min. and adjusted to the required turbidity. Counts were made of the total and viable cells in each suspension at the time that its enzymic activities were examined. Four different media were used, namely caseinogen digest broth and chemically defined media consisting of an inorganic medium with added glutamate, lactate or glucose. All experiments gave similar results and for purposes of illustration the variations in the viable counts of the suspensions obtained from glutamate medium and the changing activities of their dehydrogenases are depicted in Fig. 1. To facilitate ready comparison the results for any one suspension are expressed in terms of the corresponding result of the suspension of the 8-hour-old organism, which was arbitrarily given a value of 100. It is clear from Fig. 1 that the activity curves of the enzymes do not follow closely the curve for the viable count of the suspension. In these experiments the proportion of viable to total cells present in the various suspensions varied between 2 and 84%. The total count of these

suspensions varied between -15 and $+10\%$ of 5000×10^6 cells per ml. and may be considered to be constant, so it is clear that the cells are not uniformly active enzymically.

Fig. 1. Comparison of variation in viable count with that of the activity of dehydrogenases of suspensions of washed *Bact. coli* cells grown for different periods of time on a glutamate-inorganic medium. The results obtained with the 8-hour suspension are given the value of 100: the actual results were viable count = 2315×10^6 , and the reduction times, in minutes, for the various substrates, formate = 4, lactate = 11.5, glucose = 6.7, succinate = 13, glutamate = 4.5, alanine = 7.1, leucine = 6.3 and tryptophan = 6.3. Other results are expressed in relation to the corresponding 8-hour result. The curves are designated by the use of the initial letters (except *Gt* = glutamate) of the particular substrate at the ends of the appropriate curve.

The results given in Table I were obtained with many different suspensions of *Bact. coli* throughout the course of this work, that proved to contain approximately the same number of viable and total cells, and these results show that the activities of the enzymes examined were not solely dependent upon the proportion of viable and total cells present. It was previously known [Quastel & Wooldridge, 1927] that the activities of formic, lactic and succinic dehydrogenases were not dependent on the viability of the cells, but this table shows that this conclusion applies also to the activities of the sugar and amino-acid enzymes.

Table I. *Enzymic activity of suspensions of Bact. coli containing equal numbers of total cells and of viable organisms*

The figures represent actual reduction time in minutes. The suspensions were obtained by the growth of *Bact. coli* 47 under a variety of conditions, but the final suspensions used contained 1500×10^6 viable organisms and 5000×10^6 total cells per ml. The substrates are given at the heads of columns.

Actual viable count	Formate	Lactate	Glucose	Glutamate	Alanine	Tryptophan
1605×10^6	7	5	6.5	8	8.7	74
1595	3	11	7	4.7	9	9
1580	3.5	15.5	9.3	4	7	52
1575	4	15.5	5.5	8.5	90	8.3
1575	6.5	12	9.7	7.5	8.3	24.5
1535	2.7	3	5	3.5	5.5	19
1485	23.5	20	12.5	17	12.5	22
1475	2.4	20	6	5.5	112	—
1445	3	11.5	9	4.3	9.5	50
1445	4.5	11.7	6.5	4.3	180	133
1445	35	59	26	38	27	69
1425	3.7	5.5	3.7	6.5	10.7	5.5
1420	7.5	23	11	12	43	43
1415	3.5	11	8	6.3	16	8
1415	3.7	18	4	10	37	7

Table II. *The effect of storage at 0° upon the activity of dehydrogenases and upon the viability of a suspension of Bact. coli*

The suspension was prepared in the usual way and stored in a strength equivalent to 20×10^9 cells per ml. It was diluted fourfold for examination of the enzymes. The total count (Helber method) of the suspension remained constant, that for the 0-day suspension = 5590×10^6 , and that for the 50-day suspension = 5860×10^6 . The figures represent comparative enzymic activities expressed in terms of the 0-day results, as described for Table I.

Days at 0° ...	Enzymic activity					Actual reduction
	0	5	11	29	50	time (min.)
Formate	100	100	90	82	69	0
Lactate	100	102	79	62	44	2.7
Glucose	100	70	56	56	25	8.7
Fructose	100	96	93	82	39	7.7
Succinate	100	83	59	53	45	7
Glutamate	100	67	50	46	28	10
Alanine	100	77	62	34	0	18.5
Viable count	100	49	45	38	16	25
						1150×10^6 per ml.

The work of Yudkin [1933] suggests that certain bacterial dehydrogenases require the presence of coenzymes (boiled organism) for their maximum activity. Similar experiments to his indicate that, besides lactic and glucose dehydrogenases, succinic dehydrogenase requires a coenzyme. Dilution experiments with the suspensions of *Bact. coli* showed that the activity of the enzymes for alanine, leucine and tryptophan fell off rapidly with dilution, whilst the decline in the activity of the glutamate dehydrogenase is linear. In this respect glutamic dehydrogenase resembles formic dehydrogenase. Hence to avoid changes in enzymic activity due to "coenzyme" factors, either conditions should be chosen which do not alter these factors, or enzymes, such as those for formic and glutamic acids, independent of "coenzymes" should be examined. It was found that storage of a bacterial suspension at 0° resulted in a decline in the activity of the enzymes, but that the "coenzyme" factors which can be supplied by boiled organism are not destroyed. Sometimes lysis occurred, but often no lysis would take place over a period of two months. In Table II, figures are given showing changes in the activity of the enzymes and comparative figures for the variations in the number of viable cells. It is seen that the decline in enzymic activity of all enzymes, including that for the formic and the glutamic dehydrogenases, is less rapid than is the fall in viable count.

That there is no close correlation between viability and the activity of the glutamate enzyme of bacterial suspensions obtained from growth upon various media for different periods of time is again brought out clearly by the curves of Fig. 2.

Dehydrogenase activity in relation to the growth curve of the organism

Comparison of the number of viable organisms present in a culture medium at the time of reaping with the enzymic activity of the resulting bacterial suspension shows that the activity of most enzymes increases rapidly during the early part of the period of maximum growth, reaches its highest value during this phase and generally diminishes before the number of viable organisms present in the culture has reached its peak. The results of Fig. 3 are illustrative of many experiments carried out to test this conclusion; similar results are obtained for other dehydrogenases. They suggest that cells produced in the logarithmic phase are enzymically more active than cells present at other phases of the growth curve of the bacterial population.

Fig. 2. Relationship between the viable count (●—●) and activity of glutamate dehydrogenase (•- - -•) of suspensions of *Bact. coli*, the suspensions being obtained from organisms grown for different periods of time from the same inoculum. The graphs illustrate the results obtained with four different media, viz. (a) caseinogen digest broth, (b) glutamate inorganic medium, (c) lactate inorganic medium and (d) glucose inorganic medium.

Fig. 3. Enzymic activity and the growth curve (logarithmic) of *Bact. coli* grown on lactate medium. The various batches of culture medium were inoculated from the same suspension of washed cells and the viable counts were made just before reaping. The reduction times, in minutes, obtained with the 8-hour suspension were lactate=4.5, glucose=6, glutamate=9 and alanine=9.5. The activities of this suspension were given a value=100, and the activities of other suspensions expressed in terms of these values.

- - -• Curve giving logarithm of viable count of culture medium.
- Activity of glutamic enzyme.
- Activity of alanine enzyme.
- Activity of lactic enzyme.
- ×—× Activity of glucose enzyme.

DISCUSSION

It has been shown that standardized suspensions of washed bacterial cells exhibit marked differences in the activities of their dehydrogenases with the time of growth of the organism. This variability is not associated with the number of cells present, nor is it dependent upon the presence of some "activator" supplied by the broth, for it was equally marked when the organisms were grown upon media of simple chemical composition. Viability of the cell may enhance the enzymic activity to some extent, but most enzymes, including the amino-acid and sugar dehydrogenases, seem to be considerably independent of viability.

The enhanced activity of some bacterial suspensions is not dependent upon an ability to proliferate in the Thunberg tubes, for Sandiford & Wooldridge [1931] showed that the growth of *Bact. coli* under the conditions of these experiments was negligible, while Wooldridge *et al.* [1936] demonstrated similar variability at 45° in the activity of the dehydrogenases of *Bact. aerogenes*, an organism which will not grow at 45°. It is possible that cellular changes may take place in the Thunberg tubes, and that these may affect enzymic activity.

When the changes in enzymic activity are compared with the phase of growth of the original bacterial population from which the cells were reaped it is clear that those cells reaped during the phase of increased growth exhibit the maximum enzymic activity. Generally this activity, for all enzymes, lessens before the stationary stage sets in, and continues to fall during the stage of decline. Frequently the maximum enzymic activity appears in the early period of the phase of maximum growth and occasionally it has been shown to be present at the very beginning of this phase. These results accord with those of many others, e.g. Sherman & Albus [1923], who demonstrated during this phase a condition of "physiological youth", when the bacterial cells are more susceptible to injury. To demonstrate satisfactorily an enhanced enzymic activity during the lag phase itself by a standardized technique is no easy matter for there is always the difficulty of securing an adequate concentration of the "rejuvenated" cells so that their activity is not masked by that of the greater numbers of other cells. Furthermore, the necessity for washing the cells so that the tests can be made under standard conditions may cause greater injury, with corresponding decline in enzymic activity, during the lag phase, than during the other phases. The washing procedure appears to have some effect upon the cell, for the final suspensions, as judged by total counts, seem to contain cells of similar size, the swollen forms of the late lag period [Clark & Ruehl, 1919] being apparently absent from the bacterial suspensions prepared from cultures of short incubation. Nevertheless, in spite of the disadvantages of the technique adopted, it is clearly shown that there is a rapid rise in the activity of bacterial dehydrogenases during the early period of growth, and that this activity usually reaches a maximum during the logarithmic phase. It seems probable that this increased activity develops earlier, e.g. during the lag phase, than is shown by this technique. This method of examining the cell under standard conditions is possibly a more valuable method of analysis than that of following changes in the metabolic rate (as evidenced by ammonia or carbon dioxide production, etc.), for bacterial populations actually growing in relatively complex media are in continuously changing environment and deductions from such results will be inconclusive. The work of such authors as Walker *et al.* [1934] is valuable in demonstrating clearly that the metabolic rate of bacterial cells increases to a high value towards the end of the lag phase, but the apparent early and rapid decline of this activity would appear to be less certain and is probably associated

with the rapidly changing conditions of their experimental media. While there is general agreement [Topley & Wilson, 1936] that "the lag phase appears to be essentially a phase of rejuvenescence" there is practically no evidence suggesting what physiological changes are inherent in this "rejuvenescence". The evidence presented in this paper strongly supports the view that during the lag phase cellular structures are in a state of fluctuation and suggests that, influenced in part by environmental conditions, the activities of appropriate enzymes such as dehydrogenases are considerably increased during this and the immediately ensuing period.

SUMMARY AND CONCLUSIONS

1. The variation in the activities of dehydrogenases, exhibited by standardized suspensions of washed cells of *Bact. coli* obtained by growing the organism for different periods of time before reaping, is not associated with a variation in the number or size of the individual cells present in the different suspensions.

2. Viability of the cells may have some effect upon the enzymic activity of bacterial suspensions. This and previous work indicates that the activities of the formic, lactic and succinic enzymes are relatively independent of viability, and that the enzymic activities for glucose and the amino-acids are more affected by cellular viability. The activities of the tryptophan, alanine and leucine enzymes may vary considerably from suspension to suspension even though the number of viable cells remains about 80 % of the total.

3. The great variation in enzymic activity exhibited by organisms grown upon the same medium seems to be most directly associated with the phase of growth of the bacterial population at the time when the cells are harvested.

4. During the lag phase in the growth of a bacterial population the cell appears to be in a state of structural fluctuation which results in the development within the cell of dehydrogenase systems of a high degree of enzymic activity, which reaches a maximum, under the experimental conditions used, during the logarithmic phase; the falling off in enzymic activity which occurs towards the end of this phase continues during the stationary stage and the period of decline.

Our thanks are due to the Medical Research Council for a personal grant to one of us (V. G.) and for a grant towards the expenditure entailed in this work.

REFERENCES

- Clark & Ruehl (1919). *J. Bact.* **4**, 615.
Donald (1915). *Lancet*, ii, 1243.
Quastel & Wooldridge (1927). *Biochem. J.* **21**, 148.
Sandiford & Wooldridge (1931). *Biochem. J.* **25**, 2172.
Sherman & Albus (1923). *J. Bact.* **8**, 127.
Topley & Wilson (1936). *The Principles of Bacteriology and Immunity*. (London.)
Walker, Winslow, Huntington & Mooney (1934). *J. Bact.* **27**, 303.
Wilson (1922). *J. Bact.* **7**, 405.
Wooldridge, Knox & Glass (1936). *Biochem. J.* **30**, 926.
Yudkin (1933). *Biochem. J.* **27**, 1849.