THE PRODUCTION OF ACTIVE RESTING CELLS OF STREPTOCOCCI

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Nearly all of the recent studies relating to the nutrition of microorganisms have been directed toward raising the largest possible cell crop in a medium of known chemical composition, without regard to the physiological activity of the cells harvested from the medium. The purpose of the present study has been to produce resting cell suspensions of streptococci with sufficient activity and stability to permit metabolic studies. In this first attempt to obtain highly active cells, knowledge of the chemical composition of the medium has been sacrificed. It should not be too much to expect, however, that it will be possible to raise, in media of known composition, cells of high physiological activity.

A number of reports in the literature, including those of Farrell (1935) and Callow (1926) indicate the difficulties encountered in the preparation of physiologically active suspensions of streptococci.

METHODS

A typical strain of *Streptococcus mastitidis*, number 70b, (Lancefield group B) from the departmental stock culture collection was used in the experiments here reported. The general conclusions have, however, been confirmed with several strains of group B organisms, including two from human sources.

The Thunberg method using methylene blue as the hydrogen acceptor was employed as a criterion of cellular activity, or more precisely of dehydrogenase activity. This method was chosen with the hope of detecting mechanisms in the cells other than the pure lactic fermentation for which these organisms are known. The method also has the advantage of being rapid, and easy enough to handle a number of cultures when necessary.

The Thunberg experiments were run at 40°C. in the conventional tubes with the following quantities of reactants:

In side arm	1 ml. 1:4,000 methylene blue
In tube	2 ml. M/15 phosphate buffer pH 7.2
	1 ml. M/20 substrate
	1 ml. cell suspension in M/30 phosphate buffer. (Cells 10 times the concentration in which they grew)

The tubes were allowed to come to the temperature of the bath and evacuated. After sufficient time for temperature equilibrium to be reached, the methylene blue was added from the side arm. The time required for 90 per cent reduction of the methylene blue present was recorded (Quastel and Whetham, 1924). In

most experiments this reduction time was relatively short, but agreement between duplicate tubes was well within 10 per cent.

Methylene blue as a hydrogen acceptor for work with streptococci has been objected to on the basis of its toxicity toward this group of organisms (Farrell, 1935). It is true that a very low concentration of methylene blue is toxic to the growth of streptococci, especially in broth. However, in these experiments, with resting cell suspensions of group B streptococci the toxicity of methylene blue was not an important factor. Figure 1 indicates that, with the cell concentration used, the toxicity of methylene blue is not apparent until a concentration greater than one part in twelve thousand is reached, as indicated by a linear relationship between reduction time and methylene blue concentration. One part of methylene blue in twenty thousand final concentration was selected as a satisfactory level. In earlier experiments one part of methylene blue in

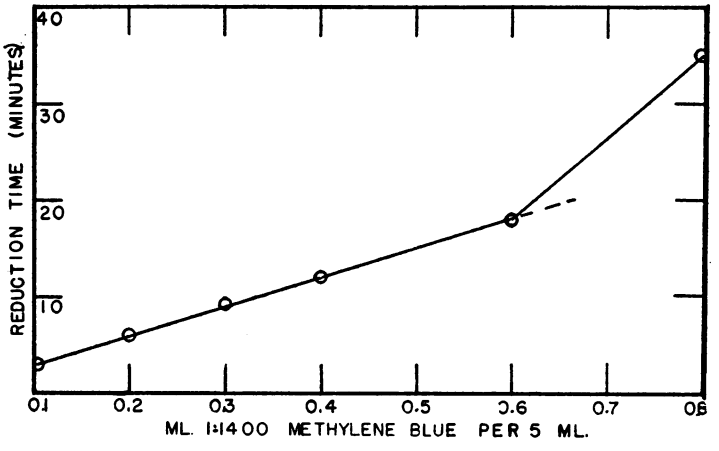

FIG. 1. EFFECT OF METHYLENE BLUE CONCENTRATION ON REDUCTION TIME

forty thousand was used and found to be satisfactory with more dilute cell suspensions.

Glucose was used as substrate in determining conditions for the production of active cells. Its use could be criticised on the basis of the complexity of the reactions which it undergoes with these organisms. On the other hand, although glucose is readily metabolized by these organisms, adverse conditions frequently affect reactions with it more readily than with other substrates (Quastel and Wooldridge, 1927). In addition, glucose permits the study of adaptive enzymes for various carbohydrates.

The amount of growth was expressed in terms of bacterial nitrogen, as determined by the micro-kjeldahl method of Pregl (Niederl and Niederl, 1938). This method was selected as giving more accurate and reproducible results with these long chain-forming organisms than would the conventional plate count (Mueller, 1935). The method is not unduly tedious for the number of determinations required and gives the added advantage of yielding results rather promptly. No

attempt has been made to determine the proportion of dead cells in the suspensions.

Preliminary studies of factors involved in the preparation of cell suspensions included the age of the culture, incubation temperature, size of inoculum, and mode of washing the cells. For a given medium, cells of satisfactory activity could be obtained by growing at 37°C. and harvesting near the end of the logarithmic growth phase. Three washes with $\frac{1}{3}$ the growth volume of M/30 phosphate, pH 7.2, produced cells with a low endogenous rate of reduction of methylene blue without material decrease of the reduction rate in the presence of substrates. In all experiments recorded in this paper the time for methylene blue reduction in the absence of substrate was over two hours. In studies with substrates which had high reduction rates, less washing could be used without the endogenous respiration accounting for too great a percentage of the experimental values. Twelve to fifteen-hour cultures grown at 37°C. from a 0.1 per cent inoculum fulfilled the conditions described above and were therefore used in the following experiments.

After washing, the cells were suspended in $\frac{1}{10}$ their growth volume and 1 ml. of the suspensions was used in the Thunberg tubes, as described above. Thus, the values for bacterial nitrogen (figs. 2–5) are milligrams per 10 ml. of growth medium, equal to mgm. per ml. of cell suspension. Dilution of the cell suspensions gave the conventional type of curves as indicated by Quastel and Wooldridge, (1924) with *Escherichia coli* and by Farrell (1935) with streptococci. The conditions in these experiments were such that doubling the cell concentration approximately halved the reduction time, and halving the cell concentration approximately doubled the reduction time.

RESULTS

A number of media, as well as the effect of varying the amount of carbohydrate, were studied before it was found that cells of predictable activity could be grown in a medium composed of Bacto-tryptone, yeast extract, phosphate buffer, and a small amount of glucose. By varying the concentration in the growth medium of each of the above constituents while the others were held constant, the effect of each on the physiological activity and stability of the cell suspensions prepared from different media was determined.

Effect of tryptone

The effect of varying the concentration of tryptone from 0 to 2 per cent in a medium containing 1 per cent yeast extract, 0.5 per cent K_2HPO_4 and 0.1 per cent glucose is shown in figure 2. The quantity of bacterial nitrogen increased linearly from 0.13 mgm. per 10 ml. of medium in the absence of tryptone to 0.26 mgm. with 2 per cent tryptone, or a twofold increase. The rate of reduction of methylene blue by resting cell suspensions was three times as great in a medium containing 1 per cent tryptone as in a medium without tryptone. The increase in quantity of cells, about 50 per cent in the presence of 1 per cent tryptone, is not sufficient to account for the increased activity. A further increase

in tryptone from 1 to 2 per cent gave approximately the same increase in growth as from 0 to 1 per cent, with only a slight increase in the methylene blue reduction rate. In subsequent experiments 1 per cent tryptone was used as a satisfactory concentration.

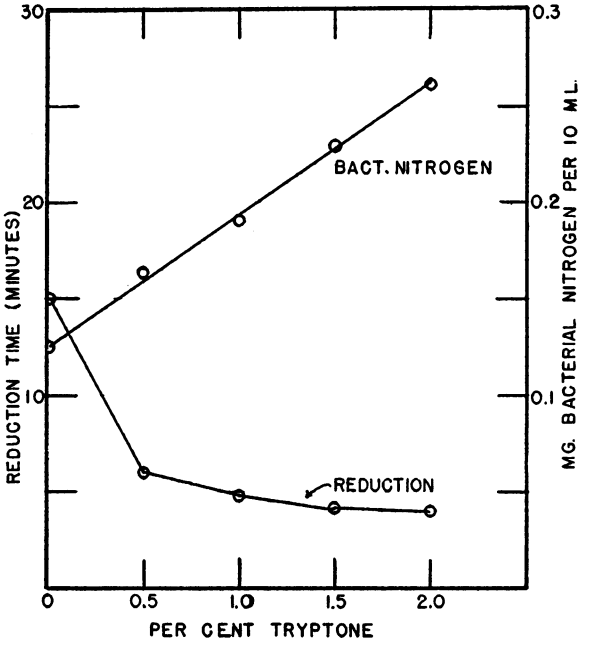

FIG. 2. EFFECT OF TRYPTONE CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP AND RATE OF METHYLENE BLUE REDUCTION BY THE CELL SUSPENSIONS HARVESTED FROM THE MEDIUM

Effect of yeast extract

The effect of varying the yeast extract concentration from 0 to 2 per cent in a medium containing one per cent tryptone, 0.5 per cent K_2HPO_4 and 0.1 per cent glucose is shown in figure 3. Only a small increase in bacterial nitrogen occurred with the increase in the yeast extract concentration, but a very marked increase in the reduction rate did occur. The addition of 0.2 per cent yeast extract, to the growth medium, increased the methylene blue reduction rate of the cell suspensions harvested from the medium by 400 per cent, whereas the growth was increased by less than 10 per cent. Further increase in the yeast extract concentration from 0.2 to 1 per cent approximately doubled the reduction rate, while the growth increased by about 15 per cent. Further additions of yeast extract, up to 2 per cent, increased neither the quantity of growth nor the activity of the cells harvested from the medium.

Upon storage of the cell suspensions in the refrigerator those cells harvested from media containing yeast extract were more stable than those from media which contained only tryptone, phosphate, and glucose (table 1). Cells har-

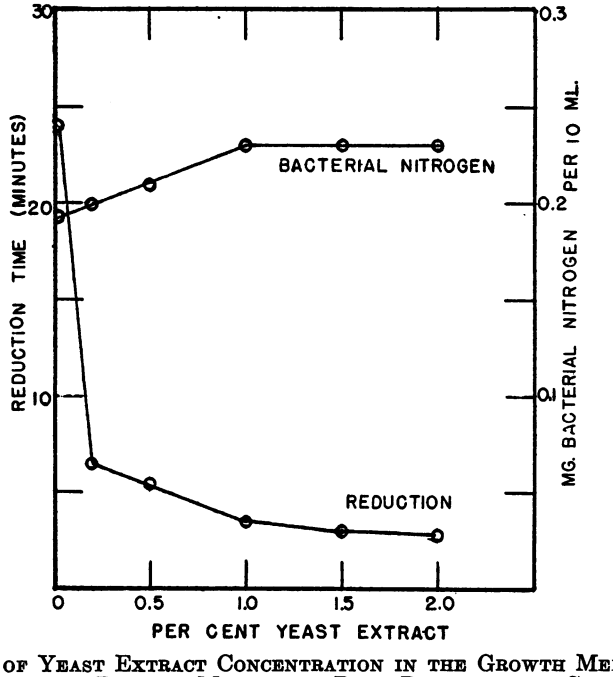

FIG. 3. EFFECT OF YEAST EXTRACT CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP AND RATE OF METHYLENE BLUE REDUCTION BY CELL SUSPENSIONS HARVESTED FROM THE MEDIUM

TABLE 1

Effect of growth in yeast extract on the stability of cells during storage

CONC. OF YEAST EX. IN MEDIUM	MINUTES FOR 90 PER CENT REDUCTION OF 1:20,000 METHYLENE BLUE		ACTIVITY RETAINED
	Immediately after centrifuging	After 24 hours at 8°C.	
Twelve-hour cells			
per cent			*per cent*
0	24.00	>60	<40
0.2	6.00	8.8	68
0.5	5.25	8.0	65
1.0	3.50	5.5	65
1.5	3.10	3.4	91
2.0	2.80	3.3	85
Twenty-four-hour cells			
0	28.0	>60	<45
0.2	10.5	23	45
0.5	8.0	19	42
1.0	5.5	10.5	52
1.5	3.6	7.0	50
2.0	3.10	6.0	50

vested from 12-hour cultures, in addition to having greater activity, were more stable than those harvested from 24-hour cultures. Therefore 12- to 15-hour cultures were used.

A concentration of 1 per cent yeast extract was considered satisfactory for most work, but occasionally 1.5 per cent was used when cells were to be stored. No attempt has been made in this work to ascertain which constituent or constituents of the yeast extract were responsible for this marked effect.

Effect of buffer

The buffer concentration in a medium containing 1 per cent each of tryptone and yeast extract and 0.1 per cent glucose was varied from 0–0.1 molar (fig. 4)

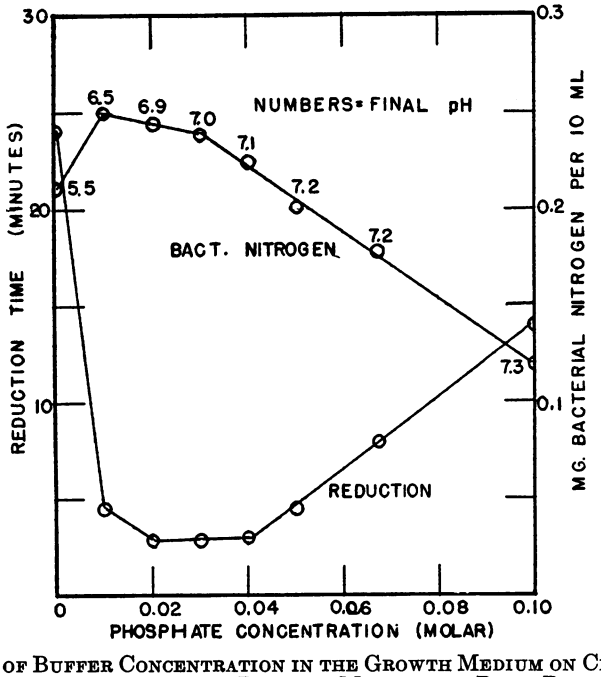

FIG. 4. EFFECT OF BUFFER CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP, FINAL pH IN THE MEDIUM, AND RATE OF METHYLENE BLUE REDUCTION BY CELLS HARVESTED FROM THE MEDIUM

Small concentrations of buffer increased the quantity of growth slightly. This is compatible with the work of Hewitt (1932) which indicates a stimulation in the rate of the lactic fermentation on the addition of phosphate. On the other hand, the stimulatory effect may be due to the buffering capacity which would keep the reaction at a favorable level for growth over a longer period of time. Concentrations of buffer above 0.04 molar showed an inhibitory effect on the growth of these group B streptococci. The methylene-blue reduction rate was increased markedly up to a concentration of 0.02 molar phosphate. Although the concentration of phosphate ions over this range may be of importance in determining the cellular activity, the increased buffering action must not be over-

looked. (The effect of the final pH in the growth medium will be discussed with the effect of the sugar content of the medium.) In the range from 0.02 to 0.04 molar phosphate the amount of growth and the reduction rate, as well as the final pH of the medium, (indicated by numbers above bacterial nitrogen curve) were approximately constant. Above 0.04 molar phosphate the cellular activity, as well as the amount of growth, decreased. Therefore, a phosphate concentration of 0.03 molar (0.5 per cent) was used for growth of group B organisms.

Effect of glucose

By varying the glucose concentration in a medium containing 1 per cent each of tryptone and yeast extract and 0.05 per cent K_2HPO_4, a marked effect of the

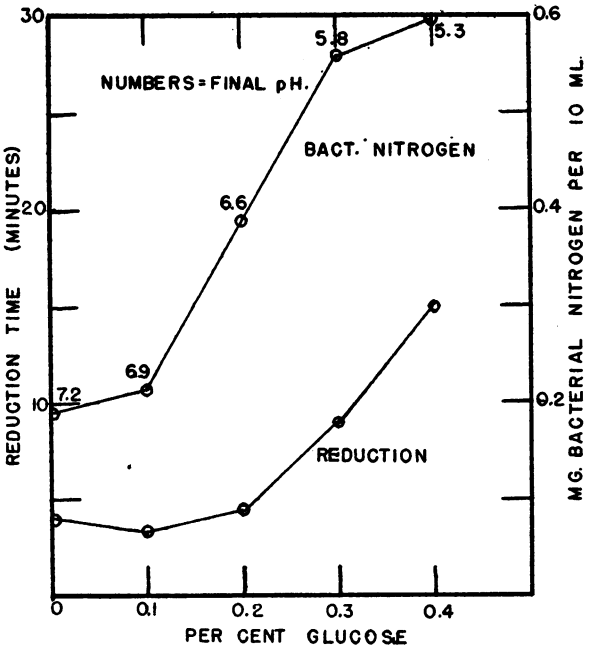

FIG. 5. EFFECT OF GLUCOSE CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP, FINAL pH IN THE MEDIUM, AND RATE OF METHYLENE BLUE REDUCTION BY THE CELLS HARVESTED FROM THE MEDIUM

final pH in the growth medium upon the activity and stability of the cells appears (fig. 5). The numbers just above the bacterial nitrogen curve indicate the pH in the growth medium from which the cells were harvested. Cells grown in a medium which became more acid than pH 6.8 had less activity than those grown in a medium which was not allowed to reach this acidity. Very great decrease in the cell activity occurred when the final pH in the growth medium fell below 6.5. As can be seen from figure 5, there is a marked decrease in the methylene blue reduction rate in spite of a great increase in the cell nitrogen. With the higher levels of glucose the endogenous respiration was increased somewhat.

DISCUSSION

The Thunberg method is applicable to the streptococci, which have strong dehydrogenase activity when the cells are properly prepared. The toxicity of methylene blue for these organisms is not so great in resting suspensions as in growing cultures. The more exacting nutritional requirements, the generally low level of synthetic powers, and the lack of an adequate aerobic mechanism among these organisms necessitate more exacting conditions for the preparation of resting cells. For example, strong aeration, as suggested by Quastel and Whetham (1925) for the removal of reducing material from suspensions of *E. coli* in the production of resting cells has been used by Farrell (1935) and Katagiri and Kitahara (1938) with streptococci and lactobacilli. The aeration, satisfactory in the preparation of resting cell suspensions of *E. coli*, is not only unnecessary but deleterious when applied to these lactic acid types. With streptococci the age of the culture is of more importance than with some types of bacteria.

The necessity of accessory factors or amino acids for the production of active cells is indicated by the stimulatory effect of higher levels of yeast extract and tryptone.

The fact that it is possible to raise resting cells of increased activity, that is, greater rate of methylene-blue reduction without increased growth, in a medium rich in yeast extract, suggests the possibility of a higher level of accessory substances per cell. Additional evidence of this possibility is offered by the observation that above a certain level, when more cells are raised per volume of medium, the activity is related to the volume of medium from which the cells were harvested and not to the quantity of cells.

The decrease in cellular activity when the final pH in the growth medium falls serves also to indicate the sensitivity of these organisms. This result recalls the observation of Lwoff and Lwoff (1937) on the sensitivity of *Haemophilus influenzae* to lowered pH in the growth medium.

It is our opinion that a number of observations on the absence of dehydrogenase activity in streptococci, and variability in activity of cell suspensions, may be traced to variation in one or another of these factors.

The use of methylene blue as a hydrogen acceptor is a method of setting up an artificial system the reaction rate of which may not be an accurate index to the metabolism of the organism. For example, it has not been shown that the conditions outlined for the production of cells with a high dehydrogenase activity, using methylene blue as the hydrogen acceptor, are necessarily the best conditions for the lactic fermentation. The criticisms offered by Barron and Jacobs (1938) as to the possibility of not observing certain dismutation reactions by this method has not been considered. This would not, however, invalidate positive results obtained by the method.

It is possible to produce streptococcus cells of high metabolic activity and low endogenous metabolism by growing them in a medium containing a high concentration of nitrogenous constituents and accessory factors and a low concen-

tration of carbohydrate, or energy source. Under these conditions the cells will cease to multiply for lack of energy source while they are in a neutral medium of high nutritional quality. Because their energy-supplying mechanism is depleted, the cells will have a low endogenous metabolism. This is more easily accomplished with streptococci than with a number of other organisms because fewer substances serve as satisfactory energy sources.

SUMMARY

Resting cell suspensions of streptococci of high physiological activity, low endogenous respiration, and fair stability have been produced. This has been accomplished by growing them in a well-buffered medium, rich in nitrogen and accessory factors, and low in carbohydrate, followed by washing in neutral phosphate buffer. It has been shown that streptococci grown under these conditions have, in resting suspensions, strong dehydrogenase activity.

The cells should be harvested near the end of the logarithmic growth phase, from a medium that is not allowed to become more acid than pH 6.8.

Methylene blue is not toxic to resting cell suspensions of group B streptococci at a concentration of 1 part in 20,000. Therefore, this concentration, or less, of methylene blue is satisfactory as a hydrogen acceptor in dehydrogenase studies.

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