ANALYSIS OF RESPIRATION DURING GERMINATION AND ENLARGEMENT OF SPORES OF BACILLUS MEGATERIUM AND OF THE FUNGUS MYROTHECIUM VERRUCARIA

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Considerable attention has been given in many publications to mathematical interpretations of growth curves. The changes in metabolic activity, as measured by respiration, which occur during the transformation of dormant cells into actively growing ones are lacking such treatment. In a theoretical analysis, Buchanan (1953) has tacitly assumed that exponential growth occurs during this transformation.

The present report is concerned specifically with an analysis of the respiration curves during germination and growth up to inception of cell division of *Bacillus megaterium* and during germination of *Myrothecium verrucaria* spores. The total O_2 uptake curves for both organisms are parabolic, being characterized by two phases of linear increase in rate for *B. megaterium* and one for *M. verrucaria*.

Methods

(*a*) *Spores.—Bacillus megaterium*, QM B1551, was grown on liver broth for 5 days at 30°C., on a reciprocal shaker. The spores were harvested by centrifugation at 4°C., washed four times at the same temperature, and preserved by lyophilization (Levinson and Sevag, 1953, for details). Suspensions of the lyophilized spores were made up as needed in 0.05 M KH_2PO_4 (pH 6.95) + 0.02 M NaCl + 0.02 M ammonium acetate.

Spores of the fungus, *Myrothecium verrucaria*, QM 460, were obtained from cultures grown at 30°C. on a medium containing 15 gm. agar; 3.0 gm. NH_4NO_3; 2.22 gm. $MgSO_4 \cdot 7H_2O$; 2.59 gm. KH_2PO_4; and 2.21 gm. K_2HPO_4 in 1,000 ml. of water. The carbon source was a 7 cm. circle of Whatman No. 1 filter paper. Spores were washed by centrifugation and suspended in the inorganic salts solution (Mandels, 1951, for details).

(*b*) *Respiration.*—Oxygen consumption was measured at 30°C. by conventional Warburg techniques. Two-tenths ml. of 10 per cent KOH was used in the center wells. For the *Bacillus*, 1.2 ml. of spore suspension containing 1.0 mg. of lyophilized spores was placed in the vessels, and 0.3 ml. of 2.5 per cent Bacto peptone + 0.1 per cent Bacto yeast extract + 0.125 M glucose dissolved in the phosphate buffer in the side arms. For *Myrothecium*, 1.0 ml. of spore suspension was placed in the vessels and 0.5 ml. of 3.0 per cent sucrose + 3.0 per cent yeast extract in inorganic salts

solution in the side arms. Dry weights of the suspension were obtained after filtering and washing aliquots in fine sintered glass crucibles.

(*c*) *Heat Shock.*—Heat shock was effected by exposure to a temperature of 60°C. for 15 minutes.

(*d*) *Germination.*—Germination of *B. megaterium* spores was determined periodically by removing samples from suspensions incubated in Warburg vessels as in (*b*). Air-dried smears were stained with 0.5 per cent aqueous methylene blue. Ungerminated, heat-resistant spores appear as unstained refractile bodies. Germinated spores are no longer heat-resistant and take up the stain (Powell, 1951; Levinson and Sevag 1953).

(*e*) *Turbidity.*—One ml. of bacterial suspension incubated in Warburg vessels as in (*b*) was added to 5.0 ml. of distilled water. Optical density of the resulting suspension was measured in a Klett-Summerson photoelectric colorimeter using a No. 56 (540 to 590 mμ) filter.

RESULTS

1. B. megaterium.—Respiratory activity increases rapidly with both heated and unheated spores in the complex medium employed (Fig. 1). Examination of the rate curves shows two distinct phases, both characterized by linear increases but of different slopes (Fig. 2). Data for the first 10 minutes indicate that there is a rapid increase in respiratory activity and that the initial respiratory activity may be close to zero and not the value obtained by extrapolation to zero time.

Observations were made to determine whether the changes which occur during germination of bacterial spores could be correlated with the phases of the respiration curve. The optical density of suspensions of heated spores drops rapidly from an initial value of 0.136 to 0.076 in 6 minutes and to 0.060 in 10 minutes. This coincides with the apparent rapid initial increase in respiration, with the microscopically observable loss in refractility, and with inception of stainability of the spores. The low level of optical density is maintained to about 80 to 90 minutes, during which period the diameter of the spores increases 2 to 3 times. These changes coincide with the first phase of linear increase in respiratory rate.

Concurrent with the second linear phase, *i.e.* from *ca.* 90 to 180 minutes—the spore cases rupture, elongation of the emerged cells occurs, and the optical density increases to about 0.20 at 180 minutes. Under the experimental conditions of spore and substrate concentrations, only limited cell division is observed and the presumed transition into exponential growth is not noted.

With the complex media employed, the main differences between heated and unheated spores are in the initial respiratory rates and in the slopes of the rate curves. In general, the microscopically observable changes in unheated spores lag about 10 minutes behind the heated spores. In less complex media than employed here, there is a much greater difference between heated and unheated

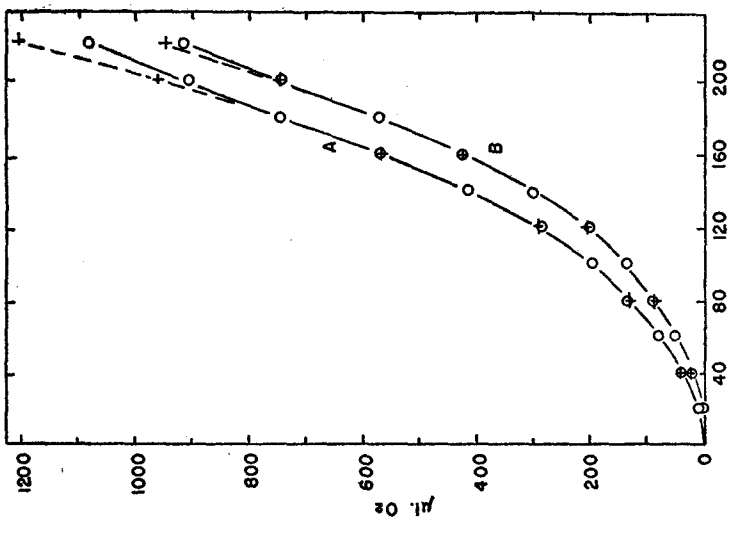

FIG. 1

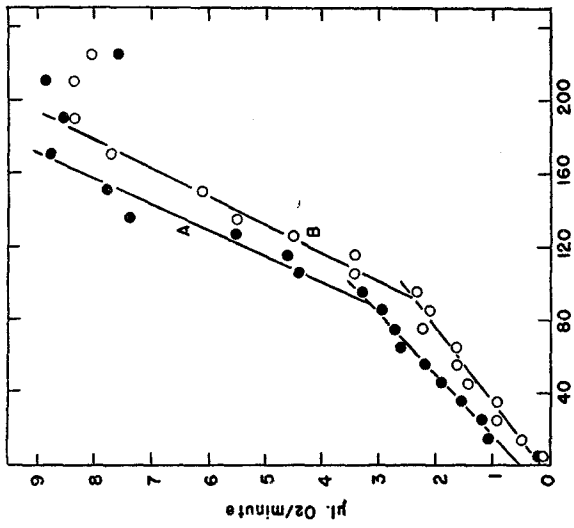

FIG. 2

FIGS. 1 and 2. Respiration during germination of *B. megaterium* spores. Curve A, heated spores; curve B, unheated spores, both 1.0 mg. per vessel. Fig. 1. Solid lines connect experimental points (circles); dashed lines connect points (crosses) calculated according to Equation $y = bt + \frac{K}{2}t^2 + C$. Constants evaluated from data in Fig. 2 by method of least squares. For curve A from 0 to 100 minutes, $b = 0.415$ μl./min., $K = 0.0311$ μl./min.^{2}, $C = 0$; from 100 to 180 minutes, $b = 3.94$, $K = 0.0742$, $C = 197$. For curve B from 0 to 100 minutes, $b = 0.154$, $K = 0.0241$, $C = 0$; from 100 to 200 minutes, $b = 2.92$, $K = 0.0640$, $C = 136$.

spores (Levinson and Hyatt, 1955). Furthermore, the second linear phase may not be observed if the bacilli fail to emerge in the medium used.

2. Myrothecium verrucaria Spores.—Rapid growth and germination occur in sucrose–yeast extract medium (Mandels and Darby, 1953). The effect of the yeast extract is to provide a complex of nutrients and accessory growth factors. Release of a trigger mechanism is not involved, since rapid growth and respiration cease upon removal of the yeast extract and suspension in sugar solution containing inorganic salts. Experimentally, relatively dense suspensions of spores are used to measure respiration. The extent of growth and germination can thus be limited by the quantity of nutrients available. Thus, the course of respiration in this medium is a function of spore concentration (Fig. 3). At both spore concentrations, the rate of respiration increases linearly with time (Fig. 4). The levelling off of the rate, followed by a decline, is due to depletion of nutrients and/or accessory factors provided by the yeast extract and cannot be ascribed to attainment of a particular stage such as protrusion of a germ tube. No break can be observed in the respiration curves at the time of germination (*i.e.* protrusion of a germ tube) which would occur at about 2 hours in the more dilute suspension. Only low germination would occur in the denser suspension.

In simpler media such as sucrose plus nutrient salts, in which relatively slow growth and germination occur, the oxygen uptake curves are more or less linear (see Fig. 4, Mandels, 1954).

DISCUSSION

Of primary interest here is an analysis of the changes in metabolic activity of spores during germination. Data show that the rate of respiration per cell (R) increases linearly with time, *i.e.*:

$$R = dy/dt = Kt + b \tag{1}$$

in which y = oxygen uptake in microliters, t = time in minutes, K = slope of the rate *vs.* time plot, and b = the y intercept. By integration of (1) we get the parabolic equation,

$$y = bt + \frac{K}{2}t^2 + C \tag{2}$$

For practical purposes, the curves (Figs. 1 and 3) can be considered to start at the origin. Hence, $C = 0$, and

$$y = bt + \frac{K}{2}t^2 \tag{3}$$

With *B. megaterium* spores two phases are evident, both of parabolic form but differing in the acceleration in respiratory activity. For the second phase of

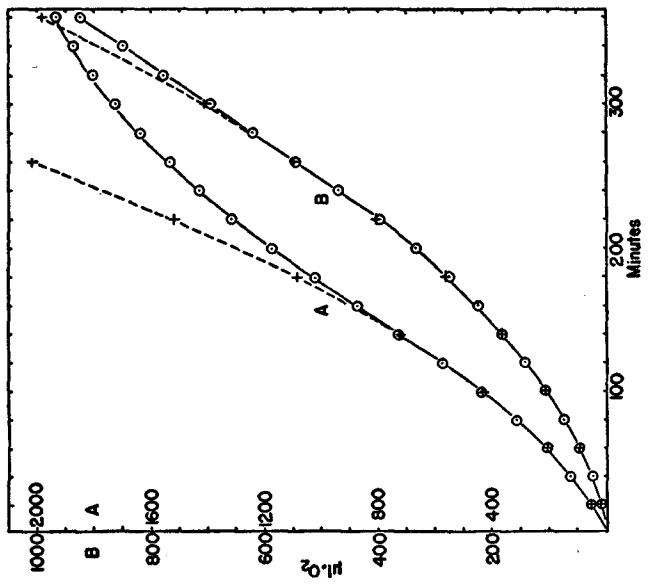

FIG. 3

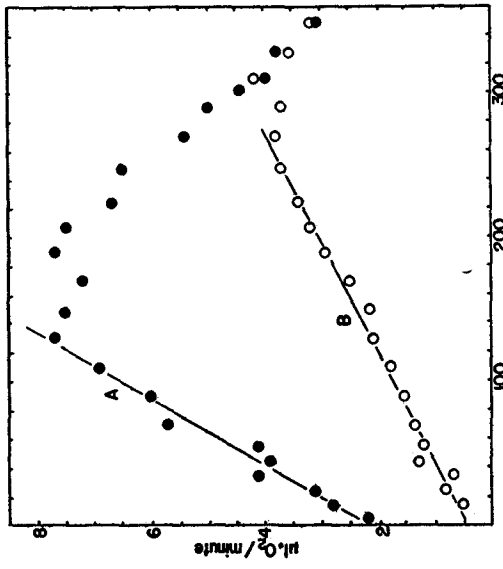

FIG. 4

FIGS. 3 and 4. Respiration during germination of *M. verrucaria* spores. Curve A, 4.3 mg.; curve B, 1.08 mg. spores per vessel (1 per cent sucrose; 1 per cent yeast extract). Fig. 3. Solid lines connect experimental points (circles); dashed lines connect points (crosses) calculated according to Equation $y = bt + \frac{K}{2}t^2$. Constants evaluated from data in Fig. 4 by method of least squares. For curve A, $b = 2.12$ μl./min.; $K = 0.0436$ μl./min.^{2}; for curve B, $b = 0.408$; $K = 0.0130$.

linear increase, the constant C of Equation 2 is equal to the oxygen consumption at the transition from the first to the second phase.

Conformance of the experimental data to Equation 2 is shown in Figs. 1 (*B. megaterium*) and 3 (*M. verrucaria*). The constants b and K were evaluated from the rate *vs.* time data by determining the intercepts and slopes by the method of least squares. Differences between the calculated and experimental curves are well within experimental error.

The physiological characteristics of constants, b and K, of the above equations are as follows. K, the acceleration in oxygen uptake, should be directly proportional to the quantity of spores and, when corrected for this, should be a measure of physiological activity. Under controlled conditions, evaluation of K can provide a quantitative measure of the metabolic capacity of the spores. The intercept b is also directly proportional to spore quantity. Physiologically, b represents the rate of respiration at zero time and hence expresses the initial respiratory capacity of the spores in the medium employed. The constants calculated (for *M. verrucaria*) are not directly proportional to spore concentration as they should be. This must be due to manipulation errors or to too dense a suspension.

We have assumed that the entire respiration curve is parabolic—that the parabolic response starts immediately upon addition of substrate, disregarding the time required for diffusion of substrate into the cells. While this condition is met at least for practical purposes in the data considered here, a more complex situation can exist. Several types of behavior can occur, at least hypothetically, prior to establishment of the parabolic response—a lag period of constant rate or one of decreasing or increasing acceleration to an equilibrium; *i.e.*, linear increase in rate. In such cases, the initial portion of the rate curve would be flat or convex or concave.

Inspection of the data for *B. megaterium* indicates that the respiration rate during the first 10 minutes probably does not follow the calculated lines as drawn (Fig. 2) but may have a rapid initial increase from zero time. If so, this period coincides with the changes resulting in the decrease in optical density and an increase in stainability. Thus, the initial portion of the rate curve for *B. megaterium* spores is probably convex with respect to the time axis (Fig. 5). While the same situation may apply to *M. verrucaria* spores, the time involved appears to be much less. Permeability changes affecting penetration of substrates may be a factor, but not diffusion into the cells through a freely permeable membrane which would take only a small fraction of a second.

Two possible interpretations of the mechanisms responsible for the form of the parabolic respiration curves can be considered. Equation 2 indicates that respiration of the spores is the resultant of two factors (1) a basal respiration of constant rate which is the initial respiratory activity and (2) some process which is a function of time and is responsible for the linear increase in rate. Assuming the dimensions of a critical cell structure(s) to increase by linear

extension at a uniform rate, then the surface area would increase proportionately with time. These relations suggest that surface phenomena such as surface located enzymes or transfer or diffusion of substrates or products may limit respiration during germination and early growth.

Alternately, it can be assumed that respiration is limited by an enzyme which is being formed at a constant rate. Mandelstam (1952) has proposed a "mass action" theory of enzyme adaptation which predicts a linear or exponential increase in enzyme. This theory has been verified experimentally with the galactozymase system of yeast (Mandelstam and Yudkin, 1952). Either linear or exponential increases in galactozymase were observed, although the experimental factors determining the type of curve obtained were not established.

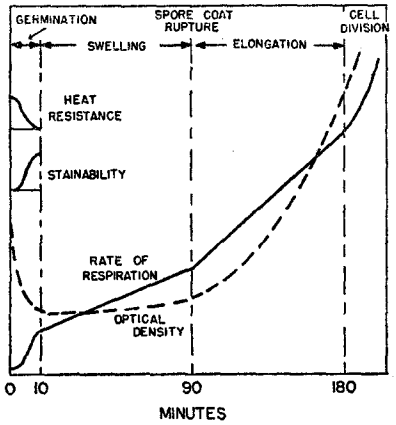

FIG. 5. Representation of changes occurring in *B. megaterium* spores up to inception of cell division. Curves for respiration rates, optical density, and stainability based on data and observation; curve for heat resistance inferred from other published and unpublished data.

Where exponential curves occurred, growth was observed. In the spore systems under consideration, growth by increase in both cell volume and dry weight occurs with *M. verrucaria*, and at least by increase in cell volume with *B. megaterium*.

Cook (1931) has measured the respiration of spores of *B. subtilis* in tryptic broth. While his data are in a form unsuitable for precise analysis, calculation shows that they do indicate linear increases in rate for boiled spores from about 1.5 to 3.4 hours and for unheated spores from about 2.5 to 5 hours. The data do not permit determination of the initial changes prior to inception of linear increase in rate of respiration. Thus, it cannot be established whether the apparent linear increase corresponds to the first or second phase found in the present study.

The only published data suitable for analysis of the respiration of germinat-

ing fungus spores which we have encountered are those with ascospores of *Neurospora*. Analysis shows the data of Goddard and Smith (1938) for heat activation of *N. tetrasperma* and of Emerson (1954) for heat or furfural activation of *N. crassa* to be consistent with a linear increase in rate of respiration.

Strictly speaking, germination is the beginning of growth or development of a resting structure. In practice, however, germination is always defined in terms of some convenient, clearly observable stage such as protrusion of a radicle (seeds) or of a germ tube (fungus spores). Germination in bacterial spores has been variously defined to include loss in heat resistance, decrease in optical density, stainability, emergence from the spore coat, or inception of cell division (Knaysi, 1948; Wynne and Foster, 1948; Hills, 1949; Powell, 1950, 1951; Levinson and Sevag, 1953; Hachisuka *et al.*, 1955). Most recent studies have used one or the other of the first three. The overlap and confusion lent by employing physiological and morphological criteria, as pointed out by Wynne and Foster (1948), obviously stem from the varied objectives of the particular study.

Physiologically, germination should be considered as the period during which the spores change from relatively inactive to actively metabolizing cells. The excellent correlation found between the phases of the respiration curve and other physiological and morphological changes occurring up to inception of cell division, permit collation of these phenomena to clarify our picture. The rapid increase in respiration found to occur simultaneously with stainability and decrease in optical density, and hence also with loss in heat resistance, lends support to the use of these phenomena as criteria of germination. If these initial changes are used to characterize germination, what terminology should be applied to the subsequent changes preceding cell division; *i.e.*, those changes such as swelling, rupture of the spore coat, and elongation occurring during the two phases of linear increase in rate of respiration? Why not simply swelling, rupture of the spore coat (where it occurs), and elongation? The temporal sequence of these phenomena is illustrated diagrammatically in Fig. 5.

The above analysis is not applicable to fungus spores as exemplified by *M. verrucaria*. Here protrusion of a germ tube must, at least tentatively, remain the criterion. It should be noted, however, that swelling and assimilation precede germ tube protrusion (Mandels and Darby, 1953), so that physiologically speaking, germination may occur earlier than is now recognized.

SUMMARY

1. There are certainly two, and probably three, stages in the development of *B. megaterium* from the spore to inception of cell division. The rapid increase in rate of respiration during the initial 10 minutes on glucose–peptone–yeast extract medium coincides with decrease in optical density and with increase in stainability. From about 10 to 100 minutes, the rate increases linearly, co-

inciding with swelling of the spores and ending at approximately the time of rupture of the spore case. From about 100 to 180 minutes, there is a second and steeper linear increase in respiration rate coinciding with cell elongation. These physiological and morphological phenomena are discussed as criteria for germination.

2. The rate of respiration of *M. verrucaria* spores also increases linearly up to about 300 minutes in sucrose–yeast extract medium. No breaks in the curves are observed during formation of the germ tubes.

3. Oxygen uptake follows the parabolic curve $y = bt + \frac{K}{2}t^2$ within the limits of experimental error for both types of spores.

4. It is postulated that metabolism during these stages of linear increase may be regulated by processes occurring at cellular or intracellular surfaces or by synthesis of a limiting enzyme at constant rate.

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