Inoculum-Dependent Division Lag of *Bacillus* Cultures and Its Relation to an Endogenous Factor(s) ("Schizokinen")

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ABSTRACT

LANKFORD, C. E. (The University of Texas, Austin), JAMES R. WALKER, JAMES B. REEVES, N. H. NABBUT, B. R. BYERS, AND R. J. JONES. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s) ("schizokinen"). J. Bacteriol. 91:1070-1079. 1966.—When cells of *Bacillus megaterium*, grown on Brain Heart Infusion Agar, were inoculated into a chemically defined medium, they exhibited a division lag which was an inverse function of inoculum size. The addition of filtrates of cultures from the same medium eliminated the inoculum-dependent component of lag, but not an inoculum-independent residual lag of constant duration. Culture filtrate of *B. subtilis* var. *niger* not only eliminated its inoculum-dependent lag but also was required to sustain exponential division. Dose-response and "growth time" bioassays, developed to measure lag-reducing activity of filtrates, demonstrated accumulation of active filtrate factor to a "critical" concentration prior to division initiation. Addition of this concentration to cultures eliminated the inoculum-dependent lag. Accumulation of the factor ceased temporarily at onset of division, but excretion was resumed later during exponential growth. Accumulation of a lag-reducing, cell-associated factor followed a similar course. Chromatographic and bioautographic analyses of culture filtrates of *B. megaterium* indicated that a single substance was primarily responsible for their activity. Results of dose-response tests for reciprocal activities of filtrates of different *Bacillus* species and strains suggested production of different factors by some, and of different quantities of similar factors by others. It is proposed that such endogenous factors which are synthesized and accumulate to a population-dependent concentration as a requisite to initiation and maintenance of division be designated as "schizokinens."

An inverse relation of the duration of division lag and inoculum size of bacterial cultures, first reported by Rahn (11) and quantitatively demonstrated by Penfold (10), remains an unexplained phenomenon. It has been postulated that such inoculum-dependent lag reflects the time required by cells to synthesize and to accumulate to a critical concentration a diffusible metabolite(s) essential to growth and division (1, 6, 11; Ecker

and Lockhart, Bacteriol. Proc., p. 165, 1960). Evidence supporting this hypothesis generally is limited to observations that filtrates of growing or mature cultures reduce or eliminate lag under certain conditions. Lodge and Hinshelwood (6) and others (1, 9) also reported that glutamate, asparagine, α -alanine, or α -ketoglutarate partially substituted for culture filtrates in reducing "early" lag (i.e., lag of inoculum cells from young cultures) of *Escherichia coli* and *Aerobacter aerogenes*, but they failed to establish a clear relationship of these metabolites to an inoculum-dependent lag or to the active filtrate component(s).

A clear definition of inoculum-dependent lag, the development of quantitative methods for measuring the potency of lag-reducing culture

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filtrates, and the demonstration of a "division activator" in pre-exponential cultures appeared to be essential to further investigation of this phenomenon. This report is devoted to these aspects of an investigation of division lag which has been underway for several years (5; Nabbut et al., *Bacteriol. Proc.*, p. 56, 1960; Walker and Lankford, *Bacteriol. Proc.*, p. 43, 1963; Byers et al., *Bacteriol. Proc.*, p. 23, 1964; Powell et al., *Bacteriol. Proc.*, p. 123, 1965; Arceneaux and Lankford, *Texas Rept. Biol. Med.* 23:630, 1965; Byers and Lankford, *Texas Rept. Biol. Med.* 23:631, 1965).

MATERIALS AND METHODS

Cultures. The cultures of *Bacillus* spp. studied were from the departmental stock culture collection. *B. subtilis* var. *niger* was the strain designated previously as *B. globigii* "UT parent" (15). *B. megaterium* R, a strain used in much of this and subsequent work, is that referred to by Rode and Foster (14) as the "Texas" strain. These strains were maintained by monthly transfers on Brain Heart Infusion Agar (Difco), and inoculum cells were provided by the second of two successive 12-hr subcultures from the stock culture to Brain Heart Infusion Agar slants.

Media and culture procedures. To minimize cell injury and leakage, filter-sterilized chemically defined basal media were used for all manipulations of inoculum cells: to suspend the cells from the infusion slant cultures, to wash them three times by centrifugation, and to prepare appropriate dilutions of the washed cell suspensions. The numbers of viable cells [or, more precisely, of colony-forming units (CFU)] in dilutions of the inoculum suspensions or of test cultures were estimated from average colony counts on four replicate plates of nutrient agar. Usually, 0.1-ml samples of the diluted suspensions were pipetted to prepared plates and spread over the surface with sterile glass L-rods, but poured dilution plates were used for some experiments. The lag of cultures of *B. megaterium* was estimated by the slope-intercept method (7); that of cultures of *B. subtilis* var. *niger* usually was indeterminate unless culture filtrates were added.

The chemically defined medium for growth studies of *B. subtilis* var. *niger* and for *Bacillus* strains other than *B. megaterium* was constituted and sterilized as described by Sergeant et al. (15). For the latter organism, the basal medium had the following quantities (in grams per liter) of reagent grade ingredients: sucrose, 10; $(\text{NH}_4)_2\text{HPO}_4$, 1.0; K_2HPO_4 , 2.5; KH_2PO_4 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.007. All ingredients except the salts of magnesium, manganese, and iron were dissolved in 970 ml of distilled water passed through a Barnstead demineralizer; this solution was adjusted to pH 7.0 with 1 N KOH and was sterilized by filtration through a Millipore filter (0.45μ) in an all-glass assembly. Solutions of filter-sterilized salts of magnesium, iron, and manganese were added just before the medium was to be dispensed aseptically to tubes or flasks. All glassware was washed rigorously, first with de-

tergent and next with acid-cleaning solution or with a solution of 0.5% each of NaClO_3 and NaNO_3 in sulfuric acid; it was then subjected to repeated rinses with distilled water and, finally, with demineralized water. The complete filter-sterilized basal media were dispensed aseptically to metal-capped sterile Pyrex tubes (22 by 175 mm), 5 or 10 ml per tube, or to Erlenmeyer flasks fitted with glass caps. Tubes or cuvettes [1-inch (2.54-cm) diameter] were used when periodic optical density determinations were to be made. The cultures were incubated at 37 C on a reciprocating shaker at eighty 3-inch (7.6-cm) strokes per minute; the tubes were slanted at an angle 60° from vertical.

Preparation of samples for bioassay. When culture filtrates were to be assayed for lag-reducing activity, cultures with visible turbidity usually were centrifuged, and their supernatant fluids were sterilized by filtration through a membrane filter. Samples taken periodically from cultures with subvisible growth generally were filtered directly. The sterile filtrates could be stored at 5 C or at -20 C for weeks without detectable loss of activity.

Optical density of cultures for dose-response assays of culture filtrates was determined at the end of an arbitrary incubation period after chilling the tubes in an ice bath to arrest growth. For "growth time" studies, optical-density readings were made periodically, usually at hourly intervals, in an instrument maintained in the incubator room. A Bausch & Lomb Spectronic-20 colorimeter set at 600 $m\mu$ or a Klett-Summerson colorimeter fitted with a no. 42 filter (400 to 465 $m\mu$) was used for this purpose.

Preparation of cell extracts. Samples of appropriate sizes were removed from large flask cultures at intervals during the culture cycle. During the lag and early stages of growth, the cells were collected on a sterile Millipore filter, washed three times with 5 ml of sterile basal medium, and then washed off the membrane with 5 or 10 ml of the medium. Dilution plate counts from this suspension indicated recovery of more than 95% of the number of cells in the original sample. After visible turbidity appeared in the cultures, the cells in 10-ml samples were separated and washed by centrifugation. The suspensions were placed in an ice bath and treated for 15 to 20 min with a 20-kc MSE (Measuring & Scientific Equipment, Ltd., London, England) model 300 sonic disintegrator with a titanium probe. Stained smears and viable-cell counts of the suspensions indicated that at least 99% of the cells had been disrupted. The cell extracts were centrifuged for 30 min at $5,000 \times g$ to remove cell debris, and the supernatant fluids were sterilized by filtration through a membrane filter and stored at -20 C. The lag-reducing activity of these filtrates was determined by the dose-response assay.

Bioautograms. A bioautographic technique was employed for detection of stimulatory areas produced by filtrates chromatographed on paper. The chemically defined media were solidified with 15 g per liter of thoroughly washed granular agar and poured into Pyrex dishes (2 by 8 by 12.5 inches) to form a base layer. A second layer seeded with 10^8 to 10^4 cells per milliliter of the test organism was poured over the base layer. The basal medium for *B. subtilis* var. *niger*

was modified for this test by reducing the glutamate concentration to 1.0 g per liter. Paper sheets on which culture filtrates had been chromatographed were cut into strips or into 1-inch (2.54-cm) squares, which were planted on the agar surface and then removed after 1 hr. The R_F values were estimated by measuring areas of stimulated colony growth of *B. megaterium* after 12 hr of incubation and of *B. subtilis* var. *niger* after 24 hr.

Chemical tests of culture filtrates. The concentration of ninhydrin-reactive substances in culture filtrates was determined by the method of Moore and Stein (8), with DL-leucine as a standard. Tests and standards were read at 570 $m\mu$ in the Spectronic-20 colorimeter. Filtrates to be tested for materials absorbing ultraviolet light at 260 $m\mu$ were examined in a Beckman DU spectrophotometer.

RESULTS

Reduction of lag by culture filtrates; inoculum-dependent and inoculum-independent components of lag. The qualitative effects of culture filtrates on growth of *B. megaterium* and *B. subtilis* var. *niger* in their respective chemically defined media revealed significant aspects of their action. Filtrates from maximal stationary-phase cultures of *B. megaterium* grown in the basal medium effected marked reduction in lag of small numbers of cells inoculated into this medium. In the representative test illustrated in Fig. 1, the lag of 60 cells was reduced from about 14 to 4 hr by a supplement of 0.01 ml of such culture filtrate per ml of medium. In this and many similar tests, the maximal cell population was not influenced significantly by the filtrate supplement. The division time was reduced only slightly (e.g., Fig. 1), or not at all, as compared with that of the unsupplemented culture.

Unsupplemented cultures of *B. subtilis* var. *niger* exhibited a pattern of growth quite different from that of *B. megaterium* (Fig. 2). Inocula of 100 cells or less of *B. subtilis* var. *niger* failed to initiate growth within 4 days or grew only after prolonged lag. Inocula of intermediate to high density (10^4 to 10^6 cells per milliliter) often initiated division within 4 to 6 hr. The cells usually were unable to continue division, however, and a second "lag" ensued, followed by a resumption of division often at a changed rate. Temporary cessation of growth sometimes occurred repeatedly during the complete culture cycle, and the number and duration of these secondary "lags" varied in different experiments. The time required to attain visible turbidity, although variable, was more consistent than the growth pattern. When filtrate of a maximal stationary-phase culture was added in sufficient concentration, the lag of a wide range of inoculum densities was reduced to a minimum of 2 to 3 hr and uninterrupted exponential growth ensued after lag

termination (Fig. 2). In these cultures, optical-density measurements of growth would have given no clue as to the effect of the filtrate in maintaining exponential growth. *B. megaterium* infrequently exhibited a single-step interruption of exponential growth in cultures unsupplemented with filtrate.

Filtrate-supplemented cultures of both *B. megaterium* and *B. subtilis* var. *niger* exhibited a residual lag of short duration, usually of 2 to 4 hr, regardless of the size of the inoculum. Higher proportions of filtrate effected slight additional reduction in this lag. In cultures of *B. megaterium* supplemented with 0.01 ml of filtrate, the lag was reduced to a minimum of about 3.3 hr (as determined by the method of least squares) over an inoculum range of 30 to 4,400,000 cells per milliliter (Fig. 3). Although the lag of unsupple-

FIG. 1. Reduction in lag of 60 cells of *Bacillus megaterium* by culture filtrate. The filtrate was collected from a maximal stationary-phase culture of *B. megaterium* in its basal medium, and 0.01 ml (16 units) was added per ml of the supplemented medium.

FIG. 2. Effect of culture filtrate on lag and growth of *Bacillus subtilis* var. *niger*. Filtrate was added to the supplemented basal medium at a concentration of about 8 to 10 units per ml. Note difference in growth patterns in the subvisible and the visible range.

FIG. 3. Inoculum-dependent and inoculum-independent components of lag of *Bacillus megaterium*. Filtrate concentration was 0.01 ml (16 units) per ml of the supplemented medium.

mented cultures inoculated with 10^6 or more cells was as short as that of the filtrate-supplemented cultures, it increased in duration as the inoculum density was decreased. The lag of two to five cells per milliliter (not shown) was found to range from 35 to 70 hr, and a lesser number of cells usually failed to grow.

The residual lag in cultures supplemented with filtrate or in cultures which received very large inocula was designated as the *inoculum-independent lag*. The increased lag of smaller inocula which is eliminated by culture filtrate was termed the *inoculum-dependent lag*. If inoculum cells in the basal medium were prepared for division and growth by syntheses, the essential products of which were retained within the cells, then lag probably would be independent of the number of inoculum cells. That component of lag which is independent of inoculum density and of filtrate supplement might be explained on this basis. In contrast, the inoculum-dependent lag indicates an interdependence of inoculum cells in terminating lag. These data evoke the hypothesis stated by Hinshelwood (3) and others that division is initiated when an endogenous, diffusible metabolite is accumulated to critical concentration.

Bioassays of stimulatory activity of culture filtrates: dose-response assay and "growth-time" assays. Before it could be determined whether a division-initiating factor indeed accumulates

during the lag phase of cultures unsupplemented with filtrates, bioassay techniques of sufficient sensitivity and accuracy were required. The most useful method developed was a dose-response assay based essentially upon a linear relation between the concentration of the filtrate stimulant and the degree to which it reduced lag. Within a limited incubation period, the optical density of a culture is a function of the duration of its lag phase, provided that its rates of growth in filtrate-supplemented and in unsupplemented cultures are identical. Since *B. megaterium* R fulfilled these requirements more adequately than *B. subtilis* var. *niger*, it was used extensively, although not exclusively, for this assay.

The dose-response assay was conducted by adding a range of concentrations of sterile culture filtrate to duplicate tubes of the basal medium. Fortunately, a stable compound of known composition was available as a standard. The 3-hydroxy-1,4-pyrones, kojic and meconic acids, as well as a variety of other compounds, were found to substitute with varying degrees of efficiency for culture filtrate in eliminating inoculum-dependent lag of *B. subtilis* var. *niger* (4, 5) and of *B. megaterium* (Walker, Ph.D. Thesis, The University of Texas, Austin, 1963). In each assay, duplicate or triplicate tubes of medium were prepared with neutralized, filter-sterilized meconic acid at 10, 25, 50, 75, and 100 μ g/ml. Tests and standards were inoculated with 100 to 200 washed cells of *B. megaterium*. After an incubation time sufficient to permit measurable growth at 25 μ g of meconic acid and nearly maximal growth at 100 μ g, growth was arrested and the optical density of tests and standards was determined. A dose-response curve for the filtrate was plotted (Fig. 4), and the amount of filtrate which would produce an optical density equivalent to that obtained with 25 μ g of meconic acid per ml was estimated. This amount was assigned a value of 1 unit of filtrate activity. That this method is valid was demonstrated by assays of twofold dilutions of culture filtrate, from 1:2 to 1:16, which yielded a straight-line function of stimulatory activity in units per milliliter in relation to filtrate dilution. Seven determinations of the activity of one filtrate sample produced values with a standard deviation of $\pm 21\%$; although not as precise as many microbiological assays, this method proved invaluable to the investigation.

The potency of filtrates of maximal stationary-phase cultures of *B. megaterium* ranged from 30 to 100 times greater than those of *B. subtilis* var. *niger* when assayed with the former organism (Fig. 4). Since similar values for relative potency of the two filtrates were obtained when the latter was used as the assay organism (Table 1), it may

FIG. 4. Dose-response bioassay for filtrate activity. Each filtrate was assayed with the homologous organism: cultures of *Bacillus megaterium* were inoculated with 200 cells per milliliter and incubated for 17.7 hr; those of *B. subtilis* var. *niger* received 10^4 cells and were incubated for 18 hr. Standards with meconic acid (not shown) produced optical density values of about 0.3 and 1.0, respectively, at concentrations of 25 and 100 $\mu\text{g/ml}$.

be inferred that *B. megaterium* accumulates a much higher concentration of a stimulatory factor(s) common to the two species, or that the factor produced by *B. megaterium* possesses greater intrinsic potency in reducing lag of both organisms.

A second assay method, designated as the "growth time assay," was found to be sensitive to about one-fifth the filtrate activity measurable by the dose-response assay. Since this method was very time-consuming, its use was limited to assays of lag-phase filtrates for which the dose-response assay was insufficiently sensitive. "Growth time" was defined for this assay as the incubation time required for a standard inoculum of *B. megaterium* to attain an optical density of 0.30. Tubes containing basal medium were supplemented with increments of undiluted filtrate and inoculated with 100 to 200 cells of *B. megaterium*. In some cases, filtrates collected during the lag phase simply were reinoculated. After visible growth appeared, the optical density of each culture was determined hourly until the growth time could be interpolated from the curve of optical density versus time. A standard curve for growth time as a function of units of filtrate activity was established for each assay with a standard filtrate, the potency of which had been determined by the dose-response method. Figure 5 illustrates the growth time as a function of both filtrate concentration and the log of its concentration. The latter plot suggests a threshold concentration of filtrate

FIG. 5. Standard for "growth time" bioassay of filtrate activity with 200 cells per milliliter of *Bacillus megaterium*. Symbols: Δ , arithmetic plot; $\circ$, semi-logarithmic plot. Growth time is time (hours) required to attain an optical density of 0.30.

below which no detectable effect on growth time is evident, an observation verified repeatedly over a wide range of inoculum densities.

Time course of accumulation of filtrate and cell-associated division stimulants. The accumulation of stimulatory activity in culture filtrates was measured throughout the growth cycle of *B. megaterium*, with particular attention to its concentration prior to and coincidental with division initiation (Fig. 6). Measurable activity appeared in the culture filtrate 1 hr or more prior to lag termination, and at the time of division initiation a level of activity was attained which remained constant through several division cycles. Net excretion of the stimulant was resumed later in the log phase, usually after three to six divisions, and its accumulation continued until the maximal stationary phase. In numerous similar tests with inocula in this size range, the same pattern was reproduced, with an initial plateau of filtrate activity within 15 to 20 units per ml and a maximal final concentration of 2,000 to 2,500 units. It is significant that the addition of 15 to 20 units of filtrate activity to fresh cultures eliminated completely the inoculum-dependent lag at this and at all lower inoculum densities. These data are believed to represent the first quantitative evidence for accumulation of a filtrate division stimu-

FIG. 6. Time course of accumulation of lag-reducing activity in relation to lag and cell division of *Bacillus megaterium*. Symbols: $\circ$, viable cell count (colony-forming units per milliliter); $\triangle$, filtrate activity (units per milliliter); $\square$, cell-associated activity (units per total number of cells per milliliter of medium).

lant by lag-phase cells prior to division initiation.

Although less success was achieved in defining the pattern of accumulation of filtrate stimulant in cultures of *B. subtilis* var. *niger* because of its low filtrate activity, the data indicated it to be qualitatively similar to that of *B. megaterium*. Slow division of an inoculum of 10^6 cells of this organism was accelerated markedly when a concentration of filtrate stimulant of 5 to 7 units per ml was attained (Fig. 7). The final concentration was only 75 units per ml in this test, about $\frac{1}{30}$ that of its terminal concentration in cultures of *B. megaterium*. The later initial production of the filtrate factor and its slow accumulation correlated with the growth pattern of *B. subtilis* var. *niger* in its basal medium (Fig. 2 and 7). It appears reasonable to speculate that the slow and erratic division of this organism may be a consequence of its inability to produce the stimulant fast enough to maintain exponential growth. If this be the case, then the concentration required to initiate and to maintain division may be population-dependent. This possibility is suggested also by the resumption of excretion of the factor in exponentially growing cultures of *B. megaterium*.

Cells of the two *Bacillus* strains were found to contain a factor(s) which eliminates inoculum-dependent lag in a manner similar to that of the filtrate stimulant. The relative activity of the cell-associated factor was determined by dose-re-

FIG. 7. Time course of accumulation of filtrate activity in relation to growth of *Bacillus subtilis* var. *niger*. Symbols: $\circ$, viable cell count (CFU) per milliliter; $\triangle$, units of filtrate activity per milliliter.

sponse assays of filtrates of extracts of washed cells collected at different stages of the culture cycle in the basal medium and subjected to sonic oscillation. A pattern typical of accumulation of the cell factor(s), in relation to the excretion of the filtrate factor in cultures of *B. megaterium*, is illustrated in Fig. 6. After an initial decrease during the first 2 hr of lag, an increase in concentration of the cell stimulant began after 3 hr, approximately paralleling the appearance and accumulation of the filtrate stimulant. Although the rate of increase of the former slowed appreciably after division initiation, it continued to increase during the early exponential phase at a rate exceeding that of the filtrate stimulant. If it be assumed that the two stimulants are identical, as subsequent evidence suggests (Byers and Lankford, Texas Rept. Biol. Med. 23:631, 1965), the cells either continue to synthesize the factor during the early exponential phase, without appreciable net excretion, or uptake of filtrate factor by the cells occurs during a period of arrested synthesis. Later in the exponential phase, accumulation of the cell factor follows that of the filtrate factor.

A lower concentration of stimulant per cell of *B. subtilis* var. *niger* made impractical its determination during the lag phase. However, its concentration in the cells relative to that of the filtrate

factor was found to parallel qualitatively that of *B. megaterium* during exponential growth (Reeves, Ph.D. Thesis, The University of Texas, Austin, 1964).

Specificity of filtrate activity. Evidence that the stimulatory activity of filtrates from maximal stationary-phase cultures of *B. megaterium* may be due primarily to a single substance was obtained from chromatographic and bioautographic analyses. Volumes of culture filtrates diluted to contain 20 units of activity were chromatographed on Whatman no. 1 filter paper, and areas of stimulation on each strip were located by the bioautographic technique with both *B. megaterium* and *B. subtilis* var. *niger* as indicator organisms. Single, ninhydrin-negative, active spots with identical R_F values (as indicated) for the two organisms were found with the following solvent systems: *n*-butanol-acetic acid-water (12:3:5), 0.25; methanol-pyridine-water (20:1:5), 0.60; isopropanol-water (4:1), 0.12; and acetone-water-urea (60 ml:40 ml:0.5 g), 0.65. Tests also were made for recovery of stimulatory activity of concentrates of filtrates chromatographed with the first two solvent systems. A culture filtrate was concentrated 50-fold by flash evaporation at 50 C, followed by precipitation of 85% of the inorganic salts with 3 volumes of ethyl alcohol, lyophilization of the supernatant fluid, and redissolution in water to 0.1 the original filtrate volume. Portions of this concentrate (which has lost none of the total filtrate activity) containing 20 and 200 units of activity were chromatographed on paper, and areas corresponding to the single active spots for each solvent were eluted with water. After concentrating these eluates by lyophilization and redissolution, dose-response assays gave values for recovery of original filtrate activity ranging from 120 to 200%. These excessive recovery values suggest elimination of an inhibitor by the concentration procedure.

Since it may be questioned whether stimulatory activity is produced by the same substance throughout the culture cycle, samples of filtrate were collected for bioautographic analysis at intervals of 4, 8, 12, and 24 hr from a 400-ml culture of *B. megaterium* initiated with 10^5 cells per milliliter. Those samples collected at 4 and 8 hr were concentrated by lyophilization and redissolution in water to $\frac{1}{50}$ their original volumes, and a volume of each containing 15 units of activity was spotted on paper. The R_F values of the single active spots from these four sequential filtrate samples were, respectively, 0.23, 0.17, 0.21, and 0.19 with the *n*-butanol-acetic acid-water solvent system, and 0.1, 0.09, 0.1, and 0.09 with isopropanol-water. These results suggest identity of the major active component throughout the culture cycle.

Certain amino acids have been reported to reduce division lag (1, 6), and amino acids are known to accumulate in the fluid phase of growing cultures. The kinetics of their accumulation in relation to that of the specific filtrate stimulant was investigated with samples collected at intervals from a 400-ml culture of *B. megaterium*. The accumulation of cellular excretory products absorbing ultraviolet light at 260 $m\mu$ also was determined (Fig. 8), and a concentrate from a maximal stationary-phase culture filtrate was analyzed for its content of amino acids in a Beckman model 120B amino acid analyzer. The amino acids constituted 1.2% of the dry weight of this filtrate for a total of 1.267 μ moles/ml, of which alanine (0.422 μ mole), valine (0.382 μ mole), and glutamic acid (0.210 μ mole) accounted for 1.014 μ moles, the remainder being glycine, serine, threonine, leucine, isoleucine, and lysine. It seems unlikely that these amino acids contribute significantly to lag reduction in culture filtrates which are diluted to 15 to 20 units per ml (0.01 ml or less per ml of medium), although certain of these may increase division rate and reduce inoculum-independent lag in higher concentrations. Moreover, it is apparent from the data in Fig. 8 that neither amino acids nor nucleic acid derivatives contribute to the stimulatory activity found in filtrates of cultures in the late lag and early log phase. These data, though not conclusive, suggest that culture filtrates contain a major, highly active component which is essential to initiation of cell division.

Species and strain specificities of filtrate stimulatory activity. The reciprocal stimulatory activity of culture filtrates of several *Bacillus* species and strains was the subject of a limited study (Jones, M.A. Thesis, The University of Texas, Austin, 1961). Filtrates from 10 cultures were

FIG. 8. Accumulation of materials in culture filtrate of *Bacillus megaterium* during growth in its basal medium. Symbols: $\circ$, CFU per milliliter; Δ , filtrate activity, units per milliliter; $\square$, optical density at 260 $m\mu \times 10^2$; $\bullet$, micromoles of ninhydrin-reactive materials per milliliter (DL-leucine standard).

produced in the basal medium for *B. subtilis* var. *niger*, and all dose-response assays were conducted in this medium with homologous filtrate, rather than meconate, as a standard; hence, neither total filtrate activities nor the unit values of activity were strictly comparable to those described heretofore. Representative data in Table 1 illustrate the variety of responses encountered under these specific cultural conditions. Whereas the culture filtrate of *B. megaterium* R was strongly stimulatory to itself and to each of the other test organisms, that of the other *B. megaterium* strain, although rather strongly stimulatory to *B. megaterium* R and to *B. cereus*, was only moderately autostimulatory and was weakly active for *B. subtilis* strains. Culture filtrates of strains of *B. subtilis* var. *niger* also were less stimulatory for the homologous culture than for certain other species. Filtrates of *B. megaterium* R and *B. subtilis* var. *niger* were roughly equivalent in their relative activities in reciprocal tests. Filtrates of *B. subtilis* strains were moderately stimulatory for this species and for certain strains of *B. subtilis* var. *niger*, but were strongly inhibitory for *B. cereus* and for the *B. megaterium* strains. It is possible that stimulatory activity in these filtrates may have been masked by the presence of an inhibitor, although interspecific inhibition by the autologous stimulant cannot be excluded. These limited data suggest both inter- and infraspecific differences in the lag-reducing factors produced by several of these organisms. Comparison of chemically purified stimulants will be required to confirm this inference.

DISCUSSION

Proof of the existence of an endogenous diffusible "division activator" would require the demonstration and clear definition of an inoculum-dependent component of lag, the accumulation in pre-exponential cultures (just prior to or coincidental with division initiation) of a specific factor to a concentration characteristic for a given

inoculum density, and that addition of this quantity of the factor to fresh cultures is sufficient to eliminate completely inoculum-dependent lag. These requirements appear to be satisfied partially by these preliminary data for a *B. megaterium* strain and, to a lesser extent, for a strain of *B. subtilis* var. *niger*. Further studies of the kinetics of accumulation of *B. megaterium* filtrate factor demonstrated that, contrary to prior assumptions (3; Ecker and Lockhart, *Bacteriol. Proc.*, p. 165, 1960), its rate of excretion per cell per hour increased linearly with time prior to division initiation (Walker and Lankford, *Bacteriol. Proc.*, p. 43, 1963). In addition, this critical concentration was shown to bear a direct relation to inoculum density. In consideration of this and other evidence to be presented in detail elsewhere, it is proposed that cells of these *Bacillus* species, and possibly microbial cells in general, synthesize and accumulate to a critical concentration a compound, a specific effect of which is to initiate and to allow or to maintain exponential division. It is proposed also that endogenous compounds of this functional nature, whether chemically similar or not, be designated as *schizokinens* (from Greek: *schizein*, to split or cleave, and *kinein*, to move). Although it cannot be determined from evidence presently available whether the primary effect of schizokinens is in initiation of cell division or of cell growth, their net effect in initiating and maintaining exponential division is considered to justify present use of this designation.

The "shift-down" of inoculum cells from a nutritionally rich, complex medium to a minimal, chemically defined medium was employed in this study to obtain maximal lag. Thus, it was possible to differentiate the two distinct components of lag described herein. It has been demonstrated (Walker, Ph.D. Thesis, The University of Texas, Austin, 1963; Arceneaux and Lankford, *Texas Rept. Biol. Med.* 23:630, 1965) in experiments to be described elsewhere that an inoculum-dependent lag characterizes small inocula trans-

TABLE 1. Reciprocal stimulatory activity of filtrates of *Bacillus* strains*

Filtrate produced by	Assay organisms					
	<i>B. megaterium</i> R	<i>B. megaterium</i>	<i>B. cereus</i>	<i>B. subtilis</i> var. <i>niger</i>	<i>B. subtilis</i> var. <i>niger</i> R	<i>B. subtilis</i> 6633
<i>B. megaterium</i> R.....	20,000	10,000	4,000	40,000	20,000	10,000
<i>B. megaterium</i>	2,000	200-400	2,000	20	20	40
<i>B. cereus</i>	1,000	200	1,000	200	2,000	200
<i>B. subtilis</i> var. <i>niger</i>	200	400	2,000	40	100	400
<i>B. subtilis</i> var. <i>niger</i> R.....	100	2,000	200	100	400	200
<i>B. subtilis</i> 6633.....	—†	—†	—†	—†	100	400

* Results expressed as units of filtrate activity per milliliter.

† Filtrate inhibitory to growth of test organism.

ferred from basal medium at any stage of the culture cycle, including the exponential phase. Since this lag is eliminated in all cases by added schizokinen, involvement of the latter during exponential division is indicated. The periodic growth of *B. subtilis* var. *niger* in its unsupplemented basal medium also suggests that a schizokinen is required at a population-dependent critical concentration, not only to initiate but to sustain division.

As yet there is insufficient evidence to distinguish among the possibilities that a critical concentration of schizokinen must be accumulated in the culture supernatant fluid, in the cells, or in both sites. However, evidence that it may be involved in transport and assimilation of metal ions, especially ferric ions (Powell et al., *Bacteriol. Proc.*, p. 123, 1965), suggests that it may perform essential functions both outside and inside the cell. Prior to this study, Sergeant et al. (15) and Lankford et al. (4) observed that autoclaved solutions of glucose and phosphate, meconic and kojic acids, and a variety of other compounds substituted with varying degrees of effectiveness for culture filtrate in reducing lag of small inocula and in maintaining exponential growth of *B. subtilis* var. *niger*. Since all active compounds possessed structural requisites for chelation of metal ions, it was proposed that they performed a function in common with a natural bacterial metabolite, probably involving assimilation of metal ions. This prediction appears to have been justified by recent isolation of a ferric ion-binding secondary hydroxamate from *B. megaterium* culture filtrates. The lag-reducing potency of 1 μg of this substance was equivalent to 5 units of filtrate schizokinen, and its R_F values indicate it to be identical with the latter (Byers and Lankford, *Texas Rept. Biol. Med.* 23:631, 1965).

That a variety of substances may reduce lag of small inocula is indicated by numerous reports. Ramsey and Lankford (12), for example, observed that growth of small inocula of certain lactic acid bacteria could be induced by an autoclaved solution of glucose and potassium phosphate, by CO_2 , pyruvate, oxaloacetate, and certain other Krebs cycle intermediates, by some reducing agents, and by kojic acid. Similar observations with propionic acid bacteria were reported by Field and Lichstein (2), but such studies have failed to take into account the relative efficiency of these substances in substituting for culture filtrates or their relative effects on the inoculum-dependent and independent components of lag. A re-examination of the problem with these bacteria appears desirable. It is possible that certain bacteria may require for growth initiation a

minimal concentration of more than one endogenous diffusible substance, possibly quite different from that (or those) required by the *Bacillus* species in this study.

The occurrence of inoculum-dependent lag has been reported for cultures of cells of protozoa, yeast, and other organisms, and it has been postulated that endogenous division control factors are involved (for example, Robertson's "allelocatalysis" hypothesis) (13). However, the extensive and conflicting literature on such phenomena in cultures of nonbacterial cells gives few clues as to their possible analogy to that which is the subject of this study.

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