# INITIATION OF GROWTH OF *BACILLUS* SPECIES IN A CHEMICALLY DEFINED MEDIUM[1]

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When separately sterilized glucose is added to a simplified culture medium, adequate in other respects, small inocula of certain microorganisms may produce visible growth only after prolonged incubation. If glucose is autoclaved in the same medium, or separately with phosphate, visible growth may appear after a relatively short period of incubation. The evidence reviewed recently by Ramsey and Lankford (1956) indicates that products of heat-accelerated interaction of reducing sugars with phosphates stimulate growth of lactic acid bacteria by initiating early cell division, without exerting a significant effect upon the subsequent growth rate or the maximum cell density. Because of the relatively complex culture media required for lactic acid bacteria, a simplified test system was sought among bacteria with less exacting nutritional demands. This condition appeared to be satisfied by certain species of *Bacillus*, which respond to autoclaved glucose-phosphate in an ammonium-salts-glutamate medium. It now appears, however, that the factor or factors stimulatory for *Bacillus* species is distinct from that which stimulates the lactic acid bacteria, although the activities of both may be essentially similar in nature. This report concerns the characteristics of the growth response of *Bacillus globigii* to autoclaved glucose-phosphate solution, as well as some properties of the active factor(s).

## EXPERIMENTAL METHODS

*Cultures.* *Bacillus globigii* strain "UT parent" was selected for most of the experimental work, although the growth response of 5 other *Bacillus* strains was comparable. These were *Bacillus globigii* strain "UT daughter," *Bacillus cereus* strain UT, *Bacillus subtilis* var. *niger* strain UT 220, *Bacillus megaterium* strain UT and *Bacillus mesentericus* strain UT. A stock culture of each strain was established on brain heart infusion agar (Difco) slants by fishing from a single typical colony. Cultures which provided the inoculum cells were started from this stock. In order to reduce complications due to spore germination, vegetative inocula for the tests were prepared from the second of 2 successive transfers incubated 12 hr on brain heart infusion agar slants. The 12-hr growth on the second slant was suspended in 20 ml of the filter sterilized basal medium, washed twice by centrifugation in this medium and resuspended in 10 ml of the same medium. The basal medium also was used to prepare decimal dilutions of the washed cell suspension. The number of viable cells in the inoculum was estimated from colony counts of duplicate pour plates of brain heart infusion agar prepared from suitable dilutions.

*The culture medium.* After an extensive series of trials, a medium of the following composition per ml was adopted for studies of the glucose-phosphate stimulant: DL-glutamic acid, 2,500 $\mu$g; $NH_4Cl$, 200 $\mu$g; glucose, 5,000 $\mu$g; $KH_2PO_4$, 660 $\mu$g; $K_2HPO_4$, 1340 $\mu$g; $MgSO_4 \cdot 7H_2O$, 200 $\mu$g; $Fe^{+++}$ (as $FeSO_4 \cdot 7H_2O$), 0.03 or 0.3[3] $\mu$g; $Mn^{++}$ (as $MnSO_4 \cdot H_2O$), 0.003 or 0.01[3] $\mu$g; and biotin, 0.001 or 0.003[3] $\mu$g. The pH was adjusted to 7.0 with NaOH, and the entire medium, except the $Mn^{++}$ salt, was sterilized by filtration through a fritted glass UF filter or an HA millipore filter. The $Mn^{++}$ salt was filtered separately and added aseptically to the sterile base. In some of the studies 1 $\mu$g per ml of nicotinic acid or of diphosphopyridine nucleotide (DPN) was added to the complete medium.

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[3] The higher values, used in some of the latter studies, appeared to give a more consistent growth response.

*The autoclaved glucose-phosphate solution.* Growth stimulation was obtained with an aqueous solution containing: glucose, 10 g; $KH_2PO_4$, 0.66 g; and $K_2HPO_4$, 1.34 g (pH 6.5) per 100 ml. Fifty ml of this solution were autoclaved at 121 C for 15 min, although longer periods were used for greater volumes. Such solutions retained their stimulatory activity when stored at 10 C. This glucose-phosphate solution was added to filter sterilized basal medium after the latter had been distributed aseptically to sterile tubes or flasks. Usually, graded amounts of the glucose-phosphate solution were added to a series of tubes, the amount added being expressed in terms of its original glucose content, e. g., 200 μg glucose-phosphate (as glucose) per ml of medium. The amount of autoclaved glucose-phosphate added was discounted when it did not exceed $\frac{1}{10}$ of that already present in the basal medium. When greater amounts of glucose-phosphate were added, the glucose and phosphates of the base were filtered separately and added to the medium so as to yield a final concentration not to exceed that stated above. The cultures were incubated at 37 C on a reciprocating shaker, with test tubes held at about a 30° angle.

*Estimation of growth response.* Turbidity measurements with the Klett-Summerson electrophotometer, equipped with the 400 to 465 mμ (blue) filter, most commonly were used to determine the growth response, although dilution plate counts in brain heart infusion agar were made to establish the phase of the culture cycle in which growth stimulation occurs. In some of the experiments the degree of growth stimulation is expressed as the extent of reduction in "growth time," which is defined as the time in hr required for a culture to attain an arbitrary degree of turbidity. In other tests, the stimulatory effect is evaluated in terms of the turbidity which develops in response to graded doses of the stimulant within a limited, arbitrary period of incubation. The relative potency of a test preparation was estimated by comparing its dose-response curve with that of a standard glucose-phosphate solution.

## EXPERIMENTAL RESULTS

*The relation of inoculum size to growth response to glucose-phosphate solution.* In preliminary studies of growth stimulation, the seed cultures of the several strains of *Bacillus* were diluted decimally from $10^{-1}$ to $10^{-6}$ and inoculated into test media in which the method of glucose sterilization was the variable factor. When glucose was autoclaved in the medium, or separately with phosphates, visible growth appeared from all inoculum concentrations within 34 hr of incubation. In the cultures to which glucose was added aseptically after separate sterilization, growth appeared with about equal promptness from the lower inoculum dilutions, but growth from the higher dilutions was delayed for 2 to 3 days, or failed to appear after prolonged incubation. The same delayed growth response was observed whether glucose was sterilized separately by autoclaving or by filtration. Evidence for a specific role of phosphates in engendering the stimulatory activity in glucose solutions was obtained by autoclaving glucose in a medium to which the phosphates were added after separate sterilization; such a medium was only slightly superior to that with glucose autoclaved separately. These observations with the 6 bacilli are parallel in all respects to those made with lactic acid bacteria (Ramsey and Lankford, 1956; Ramsey, 1953).

The relationship of inoculum size to the growth response was investigated further with *B. globigii* in aerated flask cultures, from which samples were removed periodically for turbidity determinations (figure 1). In the presence of 300 μg per ml of glucose-phosphate, the "growth time" of the cultures was found to approximate a straight line function of the log of the inoculum concentration over a range from about $1.8 \times 10^7$ to 1.8 inoculum cells per ml. In the cultures with glucose autoclaved separately, growth from $1.8 \times 10^7$ and $1.8 \times 10^6$ cells appeared as early as those with glucose-phosphate. As the inoculum concentration was reduced, the growth time was prolonged to a degree increasingly divergent from a straight line function. The culture which received 1.8 cells per ml failed to produce visible growth. Within the range of visible growth the slopes of the time-turbidity curves of all growing cultures in both media were parallel, and no significant difference was observed in the terminal turbidity of the cultures. These data suggest that glucose-phosphate acts primarily by reducing the lag, an inference since confirmed by viable cell counts of the growing cultures.

*The relation of glucose-phosphate concentration to the growth response of B. globigii.* The growth

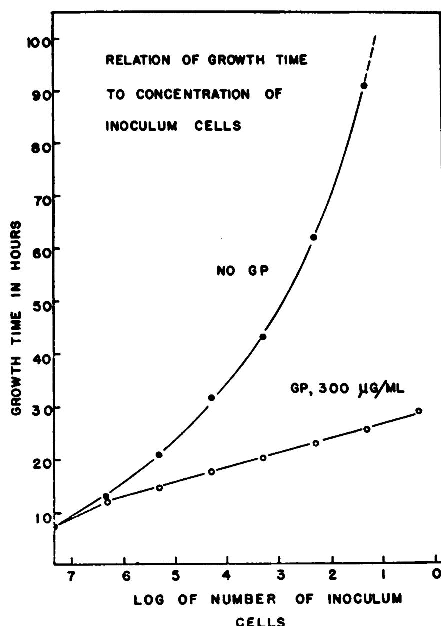


*Figure 1.* Relation of growth time of *Bacillus globigii* to the concentration of inoculum cells, in the presence and absence of glucose-phosphate (300 μg per ml). Growth time is defined as the time required for the culture to attain an arbitrary degree of turbidity, here equivalent to 100 Klett units.

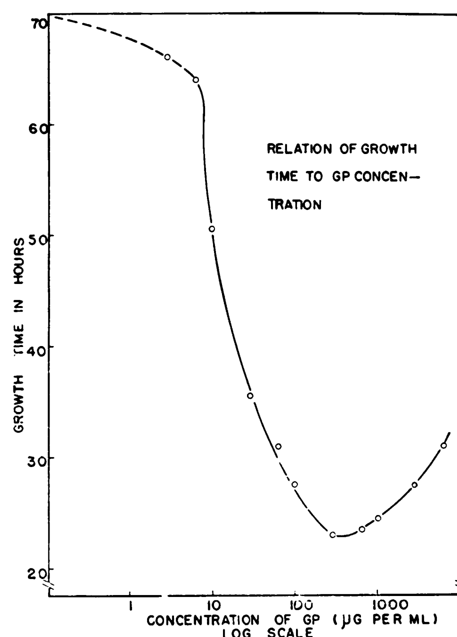


*Figure 2.* Growth time of *Bacillus globigii* in relation to glucose-phosphate concentration. The inoculum was about 190 cells per ml (about 5,700 cells per flask).

time as a function of the concentration of glucose-phosphate solution was investigated by observing the turbidity-time relationship in aerated flask cultures containing glucose-phosphate solution in final concentrations ranging from 1 to 6,000 μg per ml. As observed in the foregoing experiment, the growth rate (slope of the time-turbidity curve) and the final cell density (turbidity) of the cultures were almost identical at all concentrations of glucose-phosphate. As the glucose-phosphate concentration was increased from 6 μg to about 300 μg, the growth time decreased proportionately, although not according to a straight line relationship (figure 2). An activity optimum and a region of concentration excess were apparent in the increase in growth time at concentrations of glucose-phosphate above 300 μg per ml. If it is assumed that growth time is directly proportional to the lag, then the lag is inversely related to the glucose-phosphate concentration within the range from about 6 to 300 μg.

The relation of growth response to glucose-phosphate concentration also was determined after an arbitrary, limited time of incubation, usually about 20 to 24 hr, in aerobic and anaerobic cultures, with inocula of 500 to 2,000 cells per ml (figure 3). The dose-response curves so produced do not show a straight line relationship, because the turbidity of a culture after a fixed period of incubation is determined by the time at which growth is initiated. In cultures with low concentrations of glucose-phosphate, growth still is in the subvisible range or not even started at 20 to 24 hr, whereas cultures with higher concentrations of glucose-phosphate have attained maximum turbidity. For this reason all such dose-response curves are sigmoidal and show a pronounced foot. Their value for activity assays is therefore limited, although they have been used for this purpose for lack of a more accurate method (table 2). If the concentration range of glucose-phosphate is extended above 1,000 μg per ml, the dose-response curve becomes bell-shaped due to prolonged lag in the region of concentration excess.

The growth time of *B. globigii* is influenced by certain other components of the defined medium. Biotin, although not essential for growth, re-

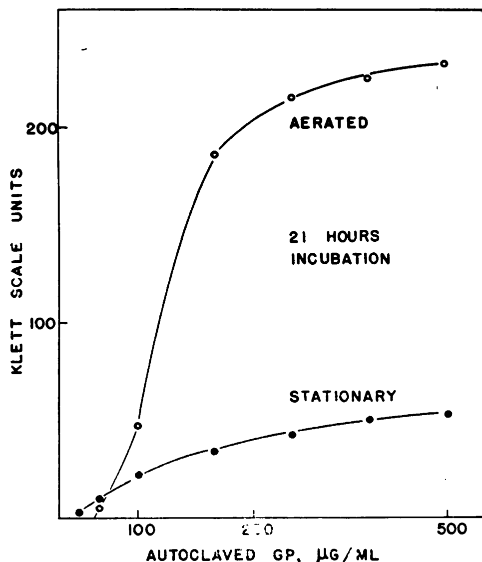


*Figure 3.* Growth response of *Bacillus globigii* in relation to dose of glucose-phosphate after 21 hr incubation. Aerated and stationary cultures. The inoculum was about 1,000 cells per ml.

duced by several hours the growth time in the presence of glucose-phosphate. It had little effect in the absence of glucose phosphate (table 1). Further reduction in growth time was effected with 1.0 µg per ml of nicotinic acid, nicotinamide or diphosphopyridine nucleotide (DPN). In the base with biotin, each of these pyridine compounds reduced the growth time to a significant degree, even in the absence of glucose-phosphate. In the presence of glucose-phosphate these compounds reduced lag by 3 to 6 hr, as compared with glucose-phosphate (figures 4 and 5). For this reason, nicotinic acid or DPN usually was added to the test medium in order to hasten growth of activity assays.

*The effect of glucose-phosphate on the growth cycle.* The effect of glucose-phosphate on cell multiplication during the stages of the growth cycle preceding the appearance of visible turbidity was investigated by periodic dilution plate counts of cultures with and without glucose-phosphate, in the presence and absence of supplements of nicotinic acid (or DPN) and of biotin, and of cultures started from small and from large inocula. In the presence of both glucose-phosphate and DPN, cell division was initiated after a stationary period of 4 to 8 hr, depending upon the size of the inoculum (figures

TABLE 1

*Growth time of Bacillus globigii as influenced by stimulatory supplements to the basal medium*

| Supplement | Growth Time* | Difference in Growth Time |
|---|---|---|
| | *hr* | |
| None | 55 | |
| GP,† 300 µg per ml | 24 | −31 |
| GP + biotin, 0.001 µg per ml | 22 | −33 |
| GP + nicotinic acid, 1.0 µg per ml | 20.5 | −34.5 |
| GP + biotin + nicotinic acid | 16 | −39 |
| Biotin, 0.001 µg per ml | 56 | +1 |
| Nicotinic acid, 1.0 µg per ml | 53.5 | −1.5 |
| Nicotinic acid + biotin | 41 | −14 |

* Inoculum: approximately 300 cells per ml of the test medium.

"Growth time" is defined as the time in hr for a culture to attain a turbidity equivalent to a Klett scale reading of 100 units, beginning from the time of inoculation.

† GP = glucose-phosphate

TABLE 2

*Stability of concentrated glucose-phosphate (GP) factor to treatment with chemical reagents*

| Treatment of GP Concentrate* (12.8 Mg Aliquots) | Loss of Activity |
|---|---|
| | % |
| Autoclaved in N HCl (in sealed tube) | 20 |
| Autoclaved at pH 7 | 0 |
| Autoclaved in N NaOH | 0 |
| Takadiastase; 24 hr at pH 4.6 | 0 |
| Boiled takadiastase | 0 |
| Methanol solution of GP with 4% $H_2SO_4$; 1 hr at 60 C | 77 |
| Acetyl chloride, 1 ml in 1 ml glacial acetic acid solution of GP† | 45 |
| 5% $H_2O_2$ for 1 hr† | 97 |
| $Br_2$ water for 30 min† | 47 |
| Na reduction in ethanol sol† | 88 |

* Approximately 4 µg per ml of untreated concentrate produced half-maximum response at 20 hr.

All solutions were readjusted to pH 7 after treatment, then filtered for assay.

† Solutions were evaporated to dryness after reaction, then redissolved in water.

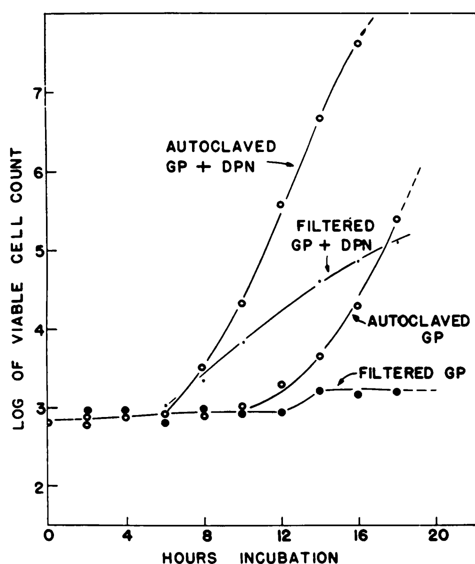


*Figure 4.* Glucose-phosphate solution and DPN in relation to growth initiation of *Bacillus globigii.* The glucose-phosphate concentration was 400 μg per ml, and the DPN concentration, 1 μg per ml, where indicated. Plate counts were made in duplicate in brain heart infusion agar. All media contained final concentrations of 0.5 per cent glucose and 0.2 per cent phosphates.

4 and 5). In cultures without DPN the stationary period was extended by an additional 3 to 6 hr. In the ensuing exponential phase, however, there appeared to be no advantage conferred by the pyridine compounds. With neither GP nor a pyridine compound present in the medium, the duration of the stationary phase ranged from 15 to 50 hr, or longer, depending upon the size of the inoculum. During this period there was observed in some cultures an increase in count corresponding to one or two cell divisions, followed by a second stationary period of extended duration (figure 4). When sustained growth eventually was initiated in some of these cultures, the subsequent growth rate and terminal cell density usually were not significantly different from those with glucose-phosphate. In certain cases, after a period of steady state growth over 6 to 10 division cycles, growth ceased abruptly and lysis began before the culture reached the stage of visible turbidity. Thus, it seems possible that glucose-phosphate serves not only to initiate cell division, but also functions in some manner to sustain continued division. This interpretation of its function may be applied to cultures containing the pyridine compounds. In the absence of glucose-phosphate, cell division in DPN-containing cultures was initiated early, followed by a steadily declining growth rate. After 4 to 7 division cycles growth ceased temporarily, to be resumed later at a more rapid rate until maximum growth was attained. Thus it would appear that glucose-phosphate functions to sustain continuous steady growth in cultures in which early cell division has been initiated by DPN or nicotinic acid. In general, these data confirm the inference drawn from the time-turbidity curves, except for the course of events in glucose-phosphate free cultures containing nicotinic acid or DPN.

*Delayed growth in the absence of glucose-phosphate.* The cause of delayed growth in the absence of glucose-phosphate has been investigated with the purpose of determining whether it involves selection of mutant cells partially or totally independent of glucose-phosphate, or whether it may be accounted for by some adaptive mechanism or physiological adjustment. Parallel sets of cultures were initiated in media with and without glucose-phosphate, with inocula ranging from about 10 to $10^7$ cells per ml. The approximate growth time was determined for

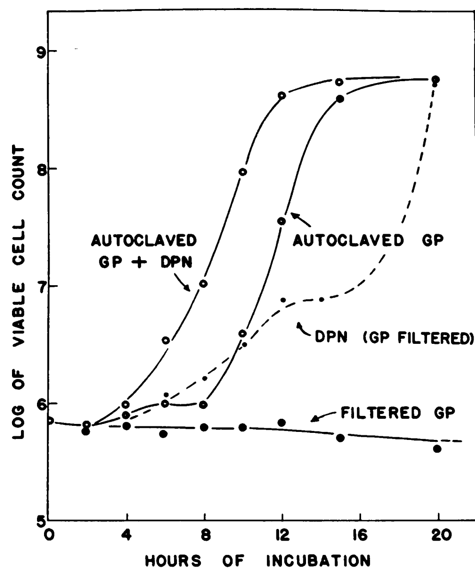


*Figure 5.* Glucose-phosphate solution and DPN in relation to growth initiation of a large inoculum. Except for the inoculum size, the conditions were the same as for figure 4.

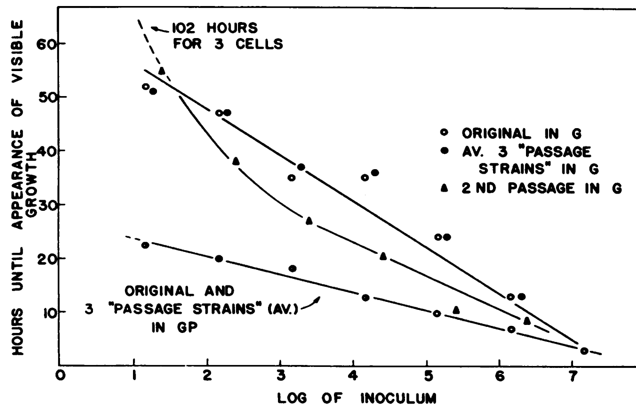


*Figure 6.* The effect of serial passage of *B. globigii* in a medium without glucose-phosphate. G refers to the basal medium without glucose-phosphate; GP refers to the basal medium plus 400 μg per ml of glucose-phosphate solution. The "passage strains" were selected from three colonies on a plate streaked from the 4th serial passage in the basal medium (G). These were deadapted by two transfers on brain heart infusion agar before inoculation to the tests illustrated. See text for further experimental details.

each culture. When visible turbidity appeared in the tubes which had received about $10^5$ inoculum cells, decimal dilutions were prepared from these for inoculation to a new series of tubes of the same medium. This procedure was repeated through four transfers in each medium, the latter in parallel with sets inoculated with "unadapted" cells. A comparison of the growth time for each dilution in glucose-phosphate free medium during the first and second passages demonstrates that some degree of temporary, incomplete adaptation does occur in the dividing cells (figure 6). Growth from intermediate inoculum concentrations appeared 5 to 10 hr earlier than in the first passage. With small inocula, however, no evidence of adaptation was apparent, and succeeding passages did not further decrease the growth time to a degree approaching that of cultures containing glucose-phosphate. There was, in fact, a comparable decrease in growth time of cultures transferred in the glucose-phosphate medium (not shown in figure 6), suggesting that the observed adaptation in both media may involve some growth limiting mechanism other than that influenced by glucose-phosphate. Evidence against mutant selection as a factor in delayed growth was obtained from parallel sets of cultures inoculated from each of 3 colonies selected at random from an agar plate streaked from the 4th passage series in the glucose-phosphate free medium. The growth times of each of these "deadapted" cultures at all inoculum dilutions, both in the presence and absence of glucose-phosphate, were almost identical with those of the original culture tested in parallel (figure 6). Therefore it seems unlikely that any permanent modification of the cells occurred during passage in the absence of glucose-phosphate.

*Properties of the glucose-phosphate factor.* Some information on the physical and chemical properties of the glucose-phosphate stimulant(s) has been obtained in the course of attempts to concentrate and purify it. Preparations and fractions subjected to physical and chemical treatments were assayed for residual activity by determining the growth response to graded doses after a limited incubation time. The factor was found to be nonvolatile, since all of the activity of a glucose-phosphate solution remained in the residue after distillation to ½ volume at 50 C under reduced pressure at initial pH values of 3, 7, and 10. More than 99 per cent of its activity was adsorbed from glucose phosphate solution at acid or neutral pH by 10 per cent (w/v) of acid washed Norite A or other activated charcoals. The activity also was removed by the anion exchange resins, Dowex 1 ($Cl^-$) and Amberlite IRA-401 ($Cl^-$), but not by the cation exchange resins, Dowex 50 ($H^+$) and Amberlite IRC-50 ($H^+$). These results indicate that the stimulatory activity is associated with the nonvolatile acid fraction of glucose-phosphate, properties which differentiate it from the nonadsorbable stimulant of lactic acid bacteria (Ramsey and Lankford, 1956).

Concentrates of the stimulatory material were prepared by adsorbing with charcoal from a solution of glucose and phosphate which had been autoclaved for 30 to 60 min. After thorough washing with water, followed by methanol, the charcoal was extracted repeatedly with a 1:4 pyridine-water solution. The nonvolatile residue was a viscous, dark brown material which, after drying, represented about 0.2 per cent of the original weight of glucose. The potency of these residues ranged between 40- to 50-fold greater than the starting material, but recovery of the original total activity was only 10 to 15 per cent. About 20 per cent of the stimulatory activity could be extracted with ether directly from glucose-phosphate solution adjusted to pH 3 or to 6.4, but not at pH 10. The potency of such extracts was approximately 300, and as little as 1.0 $\mu g$ per ml of the dry residue produced a maximum growth response. Continuous extraction with ether for 24 hr resulted in greater recovery of the glucose-phosphate factor, but its activity was less concentrated. The ether solubility of the glucose-phosphate factor for *B. globigii* further distinguishes it from the glucose-phosphate stimulant for lactic acid bacteria (Ramsey and Lankford, 1956).

The glucose-phosphate concentrate obtained by pyridine elution from charcoal was subjected to tests of its stability to certain chemical treatments. Aliquots containing 12.8 mg of solids were reacted with the test reagents, evaporated to dryness if necessary, then prepared in filtered aqueous solution at pH 7 and assayed for residual activity at concentrations up to 100-fold that required for maximum response to the untreated concentrate. Suitable toxicity controls containing untreated glucose-phosphate were included. Loss of activity as a result of the treatment was presumed to indicate reaction with the test reagent (table 2). The results of these tests suggest that the active substance is a carboxylic acid possessing at least one hydroxyl group and certain unsaturated bonds which are requisite to full activity of the factor. Evidence for or against a carbonyl group was inconclusive, although tests with ammoniacal $AgNO_3$ and Schiff's reagent were weak or negative, as was the $FeCl_3$ test for phenolic and enolic compounds. Further chemical studies have been deferred until greater purification can be achieved.

## DISCUSSION

The stimulatory activity of autoclaved glucose-phosphate solutions on growth of small inocula of *B. globigii* appears to be exerted primarily although not exclusively during the lag phase of the growth cycle. Any agent which functions to initiate cell division and aids in the maintenance of steady growth, obviously is involved in processes of fundamental importance to the cell. The rather general nature of this phenomenon is suggested by the wide variety of microorganisms, both gram-positive (Ramsey and Lankford, 1956; Field and Lichstein, 1957) and gram-negative (Fulmer *et al.*, 1931; Finkelstein and Lankford, 1957), which has been reported to grow more promptly in the presence of caramelized sugars. Unfortunately, the phase of the growth cycle responsive to the stimulatory effect not always has been defined, nor has the identity of the stimulatory substances been established. Indeed, there now is evidence for at least two different stimulatory factors in autoclaved glucose-phosphate solution. The factor(s) stimulatory for lactic acid bacteria (Ramsey and Lankford, 1956; Smiley *et al.*, 1943) and for certain gram-negative bacteria (Fulmer *et al.*, 1931) is not adsorbed by activated charcoal, whereas that active for *B. globigii* is readily adsorbable and is partially soluble in ether. However, chemical differences in the factors for lactic acid bacteria and for *B. globigii* do not preclude the possibility of a similar mechanism of action.

These stimulatory factors may possess chemical similarity or identity with certain natural substances involved in the reaction chains leading to cell division and to sustained steady growth. It is conceivable, on the other hand, that these factors may be quite dissimilar in chemical structure to the natural substances, yet serve in the same functional capacity through some common physical or chemical property, which by coincidence is shared with the latter. The first possibility was favored by Smiley *et al.* (1943), who found that pyruvic acid and acetaldehyde substituted for caramelized glucose in growth initiation of *Streptococcus salivarius*, an observation which led them to postulate that these compounds triggered the initial dehydrogenations in carbohydrate metabolism. Snell *et al.* (1948) also reported that pyruvate and reducing agents

substituted for glucose decomposition products in growth stimulation of *Lactobacillus bulgaricus*, although Ramsey (1953) has suggested that the stimulatory activity of these compounds, in relation to glucose-phosphate, is additive rather than substitutive. However, Ramsey (1953) found that some substances, including oxalacetic acid, kojic acid and $Ca^{++}$, did appear to substitute for glucose-phosphate. A Maillard type compound, N-D-glucosylglycine, was reported by Rogers *et al.* (1953) to substitute to some degree for heat degradation products of glucose in growth stimulation of *Lactobacillus gayoni*. N-D-Glucosylglycine also partially replaces glucose degradation products in growth stimulation of propionibacteria (Field and Lichstein, 1957).

The possibility that dissimilar chemical compounds may serve in the same functional capacity as a natural growth stimulant is illustrated by recent reports that ethylenediaminetetraacetic acid and certain other metal chelating agents may substitute for natural auxin, indole-3-acetic acid, in elongation of *Avena* coleoptile (Heath and Clark, 1956; Bennet-Clark, 1956). These observations led the authors to postulate that the stimulatory activity of indole acetic acid itself may be a consequence of some specific metal chelating activity. The role of glucose-phosphate factor in growth stimulation of *B. globigii* also has been approached by searching for substitutive compounds. Lankford *et al.* (1957) have found that glucose-phosphate factor may be substituted by a variety of substances diverse in chemical structure except for their common potential as metal chelating agents. This, in conjunction with evidence for an interdependence of glucose-phosphate and $Mn^{++}$ in growth initiation of *B. globigii* (Lankford *et al.*, 1957; Sergeant, 1957) suggests that glucose-phosphate, and those substances which substitute for it, may function in the assimilation of certain essential metal ions.

## SUMMARY

Autoclaved solutions of glucose and phosphate stimulate growth of certain *Bacillus* species in a filtered medium containing glucose, DL-glutamate, $NH_4^+$ and salts. This stimulation is referable primarily to a reduction in the lag phase, whereas the growth rate and the maximum cell density generally are independent of glucose-phosphate. Growth which may occur eventually in the absence of glucose-phosphate appears to be initiated as the result of a temporary "adjustment" in the inoculum cells, rather than to selection of nondependent mutant cells. The ratio of the growth time of cultures without glucose-phosphate to those with the factor increases from 1 to > 3 as the size of the inoculum is reduced, or as the concentration of glucose-phosphate is increased to an optimum of about 300 μg per ml (as glucose), above which the lag again is increased. Over a narrow range of concentration the growth response of *Bacillus globigii* is proportional to the dose of glucose-phosphate, a relationship which has been applied to activity assays.

The glucose-phosphate factor is adsorbed on charcoal and may be extracted partially with ether, properties which distinguish it from the functionally similar glucose-phosphate factor for lactic acid bacteria. Assays of residual activity of chemically treated concentrates indicate that the glucose-phosphate factor is thermostable, nonvolatile and acidic, and that its activity appears to depend upon at least one carboxyl, one hydroxyl, and unsaturated bonds.

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