STIMULATION OF GROWTH INITIATION BY HEAT DEGRADATION PRODUCTS OF GLUCOSE[1]

H. H. RAMSEY[2] AND C. E. LANKFORD

Department of Bacteriology, University of Texas, Austin, Texas

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The first report of enhanced growth of microorganisms due to heating sugars was by Fulmer and Heussleman (1927), who noted an improved response of *Saccharomyces cerevisiae* if sucrose were heated with NH_4Cl or K_2HPO_4, alone or in combination. Fulmer, Williams and Werkman (1931) extended this work and observed that the amount of stimulation was directly proportional to the degree of caramelization of the autoclaved mixture; however, removal of the brown caramel color failed to reduce the stimulatory activity.

S. Orla-Jensen (1931) and A. D. Orla-Jensen (1933) found that certain streptococci and lactobacilli would grow only after a prolonged initial stationary phase if glucose were sterilized separately from the basal medium. Prompt growth ensued if the sugar were sterilized either in the medium or separately with tap water.

The results of Fulmer, Williams and Werkman were confirmed by Smiley, Niven, and Sherman (1943) and by Niven (1944), who also noted that a stimulatory effect was produced if glucose were sterilized in a medium containing phosphate, or sterilized separately with phosphate, NH_4OH or NaOH. It was suggested that the stimulatory effect was due to the formation of acetaldehyde, pyruvate or similar substances which served as "trigger" compounds by accepting hydrogen in the initial dehydrogenation of triose phosphate.

Snell, Kitay and Hoff-Jørgensen (1948) noted that *Lactobacillus bulgaricus* required glucose degradation products for prompt growth initiation. Pyruvate was also stimulatory for the organism; however, acetaldehyde and methyl glyoxal were highly toxic. Other organisms which have been reported to respond to heat degradation products of glucose include *Streptococcus faecalis* (Rabinowitz and Snell, 1947) and *Lactobacillus leichmannii* (Hoffman *et al.*, 1949).

Growth initiation of *Lactobacillus arabinosus* and *Streptococcus faecalis* was found by Rose and Peterson (1949) to be delayed 24 hr or longer if glucose were autoclaved separately from the basal medium. The prolongation of lag was most pronounced with a light inoculum but was evident with all amounts of inocula. The stimulatory nature of the glucose degradation product could not be ascribed to a lowering of the oxidation-reduction potential nor to glucose-amino acid reaction products. The addition of preformed Maillard compounds (Maillard, 1912, 1916) lowered the Eh below that of the autoclaved glucose solution, but failed to enhance growth. However, a reducing agent such as ascorbic acid was also active in reducing the time required for the cells to enter the exponential phase of multiplication.

More recently, Snell and Lewis (1953) have reported that asparagus juice and other plant materials are highly stimulatory for *Lactobacillus fermenti* in an unheated medium. Approximately 70 per cent of the stimulation could be obtained by heating the medium and certain "heat-reaction products" of glucose (fructose, reducing agents) simulated the heat stimulation effect.

This investigation is a further study of the glucose degradation product(s) which favor prompt growth initiation, especially from small inocula. An attempt has been made to determine the optimum conditions necessary for maximum production of the stimulatory effect and to determine the phase of bacterial growth which is most affected by the factor(s).

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[2] Present address: Department of Bacteriology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma.

EXPERIMENTAL METHODS

The test organism used was *Lactobacillus fermenti* strain 36. Stock cultures were maintained as stabs in a medium consisting of glucose, 1 per cent; yeast extract (Difco), 1 per cent; and agar, 1.5 per cent. The medium used for growing inoculum cells was identical except for the omission of agar. Cultures were grown for 24 hr at 35 C prior to transfer to the test medium. When ready for inoculation the cells were centrifuged, washed once with sterile saline, recentrifuged, and resuspended in sterile saline to the original volume.

For measuring growth stimulation three methods were employed. The first consisted of turbidimetric determinations using the Klett-Summerson photoelectric colorimeter equipped with a blue filter, wavelength 400–465 mμ. Each tube received 1 drop of a 10^{-3} dilution of a 24-hr washed culture; measurement of turbidity commenced when visible growth appeared in the tubes and continued at regular intervals thereafter until two identical readings were obtained indicating maximal growth.

The second method employed the use of decimal dilutions of inoculum ranging from undiluted to a 10^{-7} dilution of the 24-hr culture. One drop of each of these dilutions was inoculated into tubes containing 10 ml of single-strength basal medium. Beginning at 10 to 12 hr after inoculation the tubes were examined for visible growth. The tubes were reexamined at regular intervals thereafter until the tube containing the highest dilution of inoculum exhibited growth in at least part of the tests.

For quantitative estimation of stimulatory activity, a typical microbiological assay procedure was devised. In order to obtain smooth, reproducible assay curves, it was necessary to supplement the basal medium with 0.05 per cent (w/v) "Tween 80." Each of a series of tubes containing increments of the heated glucose mixture or other supplements received an inoculum of 0.05 ml of a 24-hr broth culture which had been washed twice and starved an additional 6 hr in phosphate buffer. After 17 hr incubation at 35 C growth response was measured turbidimetrically or by titration with 0.0125 N NaOH.

The double strength basal medium employed throughout consisted of the following: acid-hydrolyzed casein, 10 g; glucose, 40 g; Na acetate, 12g; K_2HPO_4, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 250 mg; NaCl, 20 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $MnSO_4 \cdot 4H_2O$, 20 mg; L-cystine, 20 mg; L-tryptophan, 10 mg; asparagine, 10 mg; adenine, 20 mg; guanine, 20 mg; xanthine, 20 mg; uracil, 20 mg; *p*-aminobenzoic acid, 100 μg; pyridoxal, 100 μg; riboflavin, 500 μg; thiamin, 500 μg; Ca pantothenate, 500 μg; biotin, 10 μg; and water 1,000 ml; final pH 7.0.

The term "glucose separate" referred to in the text denotes a 20 per cent solution of glucose sterilized separately from the basal medium and added aseptically to the sterile basal medium to give a final concentration of 2 per cent. The term "glucose-phosphate mixture" refers to a solution containing 20 per cent glucose and 2 per cent phosphate (pH 6.4) salts treated in the same manner. All solutions, unless otherwise indicated, were sterilized in the autoclave at 121 C for 15 min.

RESULTS AND DISCUSSION

Optimum conditions for the production of a stimulatory effect. Using the graded inoculum method of testing for stimulation, it has been found that autoclaving glucose in the medium or separately with phosphate results in a marked enhancement of growth at all levels of inoculum, whereas sterilization of glucose separately, either by heating or by filtration, has no such effect (figure 1). Further, it would appear that sterilizing glucose with phosphate salts separately from the basal medium results in somewhat better growth than glucose sterilized in the medium. This may be attributed to two factors: First, sterilizing glucose in the medium may result in the destruction of some essential nutrient (Lankford, Swausch, and Ravel, 1947; Lankford and Lacey, 1949; Patton and Hill, 1948). Secondly, the two components are present in more concentrated amounts when sterilized separately; the concentration in the medium is only $\frac{1}{10}$ that of the glucose-phosphate mixture. It is also apparent that the response to heat-sterilized glucose is of a lower order than the response to filter-sterilized glucose; therefore, it is concluded that no appreciable production of a stimulatory effect results from heating glucose in the absence of inorganic phosphate.

The minimum concentrations for the production of the maximum stimulatory effect are 2 per cent phosphate and 3 per cent glucose. Decreasing the concentration of either ingredient

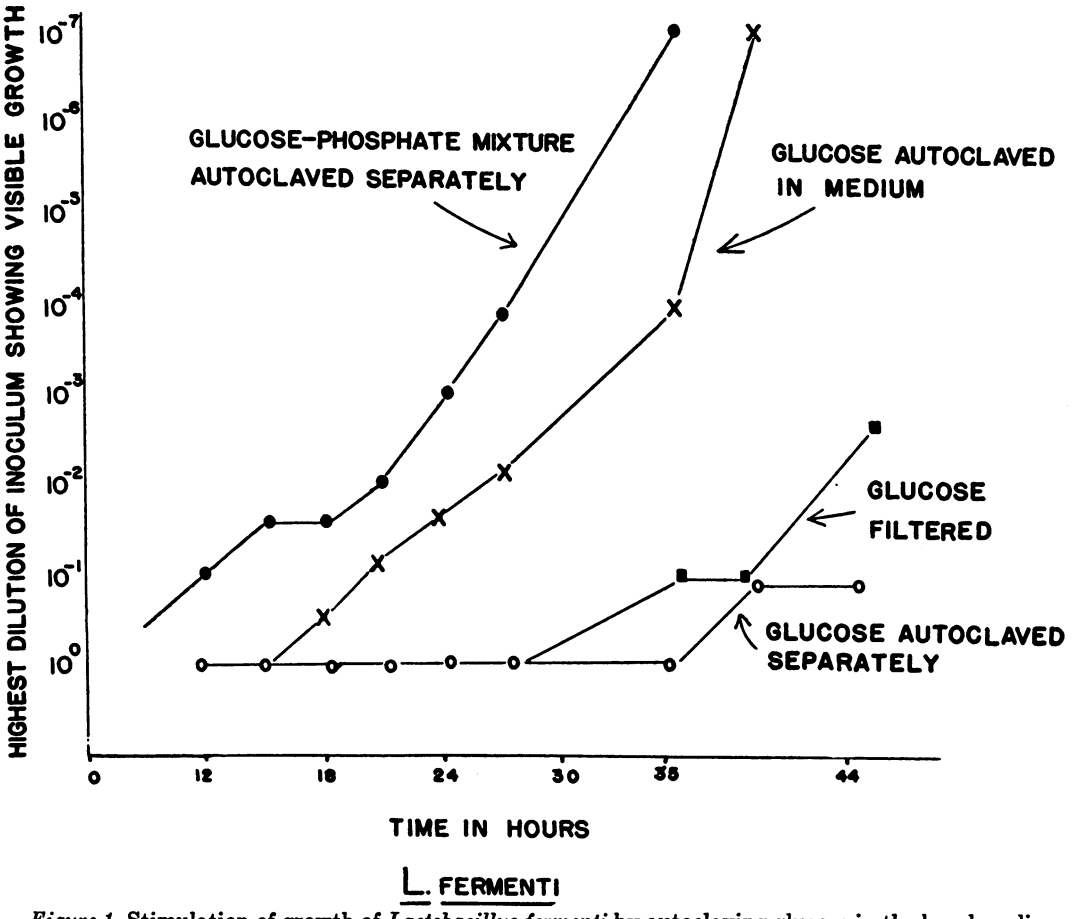

Figure 1. Stimulation of growth of *Lactobacillus fermenti* by autoclaving glucose in the basal medium or separately with phosphate.

results in a lowered activity, regardless of the concentration of the other ingredient.

The unadjusted pH of the glucose-phosphate mixture is 6.4. A comparison was made between this pH and other pH values to determine the optimum initial hydrogen ion concentration of the mixture necessary for the maximum degree of production of the stimulatory effect (figure 2). It was observed that a slight reduction in the pH tended to reduce the stimulatory activity only slightly, whereas raising the pH to neutrality or above drastically reduced the stimulatory activity of the mixture. This is believed to be significant since, at the more alkaline pH, degradation of the glucose molecule is most extensive (Zerban, 1947). In successive tests it was found that a rather sharp optimum existed around pH 6.4 with the activity dropping sharply at pH 7 and above, and more gradually at acid pH values. The authors believe this sharp optimum to be a new observation and worthy of emphasis.

In order to determine the effect of time and temperature of sterilization of the glucose-phosphate mixture upon the production of the stimulatory effect, separate mixtures containing 20 per cent glucose, 1 per cent K_2HPO_4, and 1 per cent KH_2PO_4 were autoclaved under the following conditions: 5 min at 115 C, 5 min at 121 C, 15 min at 121 C and 60 min at 121 C (table 1). It may be seen that heating the mixture for 15 min or longer at 121 C produced a higher concentration of the stimulatory substance(s) than heating for shorter periods. This effect of time and temperature of sterilization was confirmed by a plating experiment in which plates of the agar base medium, to which were added the sterile glucose-phosphate mixtures, received

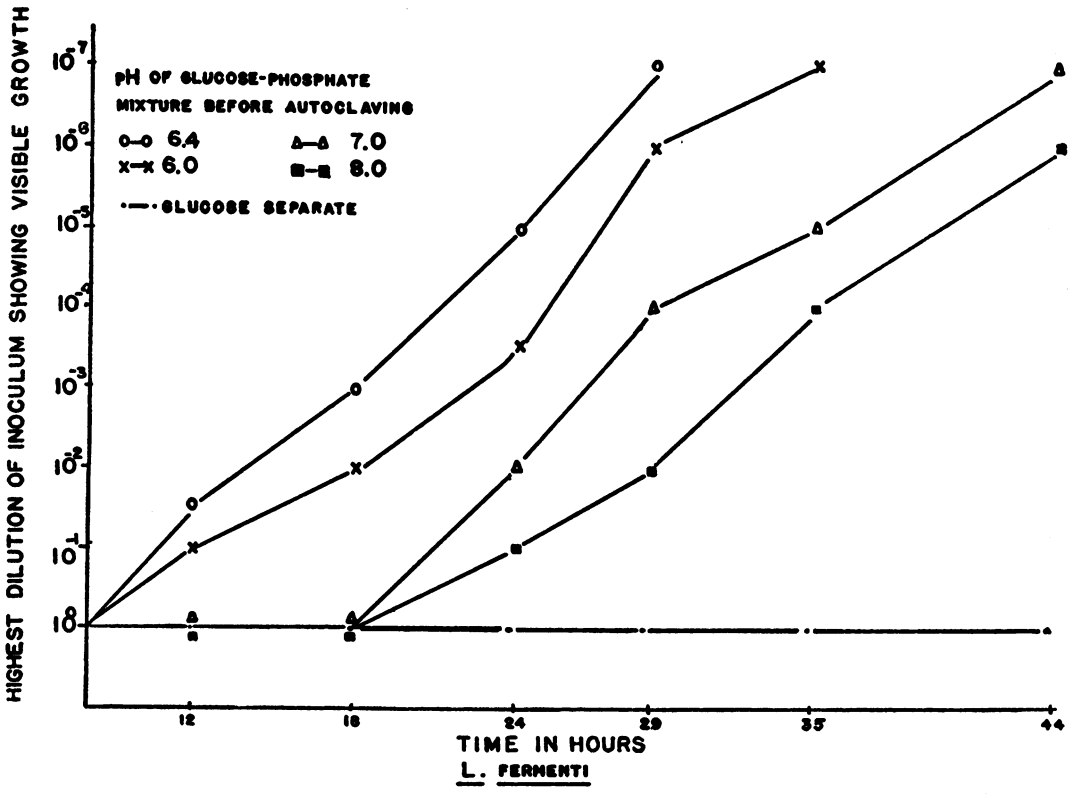

Figure 2. The effect of initial pH of the glucose-phosphate mixture on production of stimulatory substance(s).

TABLE 1

The effect of time and temperature of sterilization of the glucose-phosphate mixture on growth of Lactobacillus fermenti

Time*	Temperature*	Growth at 25 Hr†	Colonies at 48 Hr
5 min	115 C	10^{-3}	533
5 min	121 C	10^{-4}	520
15 min	121 C	10^{-5}	785
60 min	121 C	10^{-6}	945
"Glucose separate"		10^{0}	0

* Time and temperature at which each glucose-phosphate mixture autoclaved.

† Highest dilution of inoculum showing growth.

an inoculum of 1 ml of a 1:5,000,000 dilution of the washed cellls. Although differences in numbers of colonies were not striking with different autoclaving times it is significant that the factors resulting from the interaction of glucose (or its degradation products) and phosphate not only decrease markedly the time required for growth initiation, but also stimulate a greater proportion of the cells to initiate growth. This experiment, even more than the broth test, shows conclusively that the comparatively mild heat of 115 C for 5 min produces a pronounced effect on the growth response. Visible colonies appeared in 24 hr on those plates receiving the glucose-phosphate mixtures, whereas other plates gave no indication of growth. The plates containing glucose-phosphate mixtures autoclaved for 15 or 60 min showed large and easily visible colonies, whereas in the other plates the colonies were small enough to be designated "pinpoint."

The phase of growth affected by the glucose phosphate mixture. It was noted above that the primary effect of the glucose-phosphate mixture seemed to be a shortening of the initial stationary phase of growth. Using the turbidimetric assay method, it has been found that the length of the initial stationary phase is inversely proportional to the concentration of the glucose-phosphate mixture (figure 3). As little as 2 mg (measured in terms of glucose supplied in the glucose-phosphate mixture) per ml results in a shortening

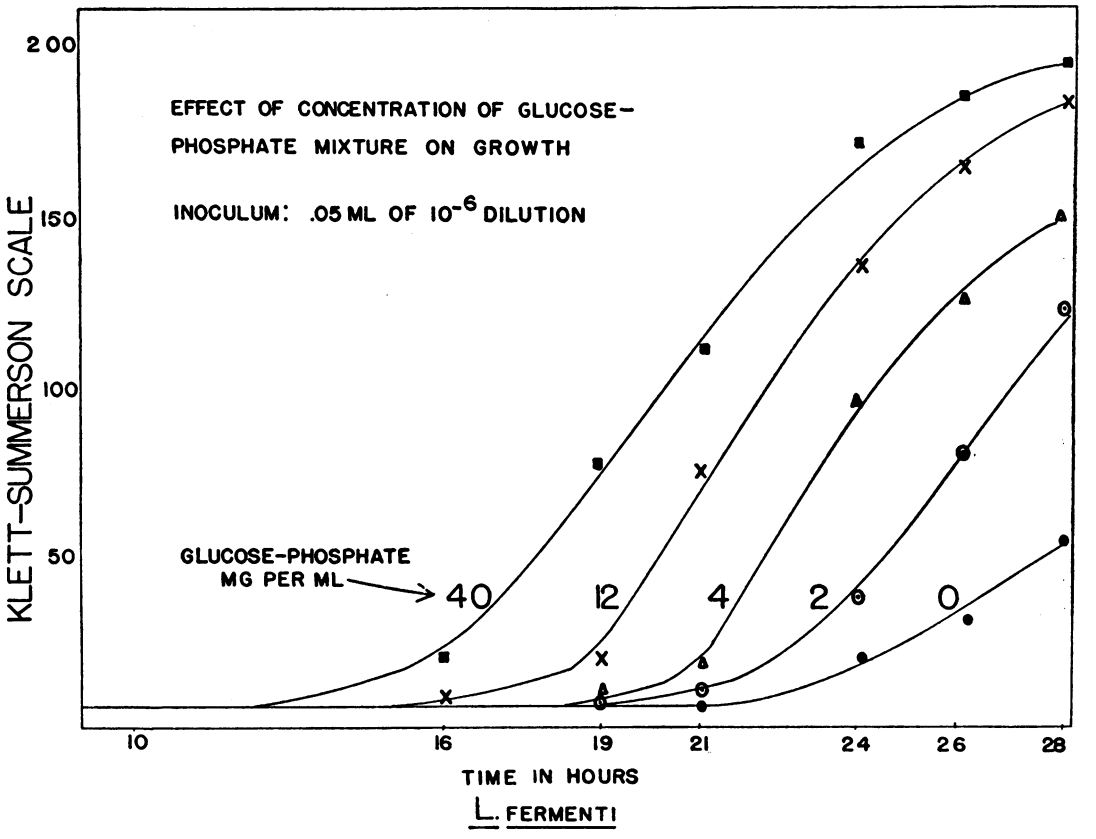

Figure 3. The relationship between concentration of active substance(s) in the glucose-phosphate mixture and lag time of *Lactobacillus fermenti*. Growth is recorded as Klett-Summerson turbidimetric units.

of the initial stationary phase by 2 hr. Higher concentrations are proportionally more effective in this manner, with 40 mg per ml resulting in a decrease in the initial stationary phase of 50 per cent. Plating experiments, not shown here, tend to substantiate the above results. Once growth is initiated, the rate of multiplication in the exponential phase is largely independent of the presence of the glucose-phosphate mixture as is the final cell crop.

Studies on the chemical nature of the glucose-phosphate factor(s). As mentioned prevously, Smiley, Niven, and Sherman (1943) concluded that the active compound which they investigated was acetaldehyde or pyruvate. In order to test the possibility that the activity described here might be due to an aldehyde or ketone, 2,4-dinitrophenylhydrazine and the specific aldehyde fixative "dimedon" (dimethyldihydroresorcinol, Eastman) were added to separate aliquots of the glucose-phosphate mixture after autoclaving to a final concentration of 0.5 per cent. No reduction in activity was observed in either case.

Using the method of Friedmann and Haugen (1943), glucose-phosphate mixtures autoclaved at various pH values were examined for an increase in keto acid concentration. All mixtures showed a significant increase in keto acid concentration; the greatest increase was found in mixtures autoclaved at alkaline pH or neutrality. Despite this appreciable production of keto acids, these compounds are not believed to be concerned in the growth-promoting activities of the glucose-phosphate mixture since activity is not decreased by addition of ketone fixatives or by acidification of the mixture followed by distillation *in vacuo*, or adsorption with "norit A" or ion-exchange resins (Dowex).

Ether extraction at pH values of 1.0 to 7.0

does not decrease the activity of the mixture. On the contrary, extracting the mixture with ether 4 times at pH 6.4 removed some toxic material and resulted in a 10-fold increase in response by the organism (figure 4). This is believed to be one of the first observations of toxicity by glucose degradation products, although numerous observations have been made on indirect toxic effects due to inactivation or binding of nutrients by aldehydes or other breakdown products of sugars (Lankford *et al.*, 1947; Patton and Hill, 1948).

Available evidence indicates that the active substance(s) in question is probably not a phosphorylated derivative of glucose since:

(1) Using the method of Lowry and Lopez (1946) and Koepsell, Johnson and Meek (1944) no reduction in free phosphate was found in the autoclaved glucose-phosphate mixture, although the reducing sugar content was decreased by 25 per cent.

(2) Incubation of the glucose-phosphate mixture with 1 per cent phosphorylase (Takadiastase, Parke-Davis) for 1 hr prior to addition to the basal medium did not reduce the stimulatory activity.

(3) Of the phosphorylated compounds tested (phosphoglyceric acid, hexose diphosphate, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate, and β-glycerol phosphate) none yielded activity comparable with that of the autoclaved glucose-phosphate mixture. It might be pointed out, however, that some of the phosphorylated compounds (e. g., phosphoglyceric acid and hexose diphosphate) stimulated growth much more than the glucose separate solution.

Attempts to concentrate and purify the active fraction of the glucose-phosphate mixture have been only partially successful. Extraction of the crude mixture at various pH values with a variety of organic solvents such as ether, chloroform, carbon tetrachloride, acetone, butanol, and benzyl alcohol have resulted in no loss of activity in the mixture. A partial purification has been accomplished by column chromatography. The use of a butanol-acetic acid-water solvent (4:1:1) on a starch column has resulted in a 20-fold concentration of the active material(s). This active fraction was devoid of both reducing sugar and inorganic phosphate. The chromatographic procedures have been hampered by

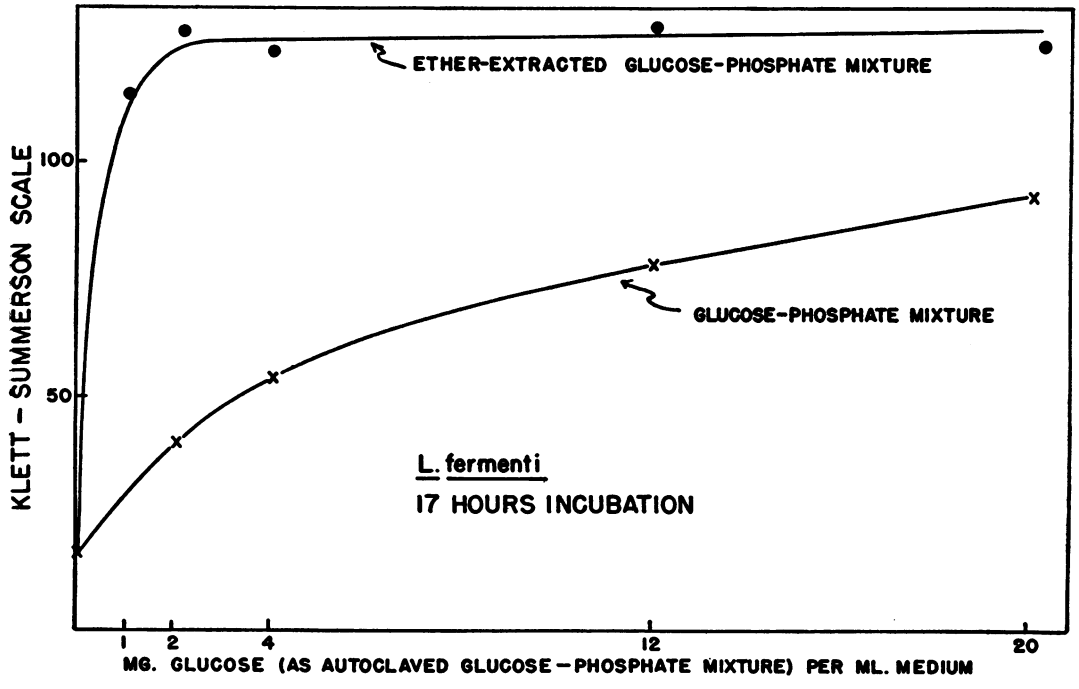

Figure 4. Quantitative response of *Lactobacillus fermenti* to untreated and ether-extracted glucose-phosphate mixture. Growth is recorded as Klett-Summerson turbidimetric units.

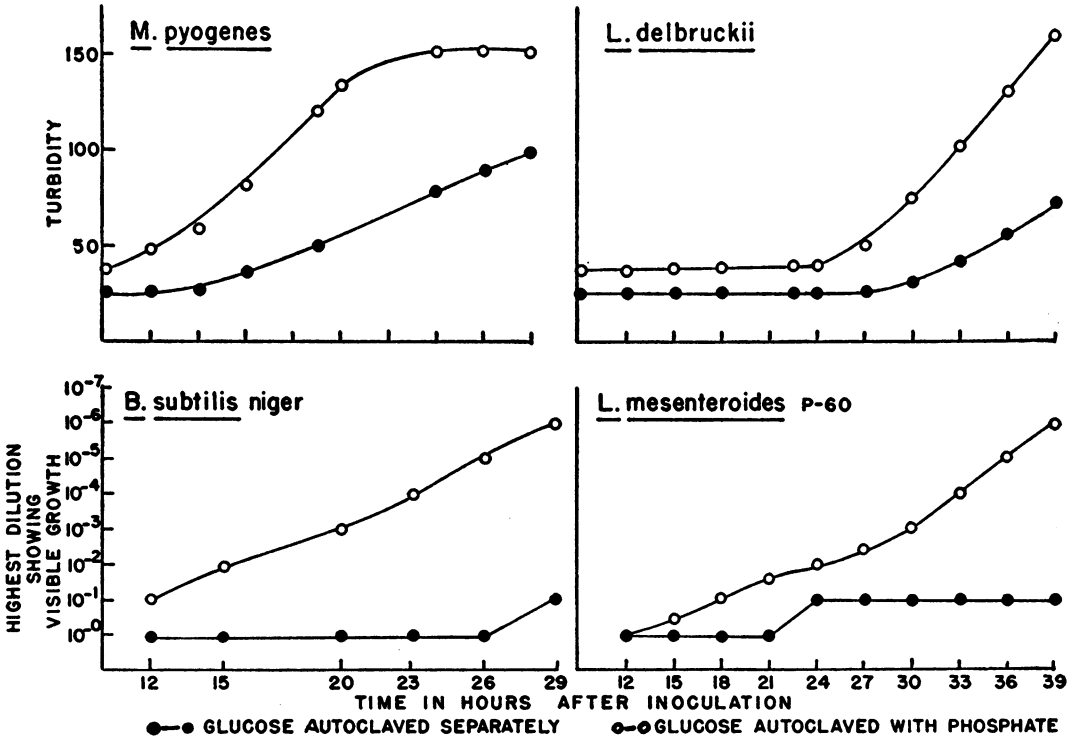

Figure 5. The effect of autoclaved glucose-phosphate mixture on growth initiation of different species.

several factors. First, when the crude glucose-phosphate mixture is evaporated and resuspended in the solvent a thick paste results which does not migrate down the column. Secondly, the glucose present in the mixture appears to interfere with the migration of the active fraction. For these reasons successful isolation and identification of the active compound(s) has not been possible.

Tests with a variety of substances have demonstrated that many different chemical compounds, as well as certain complex mixtures obtained from biologic sources, may operate to reduce the lag time in a medium containing separately sterilized glucose. In so shortening the lag time the compounds may thereby mask or replace the glucose-phosphate effect. Although tests of likely compounds were by no means exhaustive, the following list of active substances indicates the complexity of any problem dealing with growth initiation: certain phosphorylated derivatives of sugars; reducing agents; CO_2; pyruvate, oxalacetate, and other Krebs cycle compounds; glutamine and peptides of tryptic casein digest; unidentified components of peptone, yeast extract and liver; calcium (also noted by Yu and Sinnhuber, 1955); maltose and fructose (also noted by Snell and Lewis, 1953); and kojic acid.

The stimulatory effect described herein is apparently not limited to *L. fermenti*. In a limited survey of other species, *Micrococcus pyogenes* var. *aureus*, *Lactobacillus delbruckii* strain LD3, *Bacillus subtilis* var. *niger*, and *Leuconostoc mesenteroides* strain P60 initiated growth more promptly in the presence of the autoclaved glucose-phosphate mixture (figure 5); *Aerobacillus polymyxa* and *Streptococcus lactis* responded in a similar manner. The phenomenon is not universal in occurrence, however, since a number of organisms (*Pseudomonas* species, *Achromobacter* species, *Escherichia coli*, *Aerobacter aerogenes*, *Salmonella typhosa*, and *Proteus morganii* were unaffected by the presence or absence of the mixture.

SUMMARY

A compound, or compounds, is produced by the heat-catalyzed interaction of glucose and phosphate which markedly reduces the time

required for cells to enter the exponential phase of growth. The factor(s) has little or no effect on the rate of growth once multiplication commences or on the total cell crop. The compound(s) has not been identified but does not appear to be an aldehyde or ketone, a phosphorylated derivative of glucose, a volatile compound, a reducing agent, or an acidic compound.

REFERENCES

FRIEDMANN, T. E. AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., **147**, 415–442.

FULMER, E. I. AND HEUSSLEMAN, B. 1927 The production of a yeast growth stimulant by heating media under pressure. Iowa State Coll. J. Sci., **1**, 411–417.

FULMER, E. I., WILLIAMS, A. L. AND WERKMAN, C. H. 1931 The effect of sterilization of media upon their growth promoting properties toward bacteria. J. Bacteriol., **21**, 299–303.

HOFFMAN, C. E., STOKSTAD, E. L. R., HUTCHINGS, B. L., DORNBUSH, A. C., AND JUKES, T. H. 1949 Microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii*. J. Biol. Chem., **181**, 635–644.

KOEPSELL, H. J., JOHNSON, J. J., AND MEEK, J. S. 1944 Role of phosphate in pyruvic acid dissimilation by cell-free extracts of *Clostridium butylicum*. J. Biol. Chem., **154**, 535–547.

LANKFORD, C. E., SWAUSCH, C., AND RAVEL, J. M. 1947 Further studies on the effect of sterilizing glucose in culture media on growth of microorganisms; utilization of cystine by lactic acid bacteria. J. Bacteriol., **53**, 368.

LANKFORD, C. E. AND LACEY, H. 1949 Destruction of nutrient components in culture media autoclaved with glucose. Bacteriol. Proc., **1949**, 34.

LOWRY, O. H. AND LOPEZ, J. G. 1946 The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem., **162**, 421–428.

MAILLARD, L. C. 1912 Actions des acides amines sur les sucres; formation des melanoidines par voie methodiques. Compt. rend., **154**, 66–68.

MAILLARD, L. C. 1916 Syntheses des matieres humiques par action des acides amines sur les sucres reducteurs. Ann. Chim. et Phys., **5**, 258–317.

NIVEN, C. F. 1944 Nutrition of *Streptococcus lactis*. J. Bacteriol., **47**, 343–350.

ORLA-JENSEN, S. 1931 Die Abhängigkeit der Milchsäuregärung von der Art und Weise, in welcher, die Sterilisierung der Nährböden ausgeführt wird. IX^{e} Congr. intern. Larterie., Compt. rend., Copenhagen.

ORLA-JENSEN, A. D. 1933 Hitherto unknown activators for the growth of lactic acid bacteria. J. Soc. Chem. Ind., **63**, 107–110.

PATTON, A. R. AND HILL, E. G. 1948 Inactivation of nutrients by heating with glucose. Science, **107**, 68–69.

RABINOWITZ, J. C. AND SNELL, E. E. 1947 The vitamin B_6 group. XI. An improved method for assay of vitamin B_6 with *Streptococcus faecalis*. J. Biol. Chem., **169**, 631–642.

RAMSEY, H. H. AND LANKFORD, C. E. 1952 Stimulation of growth initiation by products of glucose and phosphate interaction. Bacteriol. Proc., **1952**, 165–166.

ROSE, D. AND PETERSON, R. 1949 Influence of the amino acid-dextrose reaction on growth of lactic acid bacteria. Can. J. Research, B, **27**, 428–436.

SMILEY, K. L., NIVEN, C. F., AND SHERMAN, J. M. 1943 The nutrition of *Streptococcus salivarius*. J. Bacteriol., **45**, 445–454.

SNELL, E. E., KITAY, E., AND HOFF-JØRGESEN, E. 1948 Carbohydrate utilization by a strain of *Lactobacillus bulgaricus*. Arch. Biochem., **18**, 495–510.

SNELL, N. AND LEWIS, J. C. 1953 Nutritional studies with *Lactobacillus fermenti*. J. Bacteriol., **65**, 671–677.

YU, T. C. AND SINNHUBER, R. O. 1955 Stimulatory effect of calcium on growth of *Lactobacillus fermenti*. Proc. Soc. Exptl. Biol. Med., **88**, 238–240.

ZERBAN, F. W. 1947 The color problem in sucrose manufacture. Tech. Rpt. Ser. No. 2, Sugar Research Foundation Inc., New York, N. Y.