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Influence of dextrose on the viability of *Bacillus subtilis*

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Dextrose causes an increase in viable count of *Bacillus subtilis* and the effect is greater for spores than vegetative cells. The proportion of spores in which germination is initiated is not influenced by dextrose. During germination the ability of dextrose to affect the viable count is completely lost but re-appears as the germinated spore changes into a dividing vegetative cell. When spores are incubated in broth before plating in nutrient agar there is an increase in viable count followed by a fall before the increase in count due to division is detected. When plated in dextrose agar this initial rise is not seen and there is a fall in count before onset of division. The magnitude of the increase in count caused by dextrose is dependent on the method of plating. For maximum recovery of viable organisms the combination of roll-rube method with dextrose agar is to be preferred to surface plating with or without dextrose in the counting medium.

IT has been shown previously (Richardson, 1965) that in the presence of 0·5% dextrose in the counting medium there is an increase in viable count of up to 75% for suspensions of *Bacillus subtilis* spores. The point in growth at which the presence of dextrose enables a cell or spore, otherwise non-viable, to produce a colony has been sought. Initially attempts were made to discover whether there was any difference between the magnitude of increase in count for spore, and that for vegetative cell suspensions.

Experimental

In addition to methods and materials previously described (Richardson, 1965), the following techniques were used.

Vegetative cell suspensions. These were prepared from an overnight broth culture of *B. subtilis* N.C.T.C. 8236 by the method of Adams (1966). Microscopical examination showed the suspension to be spore-free and to consist almost entirely of single cells. The suspension was used on the day of preparation. The diluent for counting vegetative cell suspensions was nutrient broth.

Roll-tube counts. These were determined using Astell roll-tubes. Tubes were incubated in an inverted position, with bungs removed to permit free diffusion of air into the tubes.

Surface viable counts. These were determined by spreading 0·5 ml inocula on the surfaces of well-dried plates, which were then incubated after a further short drying period (Roberts, 1961).

The complete counting experiment, involving the plating of 10 samples each of spores and 10 samples of vegetative cells, in roll-tubes and on plates with and without dextrose, was repeated on two further occasions.

Dextrose agar. Viable counts were compared in nutrient agar and in nutrient agar containing 0·5% dextrose. This was added to the medium before autoclaving. No change in dextrose concentration could be detected after autoclaving using Sumner's dinitrosalicylic acid reagent (Hawk, Oser & Summerson, 1952).

Dextrose effect. The observed effect of dextrose is termed the

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"dextrose effect" and is calculated from the viable count in presence of dextrose divided by the viable count in the absence of dextrose.

Change in dextrose effect during germination. Spores were incubated at 37° in nutrient broth and in 5 mM alanine solution. Samples were plated in roll-tubes without further dilution over a period of 5 hr to determine the change in dextrose effect during this time.

Influence of dextrose when present during incubation before plating. A comparison was made between the viable counts of spores incubated in nutrient broth and in nutrient broth containing dextrose, sampling at intervals for a period of 2 hr and plating in roll-tubes in dextrose-free medium. In this series of experiments dilution in broth at 37° was made before plating to inactivate the dextrose carried over to the plating medium (actual final concentration of 5–0·5 ppm was shown to have no effect on count). Samples were also plated in dextrose agar.

Nephelometry. The rates of germination and the subsequent growth pattern were followed by making nephelometric measurements on suspensions in nutrient broth (Oxoid CM1) or alanine solution (5 mM), with and without dextrose and held in a shaking water bath at 37°. Measurements were made in $\frac{3}{4}$ in tubes in an EEL nephelometer standardized to read 100 against an appropriate Perspex standard. In practice this corresponded to a spore count of about 5×10^6/ml and a check was made to establish that the meter reading varied linearly with concentration over the whole range. Eight replicate samples were incubated simultaneously and the mean values of the eight determinations were used in presenting the data graphically.

Results and discussion

It was found that $\frac{1}{4}$-strength Ringer was not satisfactory as a diluent in the vegetative cell counts; these did not show a Poisson distribution and were considered unreliable. Even with broth as a diluent, counts showed a high degree of variation and this may account for the variation in level of significance observed within the replicates from vegetative cell counts (Tables 1 and 2).

TABLE 1. THE EFFECT OF DEXTROSE IN THE COUNTING MEDIUM ON THE VIABLE COUNT OF *B. subtilis*

Method	Inoculum	Mean of ten counts in: Nutrient agar	Mean of ten counts in: Dextrose agar	Significance of difference n = 10 + 10; 18 d.f. t	Significance of difference n = 10 + 10; 18 d.f. P	Mean dextrose effect (count in presence of dextrose / count in absence of dextrose)
Roll-tubes	Spores	76	140	15·4	<0·001	1·75
		88	155	16·8	<0·001	
		63	105	6·9	<0·001	
	Vegetative cells	119	176	12·8	<0·001	1·33
		133	152	2·0	0·05–0·1	
		61	83	4·6	<0·001	
Surface plates	Spores	74	109	7·1	<0·001	1·30
		88	110	4·6	<0·001	
		76	88	2·6	0·02–0·05	
	Vegetative cells	163	153	1·2	0·2–0·3	0·92
		127	118	1·5	0·1–0·2	
		85	77	2·1	0·05–0·1	

TABLE 2. THE RELATIONSHIP BETWEEN ROLL-TUBE AND SURFACE VIABLE COUNTS OF *B. subtilis*

Medium	Inoculum	Mean of ten counts from: Roll-tubes	Mean of ten counts from: Surface plates	Significance of difference n = 10 + 10; 18 d.f. t	Significance of difference n = 10 + 10; 18 d.f. P	Mean plating effect (count in roll-tubes / count on surface plates)
Dextrose agar	Spores	140 155 105	109 110 88	5·8 8·9 2·4	<0·001 <0·001 0·02–0·05	1·29
	Vegetative cells	176 152 83	153 118 77	2·4 4·8 1·5	0·02–0·05 <0·001 0·01–0·2	1·17
Nutrient agar	Spores	76 88 63	74 88 76	0·6 0·0 2·8	0·5–0·6 >0·9 0·01–0·02	0·95
	Vegetative cells	119 133 61	163 127 85	5·2 0·7 5·2	<0·001 0·4–0·5 <0·001	0·83

EFFECT OF DEXTROSE

Magnitude. From the figures in Table 1 it is seen that when counting was by the roll-tube method, the effect of dextrose in the counting medium was greater for spores than for vegetative cells (an average increase of 75% as against 33%). With the surface viable count there was a smaller increase for spores on the dextrose medium (30%); for vegetative cells a slight decrease in count was noted. It seems possible that the lower counts obtained on dextrose plates may be due to over-crowding of colonies. Colonies on dextrose agar appeared more quickly and were much larger than colonies on nutrient agar.

Influence of dextrose on the change in opacity during growth. When the influence of dextrose on growth was observed nephelometrically the results were inconclusive. The change in opacity which occurs during initiation of spore germination was affected very little by the presence of dextrose. Minimum opacity occurred perhaps slightly sooner in the

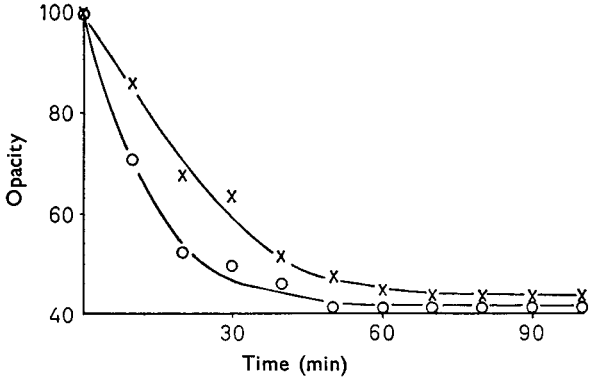

FIG. 1. The change in opacity of *B. subtilis* spore suspensions on incubation in alanine solution. ×, No dextrose; ○, dextrose 0·5%.

presence of dextrose but the % reduction in opacity was similar in each instance. This pattern occurred whether germination was induced by alanine solution (Fig. 1) or by nutrient broth (Fig. 2). The only marked

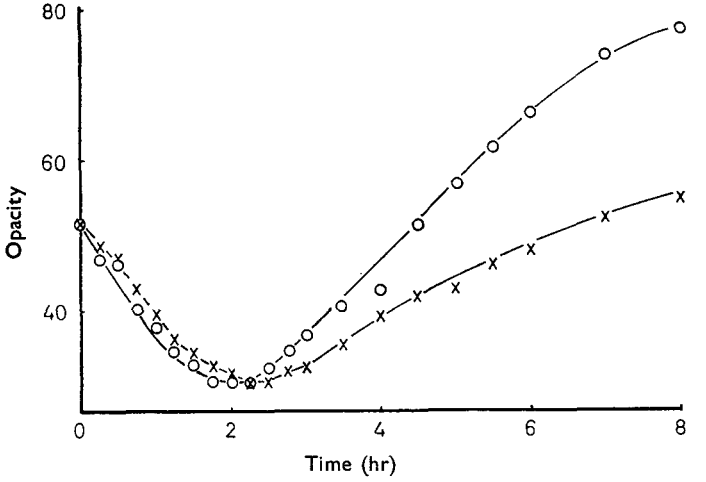

FIG. 2. The change in opacity of *B. subtilis* spore suspensions on incubation in nutrient broth. ×, No dextrose; ○, dextrose 0·5%.

difference was seen after completion of germination when there was a greater increase in opacity in the presence of dextrose. This difference was also seen with a vegetative cell inoculum (Fig. 3).

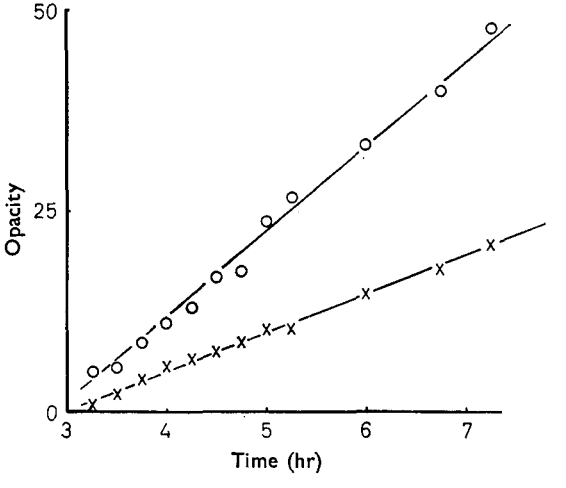

FIG. 3. The change in opacity of *B. subtilis* vegetative cell suspensions on incubation in nutrient broth. ×, No dextrose; ○, dextrose 0·5%.

From these results it is not possible to distinguish between an increase in rate of growth due to a quicker metabolic rate (including reduced

generation time) or an increase in the initial number of cells able to reproduce. The latter alternative would be shown in Fig. 2 by parallelism between the two lines but only if all potentially viable cells simultaneously became susceptible to the presence of dextrose (e.g. at a particular point in growth of a synchronous culture) and also if dextrose did not also cause an increase in rate of growth. Thus the observed effect of dextrose can only be interpreted in general terms as an acceleration in rate of increase in cell mass.

The difference in the ability of dextrose to affect the viability of spores and vegetative cells might be attributed to a simple quantitative difference in metabolism or to a more fundamental difference between the utilization of dextrose by spores and vegetative cells. To investigate this point further, the change in magnitude of dextrose effect during the processes of germination and growth was followed more closely. The clear pattern emerged (Fig. 4) that as germination proceeded the ability of dextrose to

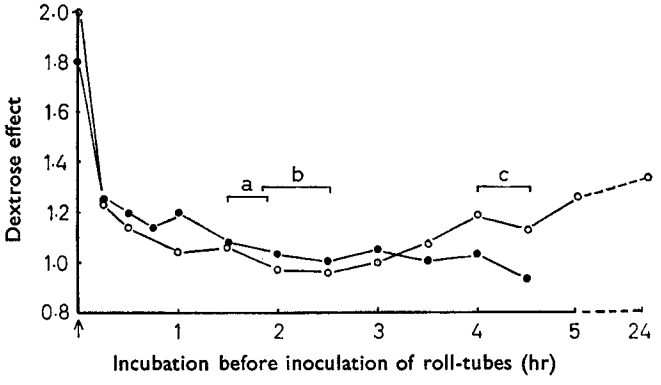

FIG. 4. The change in dextrose effect during growth of *B. subtilis* spores. ○, Incubation in broth; ●, incubation in alanine solution. (↑) Spore inoculum, (a) germination complete, (b) appearance of vegetative cells, (c) count rises due to division. Dextrose effect = count in presence of dextrose/count in absence of dextrose.

affect the count was completely lost, the value for the dextrose effect becoming 1·0. This loss occurred within 1–2 hr by which time germination was complete, although virtually no division had occurred. Subsequently, as the germinated cells began to divide, the influence of dextrose could again be detected, the dextrose effect reaching a maximum value during the first few divisions.

The same pattern was observed when incubation was in broth or dextrose broth, but, with incubation in alanine solution after the initial reduction in dextrose effect, the cells did not proceed to division and there was no subsequent gain in effect.

It seems likely therefore that two separate types of dextrose activity are involved, each associated with a different phase of growth and characterized by different magnitudes of the dextrose effect. The results as plotted in Fig. 4 show an interesting resemblance to the results of Blumenthal

(1965), who showed changes in the path of dextrose metabolism in *B. cereus* during the change from spore to vegetative cell.

Point of action of dextrose. The previous results show that dextrose must be present at or during initiation of germination in order to have maximum effect. An attempt was therefore made to discover whether there was a critical point during the germination process up to which dextrose, if present, could have some effect on the subsequent viability of the cell. To this end, spores were incubated with and without dextrose and then plated in dextrose-free medium. Mean results from five replicate experiments are shown in Fig. 5. Exposure to dextrose during initial

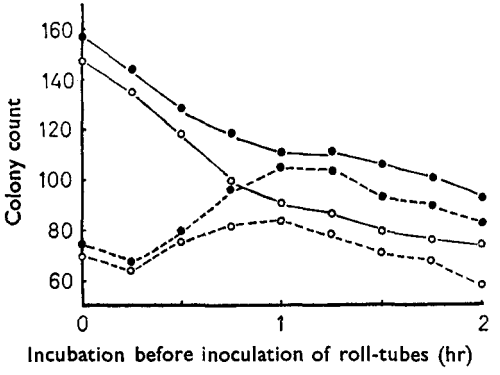

FIG. 5. The effect of dextrose during growth on the viable count of *B. subtilis* when present in the incubation medium before counting and when added to the counting medium. ●, Dextrose in incubation medium; ○, no dextrose in incubation medium; —— dextrose in counting medium; – – – no dextrose in counting medium.

incubation resulted in an increase in the subsequent viable count, becoming significant after about 30 min. The increase reached a maximum as germination was completed in about 90 min and then remained constant. However, the variation between replicates became excessive towards the end of the incubation period so that too great a reliance could not be placed on the later portions of the curves. It is felt that this excessive variation is an indication of the extent to which newly emergent cells are susceptible to environmental influences. Thus when dilution intervenes between incubation and plating there may be a removal of extracellular enzymes secreted during germination, the loss of which would adversely affect the further growth of the cell. This could also account for the fall in viable count during the early stages of germination mentioned below.

Interpretation of the change in the magnitude of the dextrose effect during germination and growth must take into account the associated changes in viable count (Fig. 5). Rather surprisingly it was found consistently during the early stages of incubation and before division could be detected microscopically, that the count showed a rise followed by a fall until the onset of division resulted in a second rise. This pattern was observed only when the cells were plated in dextrose-free medium.

When plated in dextrose agar a continuous fall occurred without the initial rise, until the fall was reversed by division. Thus the reduction in dextrose effect during the change from spore to vegetative cell is partly accounted for by an increase in count which occurs on incubation in the absence of dextrose and is not due solely to the loss of some dextrose-sensitive system during germination.

The ability of dextrose to affect viability disappears rapidly during germination. Thus it is necessary for dextrose to be present before or during initiation to achieve a maximum effect. However, dextrose does not cause activation of germination. Microscopical examination of spores germinating in alanine or broth showed that virtually all spores initiated the germination process (change in phase-contrast appearance) whether dextrose was present or not. In this respect the effect of dextrose is analogous to heat-activation in that, if the stimulus is applied before germination, the effect is observed not immediately but in the later stages of development. If the stimulus is applied later the effect is reduced (dextrose) or reversed (heat). The similarities of activation by heat and by reducing agents have previously been commented on by Keynan, Evenchik & others (1964).

If dextrose is present in the medium at the time of germination (spore inoculum) its effect is greater than if added after germination (vegetative cell inoculum). From the results it may be inferred that the presence of dextrose during initiation enables an additional number of spores to proceed through the stage of outgrowth or early division to form a viable clone. It is probable that two distinct mechanisms are involved, one taking effect during germination and the other during cell division.

EFFECT OF METHOD OF PLATING

When dextrose agar was used as the counting medium for both spores and vegetative cells the roll-tube method gave higher counts than the surface viable count. When nutrient agar was used roll-tube counts were equal to or lower than surface counts (Table 2). Thus dextrose encourages the development of submerged colonies or inhibits the development of surface colonies. The significance of this observation is not apparent but it is obvious that for highest counts of *B. subtilis* the combination of the roll-tube method and dextrose agar is required.

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