EFFECT OF STARVATION FOR GLUCOSE DURING REVERSION OF A LONG TERM ADAPTING YEAST[1]

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The mass reversion phenomenon in long term adapting yeasts was discovered by Spiegelman *et al.* (1950). The essential feature is that, whereas a galactose-grown population of cells of the gs genotype forms entirely positive colonies on galactose indicator plates, subculture of such a population on medium not containing galactose results in a sudden, mass change of the culture to a type that forms negative colonies. This change was termed reversion because the galactose-grown culture used was descended from a glucose-grown culture which likewise produced mostly negative colonies on galactose plates. Later (Spiegelman *et al.*, 1953), evidence was presented suggesting the following model for reversion:

(1) Reversion involves the dilution with growth of autocatalytic particles necessary for the formation of some enzyme(s) required for galactose fermentation.

(2) Any cell which contains one or more such particles at the time of plating will give rise to a positive colony.

(3) During growth in a galactose free medium, there is neither multiplication nor destruction of particles.

The reversion generation of a culture was defined by Campbell and Spiegelman (1956) as the number of generations of growth in galactose-free medium which that culture must undergo before 50 per cent of its cells produce negative colonies. According to the above model, the reversion generation is proportional to the logarithm of the average number of particles per cell. This constitutes the sole method for estimating this number. It has been used (Campbell and Spiegelman, 1956) to investigate the growth kinetics of the postulated particles.

The subject of the present communication is 3 cases in which the quantitative predictions of the model are not completely fulfilled. The first two were discovered in 1952–1953. It has not been clear whether they were best reconciled with previous data by negating statement (1) (which is equivalent to abandoning the entire model) or by modifying statement (2) or statement (3). We designed some experiments to decide which solution was most appropriate. They failed to show this, but led instead to the discovery of the third case.

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MATERIALS AND METHODS

The yeast strain (19.1), a respiratory deficient haploid segregant from the Winge strain 55 (*Saccharomyces chevalieri* × *S. cerevisiae* hybrid), and the yeast extract-peptone medium have been described previously (Campbell and Spiegelman, 1956). The C_1 strain of *S. chevalieri* is that used by Spiegelman *et al.* (1950, 1953). Commercial galactose (Fisher, reagent) was used for plating and growth medium. Because the galactose as purchased contains about 3 per cent glucose, 2 per cent galactose contains 0.06 per cent glucose. This is within the range previously found acceptable for plating[2] (Campbell, 1953). The plates contained, in addition to the components of the growth medium, 2 per cent agar, 0.04 per cent eosin, and 0.0025 per cent methylene blue.

The purified galactose mentioned in table 2 was made by suspending washed cells of *Torula monosa* in a 10 per cent solution of commercial galactose for 12 hr and then removing the torula by centrifugation. The resulting solution contained no detectable glucose. The N free synthetic medium of table 2 was Burkholder's (1943) medium without the $(NH_4)_2SO_4$. The

[2] Because 19.1 is respiratory deficient, some fermentable substrate other than galactose must be added to the plates to permit growth of negative or deadapted cells. This applies also in tables 1 and 2.

TABLE 1

*Reversion generation of strain 19.1 under various growth conditions**

Growth Conditions Before Reversion	Growth Conditions During Reversion	Number Determinations	Reversion Generation	Hours to Reach Reversion Generation
2% G broth	4% D broth	3	9.9	18
2% G broth in chemostat, galactose limiting; doubling time, 30–34 hr	4% D broth	2	9.6	16
2% G broth	15 hr, 0.1% D broth; remaining time, 4% D broth	8	10.0	18
2% G broth at 20 C	4% D broth	1	9.9	19
Same culture	4% D broth at 20 C	1	10.3	48
2% G synthetic	4% D broth	2	10.0	18
2% G synthetic + 0.1% asparagine	0.1% D synthetic + 0.1% asparagine	1	9.4	36
2% G synthetic + 0.1% asparagine	0.1% D synthetic + 0.1% asparagine	1	7.7	33

All cultures at 30 C unless otherwise specified.

* Abbreviations: G = galactose; D = glucose; broth = yeast extract peptone medium; synthetic = medium of Burkholder (1943).

TABLE 2

Effect of nitrogen starvation on reversion generation

Hours on N-free Synthetic Medium, 7.5 Per Cent Glucose	Reversion Generation
0	8.4
10	3.7
26.5	1.5
36.5	0.9

Zero time cells were C_1 strain which had been grown in 2 per cent purified galactose broth, harvested, and washed aseptically with sterile distilled water. Reversion generation was measured by subculturing from the starved culture into 2 per cent glucose broth, and plating at intervals onto plates containing yeast medium, eosin methylene blue, and 2 per cent purified galactose.

synthetic medium of table 1 was also that of Burkholder. Asparagine was added where indicated in order to improve the growth.

Platings were performed by placing at 48 C appropriate aliquots in 2.5 ml of 0.7 per cent agar of the same composition as the plating medium and layering immediately. After about 1 hr at room temperature, plates were inverted and incubated for 3 days at 29 C.

Cultures were kept agitated either by shaking in 250 ml flasks 240 times per min on a horizontal shaker in a 29 C room, or by aeration in 16 by 200 mm test tubes in a 30 C water bath. The culture volume was 10 to 20 ml.

EXPERIMENTAL RESULTS

Evidence for and exceptions to the simple dilution model for reversion of strain 19.1. We shall not attempt to summarize all the evidence favoring the model of Spiegelman *et al.* (1950), but shall confine ourselves to considering some aspects of it. From studies with strain C_1, Spiegelman *et al.* (1950) conclude, "The number of generations for the reversion has been . . . relatively independent of the division rate." The author's data on this point, which, though sketchy, are in substantial agreement with their conclusion, are presented in table 1.

This fact is easily explained by, and constitutes evidence for, the idea that autocatalyst is being diluted with growth. If any destruction occurs, the rate of destruction must vary as the rate of growth, within the limits of the conditions employed.

The evidence is weakened by the existence of conditions under which the reversion generation decreases markedly in the absence of growth. Table 2 shows that during prolonged nitrogen starvation, the reversion generation does so decrease. One can preserve statement (1) of the model by assuming that autocatalyst is destroyed

by this process, but there is no independent criterion for ascertaining the validity of this assumption.

A possible indication of destruction even during the normal reversion process is this: If statements (2) and (3) of the model are correct, the total number of positive cells in the culture should be a monotonically increasing function of time. The experimental curves drop soon after the 50 per cent point is reached. Their shape for strain 19.1 is shown in figure 1.

As implied in the introductory section, no critical evidence exists as to which of the three statements of the model should be modified to accommodate this fact. It was in an attempt to make such a distinction that a study of synchronous division in yeast was undertaken. It was reasoned that, if dilution of autocatalytic units plays an important role in the process, then, even under conditions where statements (2) and (3) might fail to be valid, one should observe

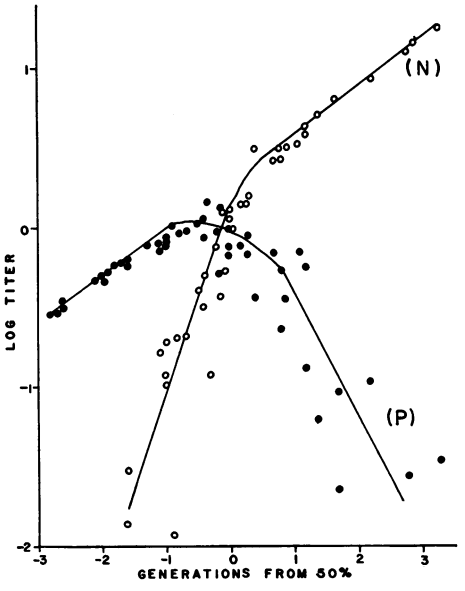

Figure 1. Pooled data from thirteen reversion experiments. Average reversion generation 9.8 (range 9.4–10.3.) Abscissa is the number of doublings before or after the 50 per cent point. Ordinate is the logarithm of the ratio of the titer of positive or negative cells (respectively) to its titer at the 50 per cent point. (*P*) positives. (*N*) negatives. Some of the early plates contained no negative colonies (out of about 500 colonies counted); no point was plotted in these cases.

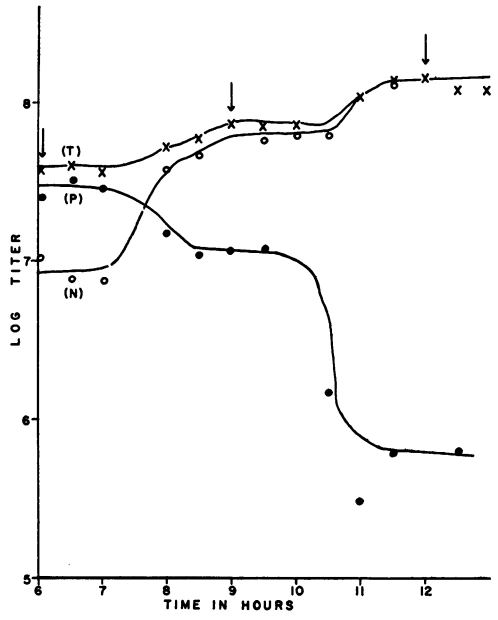

Figure 2. Typical repeated starvation experiment. Ordinate is log of titer referred to an initial growth tube. Abscissa is time after first starvation. Three hr starvation cycles. Points of dilution with fresh medium are indicated by arrows. (*T*) total colonies. (*P*) positives. (*N*) negatives.

a rapid, predictable change in the frequency of positive cells at the time of cell division. The method of repeated starvation for glucose used elsewhere (Campbell, 1957) was employed. The experiment did not answer the question for which it was designed, but led instead to the discovery of some effects of starvation for glucose on the reversion process.

Glucose starvation studies. In the first experiment, galactose-grown cells were added to 0.1 per cent glucose broth at such density that they underwent 5 or 6 doublings before starving. They were then subjected to a series of starvation cycles, as described elsewhere (Campbell, 1957). During each cycle, the population was allowed just to double before restarving. During the course of such cycles, plates were made on galactose agar and the number of positive and negative colonies tabulated.

The results of a typical experiment are shown in figure 2. The shape of the curves will be discussed later. The reversion generation in such experiments is 8.0 (6 experiments, range 7.6–

TABLE 3

Effect of glucose concentration at which starvation is performed

Experiment No.	Concentration Glucose	Generations to First Starvation	Generations During Starvation	Positives After Starvation	Reversion Generation
	%			%	
155	0.5	6.1	1.5	100	8.9
	0.091	6.1	1.4	95	8.2
	0.01	6.1	1.2	46	7.6
157	0.5	6.3	1.0	94	
	0.091	6.3	1.0	84	
	0.02	6.3	0.9	24	

8.5). If one performs the same experiment using 1 per cent glucose instead of 0.1 per cent, the population density is 10 times as large, and the reversion generation is 8.9 (4 experiments, range 8.7–9.1). Inasmuch as the normal reversion generation is 10 (table 1), this constitutes a third observation incompatible with the model of Spiegelman *et al.* (1953).

Simpler experiments designed to study the phenomenon further were then performed.

Effect of glucose concentration. Galactose-grown cultures were inoculated into 1 per cent glucose broth at such density that they would undergo about 6 generations before starving. The fact that the cell yield is proportional to the input glucose concentration (Campbell, 1957) was then utilized to allow starvation for the same growth period but from different glucose concentrations and at correspondingly different cell densities. Measured volumes of the starved culture were added to sufficient glucose broth of a particular concentration to give a calculated one doubling before starvation.[3]

[3] Sample calculation for 0.091 per cent glucose: The cell density from the 1 per cent glucose culture is 10 × what would be obtained from 0.1 per cent glucose. Therefore, to allow one doubling we must add one part of the starved culture to ten parts of 0.1 per cent glucose broth, making the actual initial concentration 0.091 per cent.

Since one allows one doubling, the final cell density is twice the amount of growth that occurs during the experiment. Hence the process is similar to the last doubling before starvation in a culture which had started at very low density and whose initial glucose concentration was twice that shown in table 3.

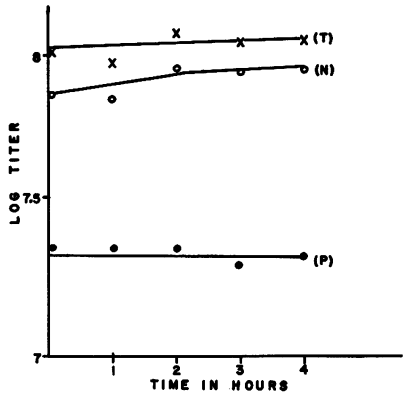

Figure 3. Constancy of composition of population after starvation. Time 0 is 6 hr after the addition of sufficient 0.02 per cent glucose to allow one doubling. The starved population to which this glucose was added had grown 6.3 generations in 1 per cent glucose broth after transfer from galactose broth. (Composition before starvation: 98 per cent positives, 2 per cent negatives.) Symbols as in figure 2.

The new cultures were then allowed to grow 6 hr, which was more than ample to obtain a doubling of the viable cell count. Table 3 shows the results of two such experiments. It is seen that the lower the concentration of glucose, the stronger the effect of starvation on the reversion generation. Because it was desirable to have a fairly large effect to study, the procedure of starvation for one generation from 0.02 per cent glucose was adopted as standard.

Time at which decrease occurs. The experiments described thus far do not distinguish whether the reduction of reversion generation occurs (a) during the period when the yeasts are passing from the fully nourished to the starved condition, or (b) afterwards, when the glucose is completely exhausted. Figure 3 shows that the proportion of positives does not indefinitely continue to diminish in the exhausted culture. This would speak against possibility (b).

Effect of varying the generation at which starvation is performed. If one inoculates a galactose-grown culture into 0.1 per cent glucose broth at such density that it starves after 8 generations, the reversion generation is reduced from 10 to about 8. If one performs the same experiment, except that the starvation occurs before the sixth generation, there is no reduction of reversion generation. Thus the time at which the

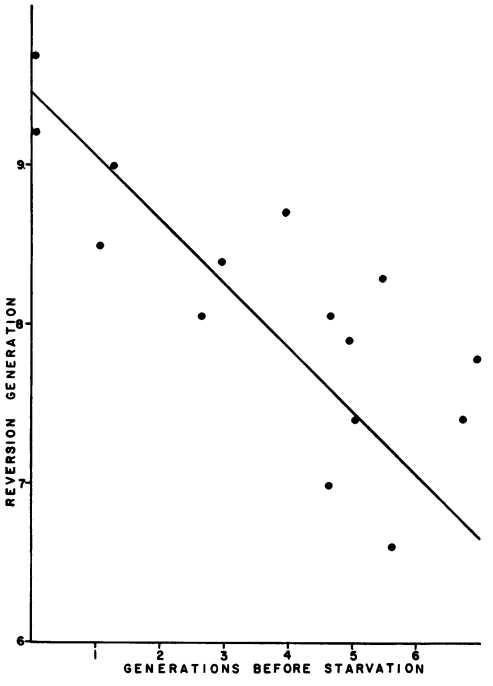

Figure 4. Reversion generation as a function of time of starvation. At all times except time 0, cells were first grown to exhaustion of 1 per cent glucose, then added to 0.02 per cent glucose broth at proper density to allow one doubling, incubated 6 hr, and diluted into 1 per cent glucose broth. (Average reversion generation of controls: 9.7.)

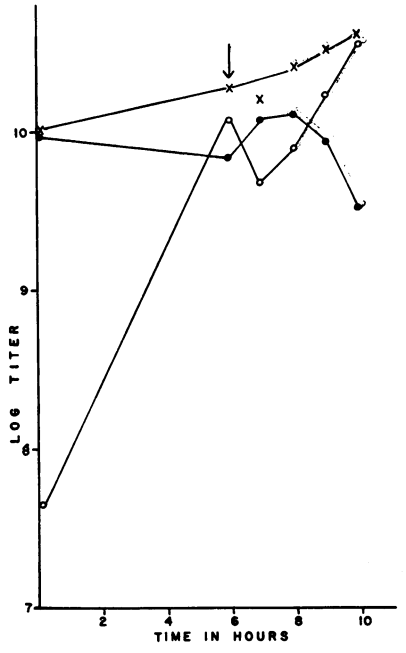

Figure 5. Effect of glucose renewal after starvation. Arrow indicates time at which starved population was diluted into fresh glucose broth. Symbols as in figure 2.

starvation is performed affects the magnitude of the observed effect.

A series of experiments in which reverting cultures were starved for one generation is summarized in figure 4. The straight line is drawn by the least squares method. Even omitting the points at time 0 from the calculation, the downward slope is significant at the 5 per cent level.

Reversibility of change from positive to negative. In some of the experiments of figure 4, negatives were present in significant numbers at the end of the starvation. In those cases, the behavior of the population on subsequent inoculation into 1 per cent glucose was always similar to that shown in figure 5. During the lag phase of the new culture, the proportion of positives first increased and then decreased.

There was no evidence of death or selection. During the 2 hr after replenishment of glucose, the viable count agreed closely with the total count. Therefore, some of the freshly formed negatives must have been changed into positives by this treatment. If a population which had been negative for several generations was treated in the same manner, no positives were produced.

DISCUSSION

There are two alternative explanations for an effect of starvation of the type observed here: (a) There might exist a certain range (c_1, c_2) of low concentrations of the limiting nutrient such that during the time that the actual concentration is between c_1 and c_2, changes occur. (b) The relevant events might not be dependent on the instantaneous external concentration of nutrient, but might result from some internal consequences of the starvation process, such as the utilization of reserves.

The results of table 3 are qualitatively compatible with (a). In the starvations performed the population density is directly proportional to the initial concentration of glucose. Assuming a Michaelis-Menten relationship for glucose utilization, this means that the time it takes for a culture to traverse the region between two given

subsaturation levels c_2 and c_1 is inversely proportional to the initial glucose concentration. Alternative (b) would predict no concentration effect.

The data of figures 4 and 5 are the most interesting with respect to the mechanism of long term adaptation. If the phenomenon involves a dilution of particles, there is no obvious reason why the particles should be more sensitive to glucose starvation at one stage of reversion than at another. Also, one would certainly not expect that a cell which had just lost its last particle should regain it when glucose is replenished.

Unfortunately, neither observation can be accepted as a serious disproof of the autocatalyst dilution model. The first is merely a quantitative observation which we do not understand—either on the dilution model or on any other. The second is complicated by the fact that, if there is destruction of particles, it is possible that such destruction will continue for some time after the cells are placed on galactose agar.

The one hypothesis that is excluded is that the inability to form a positive colony corresponds invariably to the absence of an autocatalytic particle at the time of plating.

The curves of figure 2 might seem, superficially, to support a particle dilution mechanism. The frequency of positives changes markedly only at the time of cell division. However, the curves are quite explainable without recourse to such a model. At the end of one cycle, the number of positives is constant because the glucose has been exhausted, as in figure 3. At the beginning of the next cycle, the number of positives increases as in figure 5; but before any appreciable increase can occur, the next starvation has already begun. This causes the number of positives to drop, as in table 3.

ACKNOWLEDGMENT

The data of tables 1 and 2 were obtained while working with Dr. S. Spiegelman at the University of Illinois, and were included in a thesis submitted by the author to the faculty of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

SUMMARY

Starvation for glucose during reversion decreases the reversion generation of yeast strain 19.1. The starvation is most effective if it occurs from a low glucose concentration, which in our system implied a low population density; and if it occurs late during the course of the reversion. When cultures starved in a certain manner are returned to normal growth medium, the population changes from one producing mostly negative colonies to one producing mostly positive ones.

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