347. *Physicochemical Aspects of Bacterial Growth. Part V. Influence of Magnesium on the Lag Phase in the Growth of* Bact. Lactis Aerogenes *in Synthetic Media containing Phosphate.*

By R. M. Lodge and C. N. Hinshelwood.

In an artificial medium containing glucose ammonium sulphate, and potassium phosphate, small inocula of *Bact. lactis aerogenes* will not grow in the complete absence of magnesium ions. For a given size of inoculum there is a limiting concentration of magnesium (of the order 1—20 parts per million under the conditions of the experiments) below which no growth occurs and above which it occurs normally, the final population and, to a first approximation, the rate of growth being independent

of the actual amount of magnesium in excess of the limit. If the inoculum is introduced into a magnesium-free medium and magnesium is added subsequently, there is a limiting value for the possible delay: after this no growth will ensue. The critical concentration of magnesium decreases as the size of the inoculum increases.

The explanation proves to be as follows. With little or no magnesium present there is a lag phase preceding normal logarithmic growth. During this the organisms are supposed to be synthesising a necessary intermediate growth substance. They are also dying off, at a rate which has been determined by direct viable counts. If they are all dead before the end of the lag period no growth is observable. The lag period is a function of the magnesium concentration. From a knowledge of the death rate, the mean generation time, and the critical magnesium concentration for a series of inoculum sizes, the relation between the magnesium concentration and the shortening of the lag phase which it causes can be deduced.

MANY descriptions of bacterial media include a magnesium salt as a constituent, but it has been found that this may be omitted from the glucose–phosphate medium if this is made up with the laboratory supply of distilled water (Part I, J., 1938, 1930). However, when this medium was made up with doubly distilled water, it was observed that no growth occurred with the usual size of standard inoculum in the absence of magnesium. Since there is an appreciable concentration of ammonium sulphate in the medium, and magnesium chloride can be substituted for magnesium sulphate, the effect must be caused by the magnesium and not by the sulphate ion.

Quantitative investigation of this phenomenon seemed likely to give further insight into the mechanism of bacterial growth. Experiments were therefore made to determine the effect of magnesium concentration on the growth of *Bact. lactis aerogenes* in the glucose–phosphate medium. Table I shows that, with a standard inoculum, the stationary

TABLE I.

Inoculum = 2 loops of an actively dividing culture with a count of about 400. Temp. = 40°.

$MgSO_4$, p.p.m.	Stationary population.	$MgSO_4$, p.p.m.	Stationary population.	$MgSO_4$, p.p.m.	Stationary population.	$MgSO_4$, p.p.m.	Stationary population.
0·0	0, 0, 0	2·5	480	7·5	511	20	510
0·5	0, 0	3	475, 487, 490	8	500	25	490
1·0	0, 0	4	482, 495, 487	10	470, 497	30	500
1·25	0	5	490, 500, 490	12·5	500	40	497
1·5	0, 0	6	482	15	497	50	500, 517
2·0	0, 0	7	510	17·5	500	59	450
2·0	490						

population has the value of zero for concentrations of less than 2 parts per million (p.p.m.) of magnesium sulphate and a value of about 500 for the concentrations studied above this value. Table II gives the variation in the mean generation time with the magnesium

TABLE II.

$MgSO_4$, p.p.m.	3	4	5	6	7	10	20	25	40
Mean generation time at 40°, mins.	41	37·5, 42	38, 37·5, 37	41	43	37·5	58	58	60

concentration. The growth rate does not fall with reduction in the magnesium concentration, but, on the contrary, is slightly depressed by the higher concentrations.

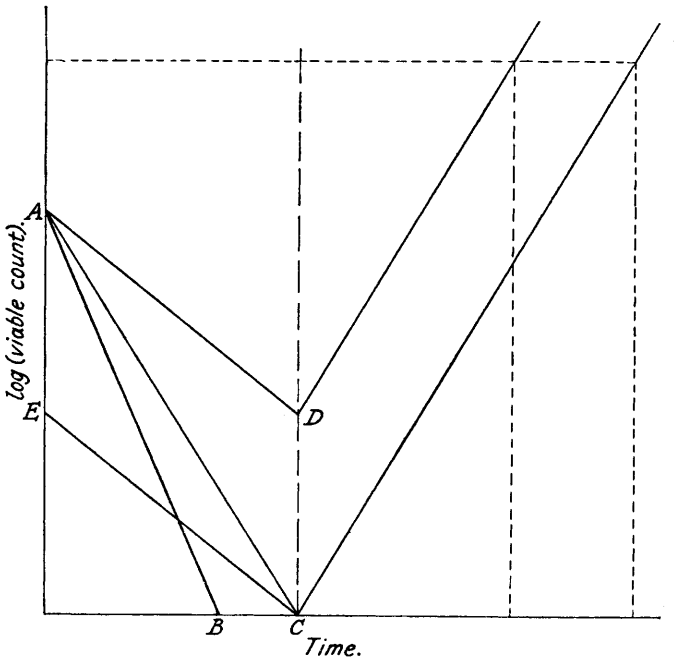
Since the stationary population has no values intermediate between zero and 500, it is clear that magnesium is not present in the rôle of an essential foodstuff, the exhaustion of which determines the onset of the stationary phase. Neither the stationary population nor, significantly, the growth rate is affected by the magnesium concentration, and it therefore appears that the above phenomenon has some connexion with the time-lag which often precedes the normal logarithmic growth phase.

Observations have shown that the organisms of the inoculum are dying off during the lag phase, and that there is a certain maximum time after inoculation of a magnesium-free medium beyond which the addition of magnesium cannot be delayed if growth is to be obtained.

Two theories may be advanced to account for the experimental results : (*a*) the death rate may be decreased or (*b*) the lag phase may be shortened by the addition of magnesium to the medium.

(*a*) Suppose that the death rate before the onset of the logarithmic phase is controlled by the magnesium concentration and that the lag phase is constant. If we take a standard inoculum, (*A*) in Fig. 1, the death rates for different magnesium concentrations may be indicated qualitatively by the lines *AB*, *AC* and *AD*. For *AB*, all the bacteria have died before the end of the lag phase, and after time (*B*) the medium is sterile; but if for higher magnesium concentrations the death rates are represented by the lines *AC* and *AD*, then enough bacteria survive the prescribed time interval (*C*) for growth to ensue quite normally.

FIG. 1.



Variable death rate.

(*b*) If the magnesium concentration controls, not the death rate, but the length of the lag phase, we have the state of affairs shown in Fig. 2. The vertical lines represent the end of the lag phase for the various concentrations of magnesium indicated; *ADC* represents the constant death rate.

The only method for distinguishing between these two hypotheses is an actual determination of the death rate of the inoculum in the presence and in the absence of magnesium. This was done by making viable counts at various intervals after inoculation. The method used was that described by Wilson (*J. Bact.*, 1922, **7**, 405). Table III shows that the

TABLE III.

Time from inoculation, mins.	0	278	458
Viable count (10^4 per c.c.) with 10 p.p.m. of $MgSO_4$ ...	47	11	7
Viable count (10^4 per c.c.) with no $MgSO_4$	50	11	7

All counts performed in duplicate. Temperature = 40·0°.

death rates are identical whether magnesium is present or not. This must mean that the magnesium controls the length of the lag phase.

Since, when the magnesium concentration is small enough, there comes a point where the inoculum all dies before division occurs, it follows that if a given magnesium-free

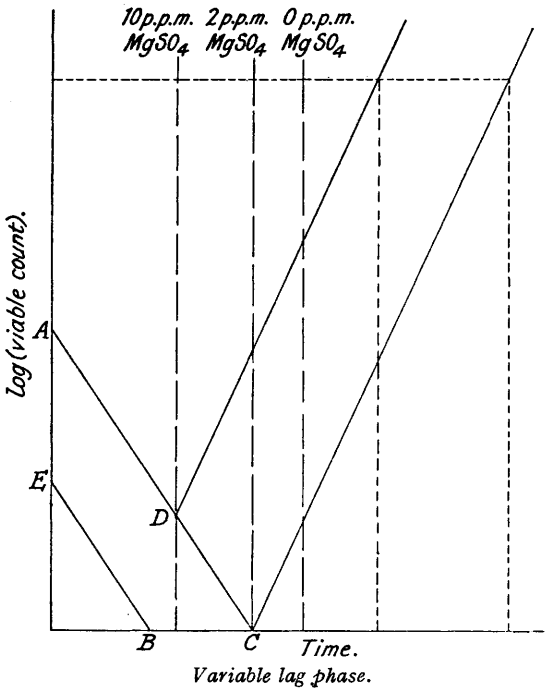
medium is seeded with a constant inoculum, and the magnesium is added afterwards at various intervals, there will be a critical time, after which no growth will occur, but before which growth will be normal though delayed. This was tested experimentally. Table IV

TABLE IV.

T_0, hrs.	T_1, hrs. (experimental value).	T_1, hrs. (calc.).	T_0, hrs.	T_1, hrs. (experimental value).	T_1, hrs. (calc.).
0	12 (= T)	(12)	24·7	36	42
1·5	12	14	36	47	58
12	21·7	27	48	63·5	73
13·5	21·7	29	69·5	92	∞
18·3	27·5—31	35	80	∞ (*i.e.*, no growth)	∞

The initial inoculum corresponded to a viable count of $2·0 \times 10^7$ bacteria per tube.

FIG. 2.



shows that, under the conditions prevailing, if the addition of magnesium is delayed for 70—80 hours after inoculation, no growth occurs, and, even with shorter delays, the time required for the attainment of a visible stage of growth begins to increase considerably. These results can be directly correlated with the death rate already measured.

The death of the organisms follows the law $n = n_0e^{-lt}$, where n_0 is the initial number of organisms, n the number alive at time t, and l a constant. From the results given in Table III for the viable counts, the mean value of l is calculated to be 0·0048 min.$^{-1}$. Suppose a medium is inoculated with magnesium present and reaches a given stage of growth in time T. If the same medium is inoculated and left without magnesium for time T_0, the number of organisms will fall in the ratio $n/n_0 = e^{-lT_0}$. When the magnesium is now added, the time, T_1, to the same stage of growth will be increased beyond T by the time for the ratio n/n_0 to be restored to its original value of unity. This extra time can be calculated from the growth equation $n_0/n = e^{kT_1}$, where $k = \ln 2/(\text{mean generation time})$, and it increases with T_0.

The logarithmic law, however, does not hold indefinitely. When the calculated number of organisms left alive is less than one, it means that complete sterility has really

been reached: and even before this the statistical law may have broken down. There may also be a slight variability in the resistance of the organisms, so that the time to complete sterility is not in principle quite accurately definable. The differences in times required to reduce the original numbers to, say, 10, 1, and 0·1 respectively, however, do not, in fact, vary much in comparison with the times themselves, so that we will regard the time to sterility as given approximately by that to reduce the number of organisms left alive to one per culture tube, *i.e.*, 0·05 bacterium per c.c. On this basis, the mean generation time being taken as 40 minutes, it is calculated that there should be no growth after about 60 hours' deprivation of magnesium. Before this, the time between the addition of the magnesium and growth should begin to increase at the rate of about 3 hours for every 10 hours delay in the addition of the magnesium. The initial inoculum corresponded to 2×10^7 organisms per tube of 20 c.c.

Having regard to the variability of the mean generation time and the comparative inaccuracy of viable counts, the agreement (Table IV) is quite close enough to show that the general principle assumed is correct.

An adaptation of this principle allows us to obtain information about the variation of the length of the lag phase with the magnesium concentration. We make a series of determinations of the limiting concentrations of magnesium c_1, c_2 . . ., which just allow growth of inocula n_1, n_2. . . . If n_1 is greater than n_2, c_1 will be less than c_2. Suppose the lag phase in the absence of magnesium is L_0, for a concentration c_1 it is $(L_0 - t_1)$ and for c_2 is $(L_0 - t_2)$, then, as is evident from Fig. 2, $(t_2 - t_1)$ is the time required for the viable count to drop from n_1 to n_2; *i.e.*, $\ln n_2/n_1 = e^{-k(t_2 - t_1)}$. Since l, n_1, and n_2 are known, $(t_2 - t_1)$ can be calculated. This gives the difference in lag phase for the two concentrations c_1 and c_2. Table V gives the results from which the limits for inocula of various sizes are

TABLE V.

c = Concn. of $MgSO_4$ in p.p.m.; n_s = stationary count (hæmocytometer reading). Inocula given in actual numbers of viable bacteria added to 20 c.c. of medium. Numbers in parentheses are values relative to the first as standard.

Inoculum = $1{\cdot}2 \times 10^7$ (1·0).

c =	1	2	3	7	9
n_s =	0	0	490	490	495

Inoculum = $0{\cdot}4 \times 10^7$ (1/3).

c =	6	8	10
n_s =	0	496	500

Inoculum = $0{\cdot}3 \times 10^7$ (1/4).

c =	2	4	8	9	11	12	17
n_s =	0	0	0	0	500	497	495

Inoculum = $1{\cdot}2 \times 10^6$ (1/10).

c =	14	16	18
n_s =	0	0	506

Inoculum = $7{\cdot}5 \times 10^5$ (1/16).

c =	4	8	12	17	20	21	23	25	30
n_s =	0	0	0	0	0	0	500	490	500

calculated, and Table VI the estimated changes in the lag, and Fig. 3 shows them plotted against c.

TABLE VI.

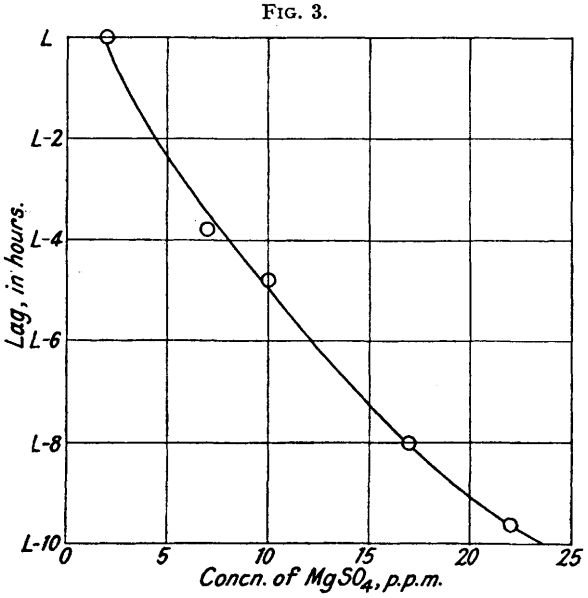
Inoculum size (relative)	1	1/3	1/4	1/10	1/16
$MgSO_4$ at limit, p.p.m.	2	7	10	17	22
Shortening of lag (in hrs.) by increasing [$MgSO_4$] above 2 p.p.m.	0	3·8	4·8	8·0	9·6

Pett and Wynne (*Biochem. J.*, 1933, **27**, 1660) have stated that magnesium stands apart from all other ions as a phosphatase activator. It seems that magnesium must play a fundamental part in the normal functional activities of these enzymes (Bamann and Salzer, *Ber.*, 1937, **70**, 1263). Clark (*Trans. Roy. Soc. Canada*, 1938, **32**, III, 1) points out that all substances which act as enzyme activators only do so in low concentrations, and that when their concentrations are increased beyond a certain limit, these same substances have an inhibitory effect. In view of this, it is simple to see how the magnesium concentration can control the length of the lag phase. Suppose that the phosphatase controls the formation of a substance intermediate in complexity between the foodstuff provided and the bacterial protoplasm, and that this intermediate has to be present in a certain minimum concentration before division can occur. Its rate of synthesis,

and thus the time needed to reach the necessary concentration in the cell, will vary with the magnesium concentration. Fig. 3 seems quite naturally to express this variation.

To obtain preliminary evidence bearing on an idea—based at the moment only on speculation—that there might be an analogy between the termination of the lag phase of non-sporing bacteria and the germination of spores, experiments were made to determine whether the germination of spores of *B. mesentericus* was appreciably influenced by the magnesium content of the medium. *B. mesentericus* was grown in bouillon to which some

FIG. 3.



Influence of maguesium concentration on lag phase.

glucose had been added. After about 3 days, nearly all the organisms had turned into the spore form. The spores were centrifuged off, washed with normal saline, and then suspended in the glucose–phosphate medium described above. The rate of germination was observed by counting in the hæmocytometer. If the organisms are not stained there is no great difficulty in distinguishing the spores from the bacteria. Presence or absence of magnesium in the medium was found to make little difference to the rate of germination. In this respect therefore there appears to be no evidence of an analogy.

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