364. *Physicochemical Aspects of Bacterial Growth. Part II. Quantitative Dependence of the Growth Rate of* Bact. Lactis Aerogenes *on the Carbon Dioxide Content of the Gas Atmosphere.*

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In a synthetic medium, *Bact. lactis aerogenes* will not grow in the complete absence of carbon dioxide. A quantitative investigation of the relation between growth rate and the concentration of carbon dioxide shows that the rate increases rapidly to a limiting value, reached when the concentration of carbon dioxide in the gas phase is about 0·15%.

GLADSTONE, FILDES, and RICHARDSON (*Brit. J. Exp. Path.*, 1935, **16**, 335) investigated the effect on nine different organisms of bubbling streams of carbon dioxide-free air through

freshly inoculated media, and found that in most cases growth was inhibited, whilst in those where it was not, they suggested that the gas stream had failed to remove completely the carbon dioxide produced in cell respiration.

Since the effect was shown not to depend upon changes of p_H, we have here a good opportunity for making a quantitative study of a fundamentally important growth reaction. Having found that *Bacterium lactis aerogenes* also fails to grow in the phosphate–glucose medium in the complete absence of carbon dioxide, we decided to determine the relation between the actual growth rate and the percentage of carbon dioxide in an air stream bubbled through the medium.

The principle of the experiments was as follows. Preliminary observations showed that the optimum concentration of carbon dioxide was quite small; the required gas atmospheres were therefore maintained by drawing through the culture tube mixtures of pure air and air containing a small fixed percentage of carbon dioxide, the proportions being varied and measured by calibrated capillary flow-meters of the usual type. Since the flow-meters employed had a large range of sensitivity, accurate measurement of the small carbon dioxide percentages was possible.

The growth curves are shown in Fig. 2, and the relations between growth rate and carbon dioxide concentration are shown in Figs. 3 and 4 and Table I.

FIG. 1.

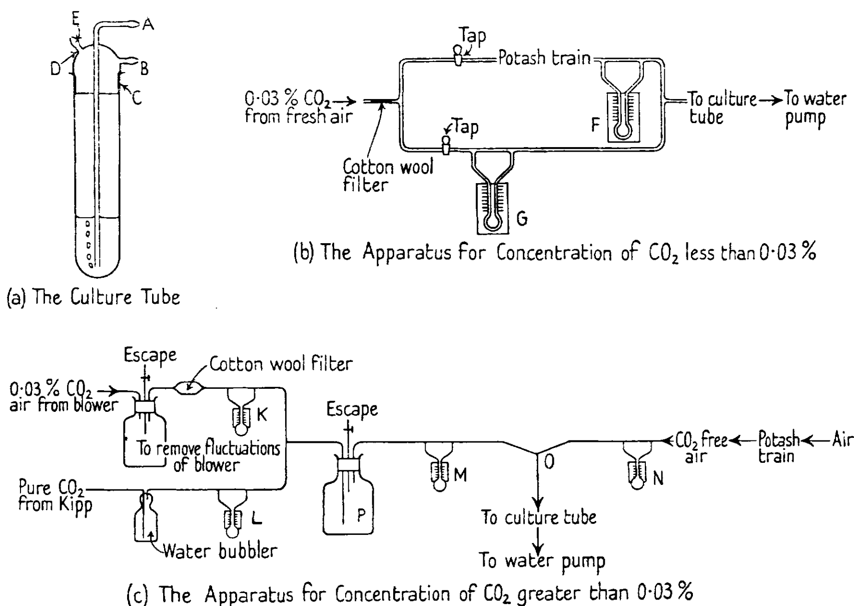


TABLE I.

The Dependence of Growth Rate on Carbon Dioxide Concentration.

(T is the time, in mins., for the count to reach 100; and T_g is the mean generation time, in mins.)

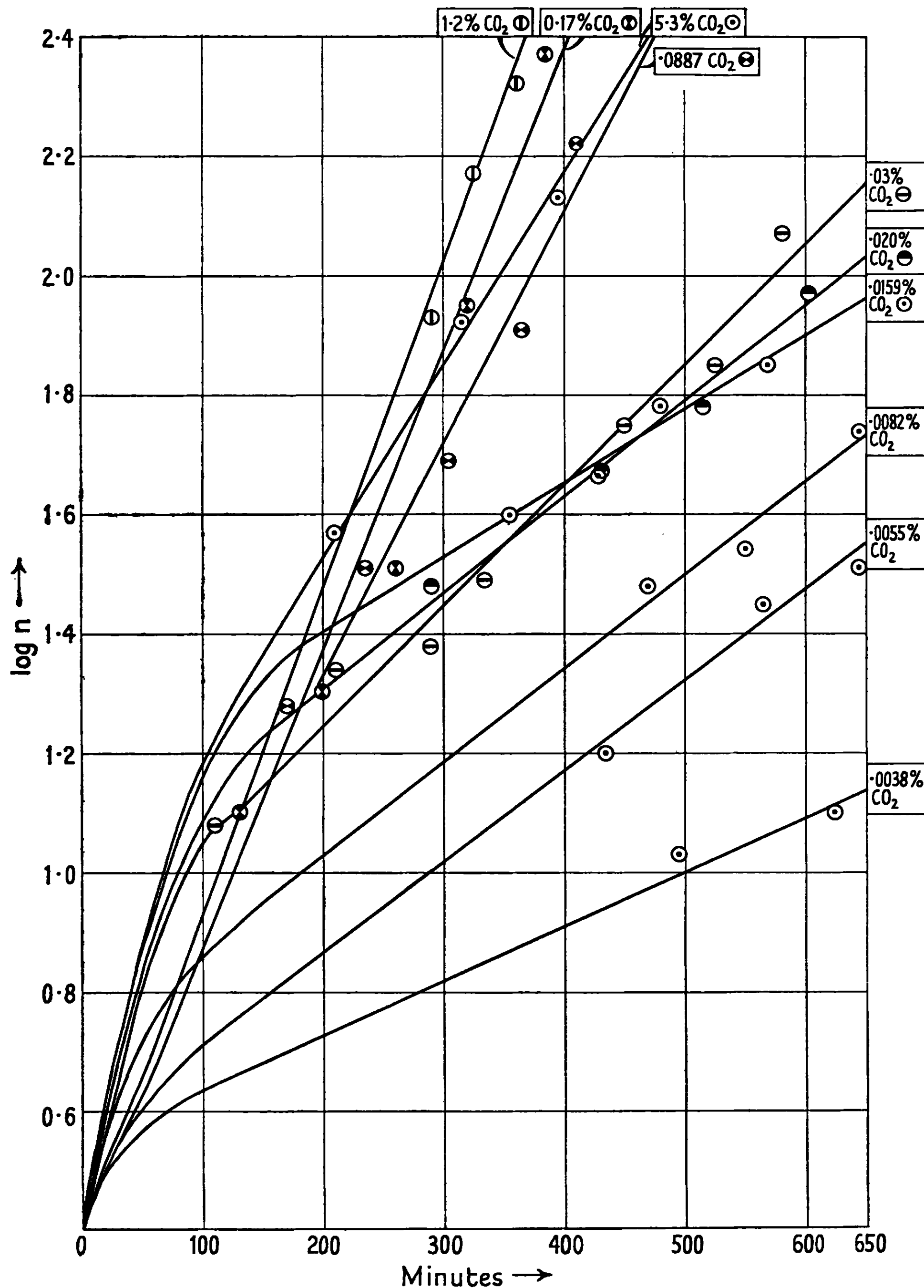
CO_2, %, in gas phase	5·3	1·2	0·17	0·088	0·030	0·020	0·0159	0·0082	0·0055	0·0038
T	347	295	330	372	575	630	680	825	950	1350
$500/T$	1·44	1·69	1·51	1·34	0·87	0·79	0·73	0·60	0·53	0·37
T_g	92·5	55	55	72·5	145	175	240	192	195	330

Before these are discussed the experimental method will be described in greater detail, and, in particular, the standardisation of the inoculum will be explained. This is essential if reproducible results are to be obtained.

The apparatus is shown in Fig. 1. The culture tube consisted of two parts fitting together at the ground joint C, the upper part carrying the tube A, by which the gas stream entered, and

B, by which it was sucked out. Samples were taken through *D* at intervals during growth by means of long Pasteur pipettes, *D* normally being closed by a ground glass cap. In preparing

FIG. 2.



this culture tube, rigidly aseptic technique was used, the large luminous flame of a hand blow-pipe proving a useful auxiliary in this part of the work. For most experiments the percentage of carbon dioxide required was less than the 0·03% present in ordinary air. A supply of gas

FIG. 3.

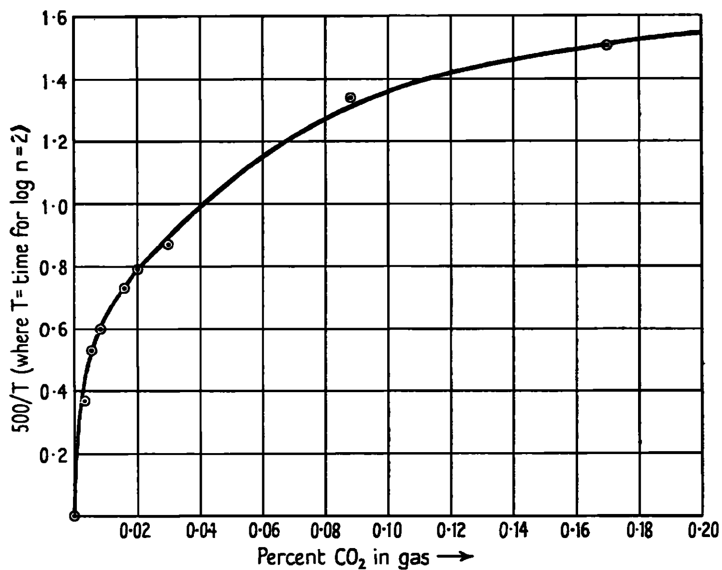
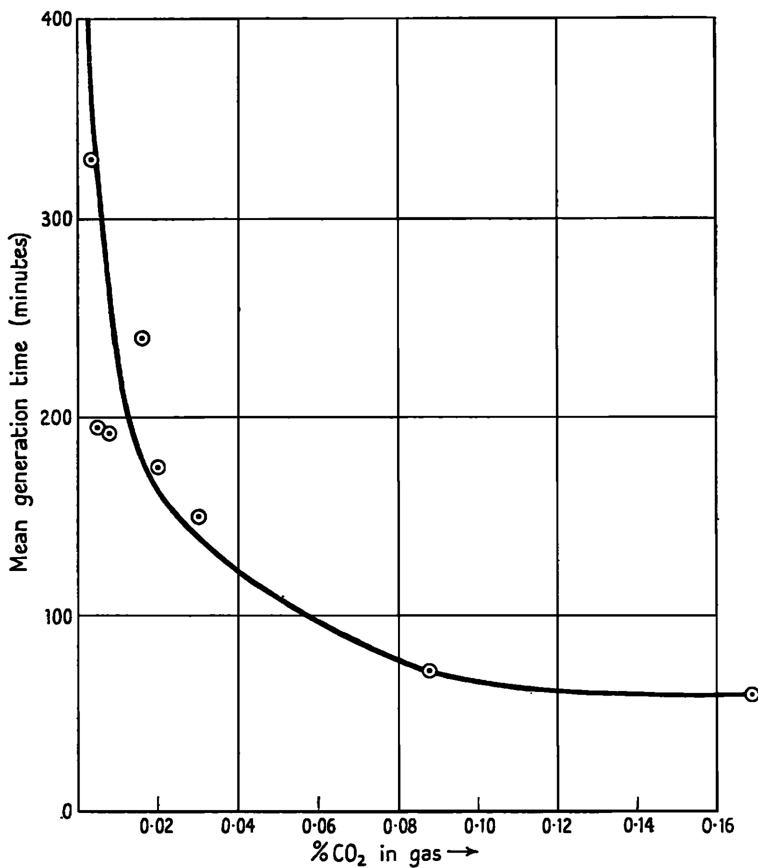


FIG. 4.



of this content entered the apparatus by a long tube leading to the fresh air outside the laboratory, being filtered through cotton-wool and divided into two parts. One part, measured by the flow-meter *F*, was freed from carbon dioxide by an efficient potash train, and the other, still containing its 0·03% of carbon dioxide, was measured at *G* so that it could be mixed with the pure air in any desired proportion. The rates of flow, and thus the proportions, could be adjusted by means of the taps shown in the diagram. When concentrations of carbon dioxide greater than 0·03% were required, the arrangement shown in Fig. 1*c* was used. Ordinary air and pure carbon dioxide were mixed to give a gas containing about 10% of the latter as calculated from readings of the flow-meters *K* and *L*. Most of this gas was allowed to escape from the bottle *P*, but a small fraction of it passed through the fine flow-meter *M* and was mixed in the required proportion at *O* with the carbon dioxide-free air measured by *N*. In all the experiments the total rate of flow through the culture tube was maintained at 20 divisions of the flow-meter *N*, or the equivalent of this reading on one of the others. This corresponded to a fairly brisk bubbling of the gas through the solution. A large bottle interposed between the filter-pump operating the stream and the rest of the apparatus served to keep the readings of the flow-meters steady. To prevent evaporation from the culture tube, the air stream was first drawn through a tube of sterile water at the same temperature.

A standard technique of inoculation was found to be essential. The stock culture was grown in bouillon: this was not used directly for the experiments, but two loops of it were used for inoculating a medium consisting of 10 c.c. each of glucose and phosphate solutions with 0·3 c.c. of magnesium sulphate (see Part I), which was that used in the growth experiments themselves. This subculture was allowed just to grow to a stationary concentration, n, of about 350, which was counted. A small sterile pipette of known volume (about 0·1 c.c.) was then used for inoculating the new medium. It was important not to remove the first culture from the thermostat during this time, since chilling was found to have an adverse effect on subsequent growth. The initial count of the new medium was known approximately from the total count of the old and the dilution ratio, and was nearly equal to the viable count, as shown by the fact that in appropriately chosen circumstances the new growth curve of $\log n$ against t could be extrapolated back to cut the $\log n$ axis at a point close to that indicated by the estimated initial value of n.

The inocula used were rather large. This had the disadvantage that the initial stages of the growth curves were still influenced to some extent by the previous history of the culture: but the practical convenience was considerable, since measurable growth appeared in a shorter time, and the results were more reproducible and more certain. When the growth period is unduly protracted, quantitative results become more difficult to obtain.

The preparation of the inoculum has a great effect on growth rate, and the method adopted was based upon the following preliminary observations.

The growth rate of the organism used could be doubled by repeatedly sub-culturing in the artificial medium, an example of the phenomenon of "training" (cf. Graham-Smith, *J. Hyg. Camb.*, 1921, **19**, 133), which is illustrated in Table II. Therefore, the inoculum used in the

TABLE II.

The Effect of "Training" on Growth Rate.

CO_2, %,	T.		$500/T$.		T_g.	
in gas.	Trained.	Untrained.	Trained.	Untrained.	Trained.	Untrained.
0·17	257	330	1·94	1·51	28	55
0·088	330	372	1·51	1·34	60	72·5
0·0082	495	825	1·01	0·60	90	192
0·0038	845	1350	0·59	0·37	190	330

final experiments was always freshly standardised as decribed above. This method had the advantage that the organisms, having only just reached their stationary concentration, showed no lag period. In the artificial medium, quite long and erratic lag periods occurred with older organisms or cultures of which the temperatures had not been controlled.

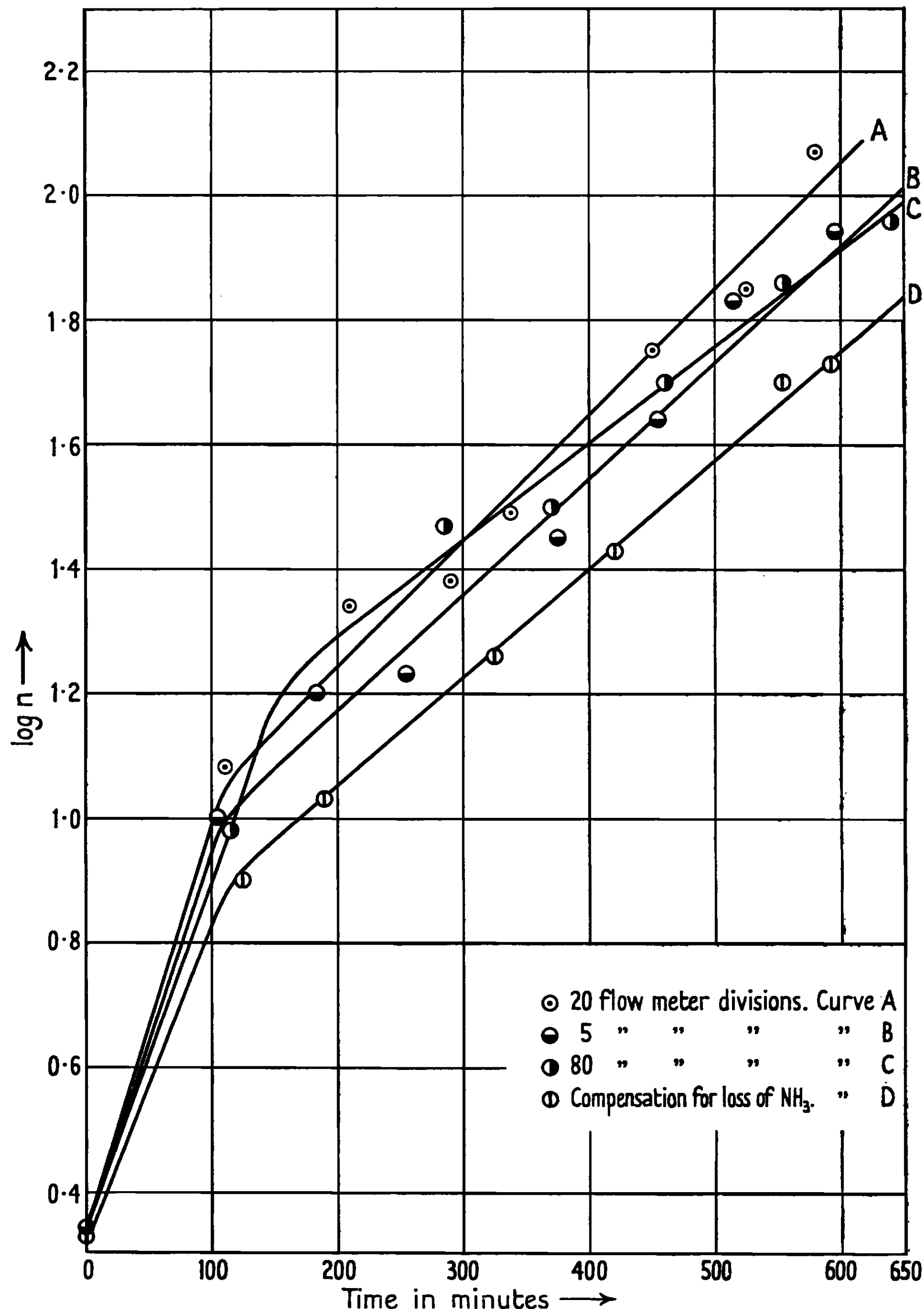
A stream of pure carbon dioxide passing through the culture medium, so far from having a stimulating effect on growth, retards it, the average value of the mean generation time for 9 experiments in which carbon dioxide was passed being 85 minutes, with a mean deviation of 6 minutes, whilst without carbon dioxide the average value for 6 experiments was 56 minutes with a mean deviation of 4 minutes.

This adverse effect, however, does not appear unless the concentration of carbon dioxide is

quite high. In the region of low concentrations the growth rate increases rapidly with the amount of carbon dioxide to a practically constant limiting value.

With regard to the main series of experiments summarised in Figs. 3 and 4, the question

FIG. 5.



arises how far the results are a function of the actual rate of bubbling of the gas stream through the medium. Fig. 5 shows the growth curves corresponding to flow-meter readings of 5, 20, and 80 respectively, whence it appears that a 16-fold variation in the rate of the gas stream has

little if any influence on the growth. This proves that equilibrium is established between the carbon dioxide in the gas and that dissolved in the solution.

Under normal conditions, *Bact. lactis aerogenes* in the artificial medium gives a strictly linear growth curve (cf. Part I, Fig. 2; Part II, Fig. 3) during the period of active multiplication. In the experiments with amounts of carbon dioxide insufficient for maximum growth, the rate is greater in the initial stages than that to which it settles down later (Fig. 2). This suggests that, although the carbon dioxide in the gas is in equilibrium with that in solution, the latter is not in equilibrium with that retained inside the cells of the inoculum when they are transferred from the old medium to the new. The cells, therefore, grow at a rate corresponding to a greater carbon dioxide concentration until the excess has either escaped by diffusion or been diluted by repeated division of the original cells. It must be admitted that the abnormal initial part of the curve would be much less in evidence if a smaller inoculum were used : but the practical difficulties of this procedure have already been indicated.

The nature of the curves made it advisable to record the growth rates in two ways : the first by the mean generation times, read off from the curves after the initial disturbance is over, and the second by the times required to reach a count of 100, which corresponds to a point well beyond the bend. Although the former is more theoretically significant, the latter is more reliable practically. The two, however, lead to very similar curves.

The growth medium used has a minute partial pressure of ammonia, which may therefore be swept away by the gas stream. When, however, this loss was compensated for by bringing the gas into equilibrium with a similar solution at the same temperature before it entered the culture tube, there was no effect on growth, as shown in Fig. 5.

In Table II are recorded some results which show how the whole curve of growth rate against carbon dioxide concentration is more or less uniformly raised when the standard inoculum is replaced by one sub-cultured three times instead of once in the artificial medium.

Fig. 3 shows the reciprocal of the time to reach a count of 100 (the times $\times$ 500 to give convenient units) plotted against carbon dioxide concentration in the gas phase; and Fig. 4 shows a similar curve for the mean generation time. It appears that above a concentration of about 0·15% there is little increase in growth rate. Presumably, those active centres providing the seat for the biochemical processes in which carbon dioxide performs its essential function have become saturated with that gas. At a concentration of 5% the carbon dioxide is exerting the adverse effect already referred to.

When the amount of carbon dioxide in the gas phase is 0·15% the amount dissolved in pure water at 40° is 4×10^{-8} g.-mol. per c.c. The growth medium is slightly on the alkaline side, so that the total solubility may be increased, but any excess of gas dissolved will not be present as free carbon dioxide but as carbonate or bicarbonate ion. These, as Gladstone, Fildes, and Richardson showed, will not replace carbon dioxide itself. By taking the solubility in water, therefore, we shall probably have a roughly correct idea of the amount of the active agent in solution. The volume of an organism is of the order 10^{-12} c.c. If the carbon dioxide pervaded the system without being selectively taken up, the number of molecules within the confines of the cell would be $2{\cdot}4 \times 10^{4}$ when saturation is reached. This concentration is about 10,000 times smaller than that of the glucose in the normal growth medium.

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