LXI. STUDIES IN GAS PRODUCTION BY BACTERIA.

II. DENITRIFICATION AND BACTERIAL GROWTH PHASES.

By BLODWEN LLOYD and JOHN ARNOLD CRANSTON.

From the Departments of Bacteriology and Physical Chemistry, The Royal Technical College, Glasgow.

(*Received February 14th, 1930.*)

The bacterial reduction of nitrates and nitrites to free nitrogen has long been known; it has been the subject of interest in such diverse branches of science as marine biology, sewage research, and agriculture. Despite its importance, comparatively little accurate investigation appears to have been carried out: especially is there a lack of quantitative data as to the rate at which denitrification takes place.

Baur [1902] found that 5 % nitrate or nitrite in a broth culture of *B. actinopelte* was completely decomposed in 10–14 days at 15° to 20°; Drew [1911] stated that at 29° *B. calcis* destroyed 0·5 g. of KNO_3 in 86 hours. Apparently the only record of stages in the denitrification is that of Lumia [1915], who found that under favourable conditions there was after 1½ days sudden gas production at a rapidly increasing rate, and an equally rapid slowing down towards the end. He also found that with varying conditions it was the speed of the reaction that was affected and not the total denitrification expressed either in terms of the gas produced or of the amount of nitrate destroyed. Korsakova [1927], using *B. denitrificans fluorescens* in a nitrate medium, estimated periodically the quantity of nitrogen produced and the amounts of nitrate and nitrite present. The intervals between estimations were, however, too long to show the progress of the denitrification in detail.

It is apparent from the above record that little work of value has been done on the rate of denitrification. The numerous factors that affect the rate do not seem to have been appreciated; otherwise the isolated items of information given would have been recognised as haphazard. With the exception of Carapelle [1908], who stated that the activity of bacteria possessing reducing powers is more marked in young than in old cultures, no one seems to have correlated the rate of reduction with the age of the culture. Still less has it been realised that in denitrification the rate of gas production may

under certain circumstances be taken as a measure of the number of viable organisms present; or that there is a delay in gas production which can be correlated with the lag phase of bacterial growth, and which furnishes a simple method of studying quantitatively the factors affecting the duration of the lag phase. The rate of denitrification is greater in a young culture than in an old one for two reasons: (i) the number of bacterial cells is greater in a young culture than in a senescent one; (ii) the majority of cells in a young culture are in a state of active cell-division, so that each attacks the nitrate more rapidly than a cell from an older culture.

The utility of denitrification to the organism is primarily a respiratory one, and therefore the rate of denitrification, the rate of respiration, and the rate of bacterial growth are interdependent; and a curve showing the production of nitrogen with time for a given organism under given conditions should be capable of correlation with the growth curve of the culture.

In the preceding paper [Cranston, 1930], methods are described for measuring the rate of gas production by bacteria when cultivated in a liquid medium. For the following work, the device found most convenient and simple to use was that of attaching a 10 cc. culture tube with a narrow neck to a capillary tube containing a thread of mercury; it was employed in the work described in this paper, which is the outcome of studying the rate of denitrification of over 90 cultures.

General form of the denitrification curve.

When the volume of the gas absorbed or produced during denitrification is plotted against the time from seeding, a curve of the general type shown in Fig. 1 is obtained; such a curve will be referred to throughout this paper as a "denitrification curve." The rate of change in volume varies in magnitude and sign so that as many as nine stages can be differentiated. From this curve another may be obtained by plotting the increments of volume in selected intervals of time against the mean time from inoculation. Except for the initial contraction *OC* and the final contraction *JK*, the curve will represent the rate of gas evolution at various stages in the growth of the culture; such a curve will be referred to throughout this paper as a "rate of denitrification curve."

The initial contraction indicated by the portion *OC* of the curve is due to the absorption of atmospheric oxygen present in the air space between the surface of the medium and the mercury thread in the capillary tube; it is effected partly by the oxidation of the medium and partly by the respiration of the bacteria. The control experiments discussed later show that the contraction in this phase along *OA* is due almost entirely to absorption by the medium, while the portion *AC* is due partly to aerobic bacterial respiration. The final contraction *JK*, which when it occurs is usually of very small magnitude, can be attributed to the same causes.

The rate of denitrification curve (Fig. 2, p. 534) shows general similarity to

a bacterial growth curve, and this resemblance suggests that the two may be correlated. A growth curve can be dissected to represent various phases of bacterial growth: it will be appropriate to discuss these before dealing with the interpretation of the rate of denitrification curve.

Growth phases in bacterial cultures.

The rate of growth of bacteria in a sub-culture varies greatly with time [Buchanan and Fulmer, 1928]. It has been established that in general there is no appreciable initial increase in the numbers of bacteria [Lane-Claypon, 1909; Penfold, 1914; Ledingham and Penfold, 1914]. After germination has begun, cell-division proceeds slowly at first and then more and more rapidly until such time as the bacteria are dividing at a uniform rate, *i.e.* until they are increasing in number geometrically over equal intervals of time. The rate

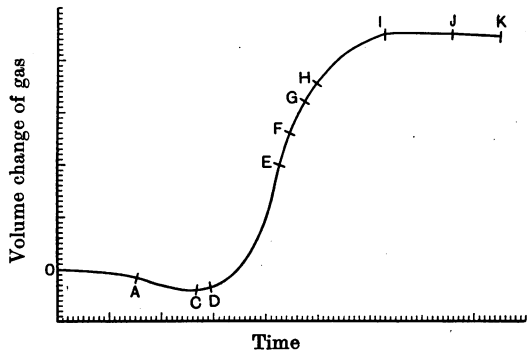

Fig. 1. General form of a denitrification curve, marked off into sections which are correlated with bacterial growth phases.

of increase then diminishes progressively until the numbers of bacteria remain constant, after which there is a diminution in numbers until the culture dies off. Buchanan differentiates seven such phases as follows.

1. *Initial stationary phase.* During this phase the number of bacteria remains constant. The duration of the phase varies with the experimental conditions, and with the organism used.

2. *Phase of positive growth acceleration.* Here the cells are beginning to divide, and the average rate of increase in numbers increases with time. This phase extends over a period of time because all the cells do not germinate at once. When all the viable cells of the original inoculum have germinated they continue to divide regularly, and the rate of increase is then constant; this gives rise to the third phase.

The term "lag phase" has been defined as covering the period which elapses between "the time of seeding and the time at which maximum rate of growth begins" [Chesney, 1916]. Buchanan, however, in a desire to differentiate between these two phases has suggested that the term should be reserved specifically to indicate the second phase, viz. "that period elapsing between the beginning of multiplication and the beginning of the maximum rate of increase per

organism." It appears to us unfortunate that he did not suggest the use of the term for the first phase instead of the second. The word "lag" is more suggestive of the first phase, and indeed in discussions of theories of the lag phase, it has been so frequently used by various workers, including Buchanan himself, to mean the stationary phase that this meaning has been sanctioned by custom.

3. *Logarithmic growth phase.* During this phase the rate of increase per organism remains constant.

4. *Phase of negative growth acceleration.* Here the rate of increase decreases, although the number of bacteria increases.

5. *Maximum stationary phase.* In this phase the number of bacteria remains constant.

6. *Phase of accelerated death.* During this phase the number of bacteria is decreasing, slowly at first and then with increasing rapidity.

7. *Logarithmic death phase.* Here the rate of decrease per organism remains constant. Buchanan and Fulmer [1928] state that such decrease is seldom logarithmic.

Comparison of growth phases with phases of gas evolution.

It is generally agreed that the significance of bacterial denitrification is a respiratory one, *i.e.* the oxygen available from the nitrate may be supplementary to, or may be a substitute for, free atmospheric oxygen. Most writers find that the presence of free oxygen retards denitrification [Kunnemann, 1898; Caron, 1912] and in some cases may even inhibit it completely [Gran, 1901; Ampola, 1907]. It does not seem to be clearly recognised, except perhaps by Korsakova [1927], that the reduction of nitrate to nitrogen occurs by stages with the formation of intermediate compounds, and that gas is produced only in the last stage. Denitrification is therefore an indication of the completeness with which oxygen is utilised, and this in turn depends on the amount of nitrate present. Statements regarding inhibiting factors should therefore only be made when the concentration of the nitrate is taken into account. This point is discussed in detail later in the paper when experimental data enable it to be done quantitatively.

The denitrifying consequence of bacterial respiration should lead to the establishment of some connection between the rate of gas evolution and the number of viable organisms present: but if that relationship is to be made quantitative, the following conditions must be satisfied.

1. The organism must depend solely upon the nitrate or nitrite for its oxygen supply.

2. The gas evolved is pure nitrogen.

3. All the bacteria consume oxygen at the same rate, this rate being independent of the age of the individual organism.

4. No time lag must occur between the absorption of oxygen and the production of nitrogen.

Condition 1 can be controlled experimentally, and Condition 2 can be arranged by selecting an organism known to produce nitrogen only. No

evidence is available regarding Condition 3. Even if the rate of consumption of oxygen is not proportional to the number of bacteria present, it is at least a measure of their aggregate metabolic activity, which is perhaps as significant. Condition 4 does not hold throughout the whole course of denitrification. Korsakova [1927] found by analyses performed during the course of denitrification that the nitrogen could not be completely accounted for as nitrate, nitrite, and free nitrogen, and therefore concluded that some of it existed as an intermediate compound. Ampola and Ulpiani [1899] suggested that hyponitrite is formed as an intermediate product in denitrification, but were unable to demonstrate its existence during gas production. The authors show later on in this paper that their results can be quantitatively accounted for on the assumption that this intermediate compound is the hyponitrite. Thus the reduction of potassium nitrite on this supposition would occur in two stages.

$$2KNO_2 - 2O = K_2N_2O_2$$
$$K_2N_2O_2 - O = K_2O + N_2.$$

A large proportion of the oxygen may thus be utilised before nitrogen is evolved at all. Under the conditions of the authors' experiments, these reactions do not occur simultaneously, but the nitrogen is given off only after the nitrite has been completely converted into an intermediate compound.

The number of bacteria present in a nitrite-broth culture is therefore proportional to the rate of gas evolution only through part of the life of the culture, viz. soon after gas evolution has begun. It is characteristic of denitrification curves that soon after gas is evolved, it is produced at its maximum rate. This seems to show that gas is not evolved till the later stages of the phase of logarithmic growth. The authors propose later to find if potassium hyponitrite can be used instead of potassium nitrite as a source of oxygen for the bacteria; the amount of nitrogen evolved would then be proportional throughout to the amount of oxygen consumed, and thus the earlier growth phases could be studied directly.

The growth phases detailed above may best be compared with the phases of gas evolution by considering the rate of denitrification curve given in Fig. 2.

The initial stationary phase is represented by *AC*. Experiments to be described later show some of the factors which determine its duration.

The phase of positive growth acceleration is represented by *CD*. As explained above, the production of nitrogen from nitrite is delayed through the formation of an intermediate compound; this occurs during the present phase so that the initial growth of bacteria is not accompanied by gas evolution. Ordinarily, therefore, the growth during this phase cannot be studied by gas production.

The logarithmic growth phase is represented by *DE*. In most of the experiments gas is first evolved during this phase, but whether it occurs at the early or the late part of the phase cannot be determined from the data of gas evolution alone.

The phase of negative growth acceleration, the maximum stationary phase, and *the phase of accelerated death,* represented by *EF*, *FG* and *GH* respectively, are usually of short duration. An investigation of these phases would require readings taken at intervals not greater than half an hour, and to attain any accuracy the volume changes would have to be magnified by using not less than 100 cc. of broth culture.

The logarithmic death phase is represented by *HI*. In most cases the numbers of bacteria were found to decrease logarithmically over measurable periods of time. In one experiment this period extended over 5 days [Cranston and Lloyd, 1930].

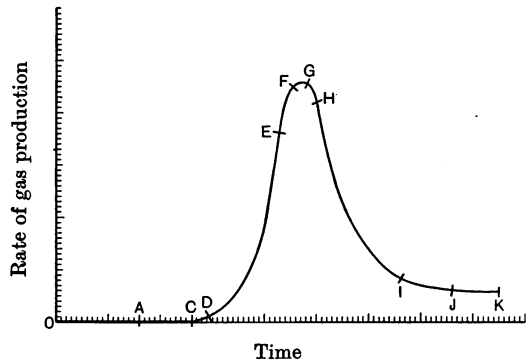

Fig. 2. General form of a rate of denitrification curve, marked off into sections which are correlated with bacterial growth phases.

The phase of slow decline, represented by *IJ*, is shown by nearly all cultures. Some experiments that throw light on these phases are described below.

Experimental.

For the following work, an actively denitrifying marine organism was selected. The strain employed was isolated from Loch Striven in the Clyde Sea Area at a depth of 20 fathoms. The organism will be described in detail in a forthcoming paper; a culture has been lodged with the National Collection of Type Cultures at the Lister Institute. The following are its principal characteristics.

Designation. Laboratory organism XIV.

Morphology. Vibrio, single, sometimes forming short chains, gram-negative, actively motile, length about 1·5μ.

Agar cultures. Slope cultures, thin coherent wrinkled films; surface colonies on agar plates up to 5 mm. in diameter, with wrinkles often radially arranged.

Gelatin cultures. No liquefaction.

Broth cultures. Uniform turbidity, with coherent wrinkled surface pellicle, the nature of the pellicle varying with the temperature of incubation; surface films absent from a young nitrate or nitrite broth.

Optimum temperature. 35°.

Size of the inoculum.

The general procedure adopted in making an inoculation was designed to make the seeding as constant in amount as possible. In order to obtain some estimate of the number of bacteria used in an inoculation, the direct method of counting by means of a Thoma haemacytometer was employed. One loopful of a broth culture actively producing gas was transferred to approximately 10 cc. of the sub-culture. In the parent culture the average number of bacteria counted in unit volume, $1/20 \times 1/20 \times 1/10$ mm.^{3}, was 40, which is equivalent to 160×10^6 per cc. In the sub-culture, only one bacterium was seen in 40 unit volumes examined, equivalent to 10^5 per cc. The dilution performed by transferring one loopful to 10 cc. was thus approximately 1 in 1600. This figure was checked by ascertaining the average weight of a loopful of broth to be 5 mg., which in 10 cc. gives a dilution of 1 in 2000.

The seeding was thus of the order of 1,000,000 bacteria.

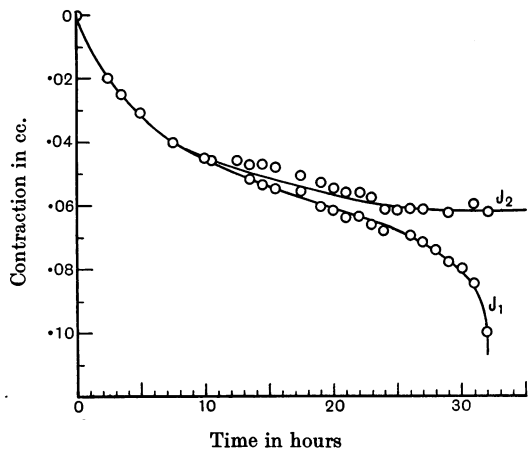

Fig. 3. Curves showing absorption of oxygen by a broth culture *J* 1, and a sterile control *J* 2.

A. *The initial contraction phase.*

The marked effect of aeration on denitrification has already been alluded to. It may be studied quantitatively by determining the factors that give rise to the initial contraction phase.

(*a*) *In absence of nitrates and nitrites.* When a culture tube of about 10 cc. capacity is filled with a broth medium and connected up to a capillary tube as explained in method 2 of the previous paper, there is an air space of about 1 cc. between the surface of the broth and the end of the mercury thread in the capillary tube. Unless oxygen is excluded from this space, there is a slow contraction in volume due to oxidation of the medium. Some actual experimental results are shown in Fig. 3. *J* 2 was a nitrite-free sterile broth control, in which the rate of contraction slowed down as the surface of the broth became oxidised. *J* 1 was a nitrite-free culture, so that after the initial

lag phase was over, the demand for oxygen for bacterial respiration suddenly increased; this circumstance accounts for the sudden drop in the curve. This is verified in curve $L3$ (Fig. 5), where effects were intentionally magnified by having an air space of about 5 cc. above the broth.

The initial rate of the contraction has been found to be approximately proportional to the surface of the broth exposed. For sterile broths, the rate diminishes with time, unless the connection to the capillary tube is opened and a fresh supply of air admitted. In this case the rate of contraction first increases and then diminishes slowly. The total amount of the contraction depends of course on the amount of oxygen available above the broth. In an open tube the absorption continues until the broth is completely oxidised; in Giltay's medium a dark brown colour is then produced.

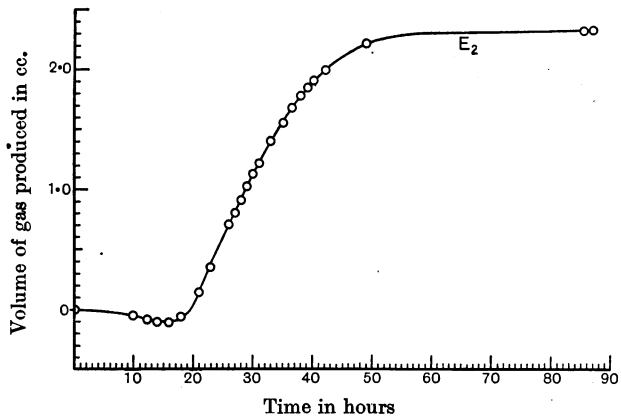

Fig. 4. A typical denitrification curve.

(*b*) *In presence of nitrites.* When a sub-culture is freshly made into a nitrite broth, the initial rate of contraction is similar to that of inoculated controls; apparently the oxygen demands of the bacteria are very small in the early part of the lag phase, and are met by the atmospheric and dissolved oxygen, and by oxygen obtained in the first stage of the reduction of the nitrite. Only when the bacteria reach the phase of rapid growth does the oxygen from these sources become suddenly inadequate, and the intermediate compound is attacked. It is for this reason that the denitrification curve of a healthy culture shows such a sharp point of inflection at the beginning of gas evolution; and attains the maximum rate of gas evolution so rapidly. Fig. 4 shows such a curve obtained from an 8 cc. broth culture ($E2$) with 25 mg. of KNO_2 added.

If a large supply of atmospheric oxygen is available, nitrate may hardly be attacked at all ($L3$, Fig. 5). Experiment shows, however, that some nitrogen is evolved even when a broth culture is spread out in a thin layer to expose a large surface for the absorption of oxygen.

(*c*) *Prevention of the initial contraction.* When the air above the broth and in the capillary tube was displaced by coal gas, the contraction took place in both sterile and inoculated broths, and was apparently due to the solution of some of the gases. No growth started in the inoculated broth, probably because of the toxic effect of the dissolved gases.

The effect of replacing air by nitrogen was next tried. 8 cc. of Giltay's medium containing 0·25 % KNO_3 were placed in tubes of 12 cc. capacity, thus giving a large air space to magnify any effects when atmospheric oxygen was not excluded. Six broth tubes were observed under the following conditions.

Designation	Inoculated	Nitrate mg.	Nitrite mg.	Atmosphere
*L*1	0	20	25	Air
*L*2	0	20	25	Nitrogen
*L*3	+	20	0	Air
*L*4	+	20	0	Nitrogen
*L*5	+	20	25	Air
*L*6	+	20	25	Nitrogen

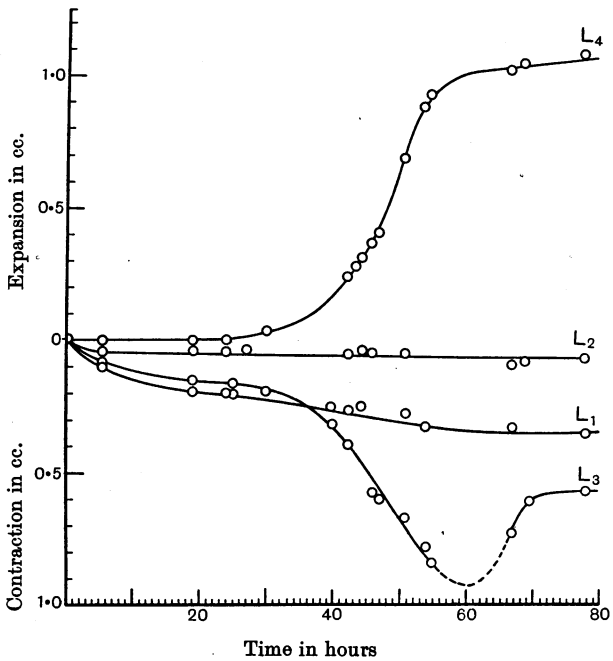

Fig. 5. Curves illustrating the suppression of the initial contraction in cultures and sterile controls on replacing air by nitrogen.

Thus *L*1 served as a control to *L*2 to determine the effect of atmospheric oxygen on sterile broth, *L*3 as a control to *L*4 to determine the effect of atmospheric oxygen on a culture, and *L*1 as a control to *L*3 to determine the contraction in the initial stages due to bacterial respiration. The denitrification curves of *L*1, *L*2, *L*3 and *L*4 are shown in Fig. 5.

No gas evolution occurred in $L5$ and $L6$, apparently because the oxygen requirements of the bacteria were satisfied by the initial stages of the reduction of the nitrate and nitrite present. In $L2$ and $L4$, which were in a nitrogen atmosphere, there was practically no contraction; but in $L1$ and $L3$, which had air, there were large contractions. On comparing the sterile control $L1$ with the nitrite-free culture $L3$, it is seen that both contracted in a similar way for the first 25 hours; the bacterial lag phase in $L3$ was then over, and a period of rapid contraction set in for the next 25 hours until the nitrate was attacked with evolution of gas, the small expansion being due to the fact that the oxygen requirements had already been partly satisfied by the oxygen of the atmosphere. $L4$, on the other hand, experienced no contraction, but at the end of 25 hours showed the expansion due to bacterial decomposition of the nitrate.

To sum up, it is found that in the absence of free oxygen there is no initial contraction; in the presence of oxygen there is an initial contraction, of a two-fold nature:

(*a*) a consumption of oxygen due to oxidation of the medium, which slows down with time;

(*b*) a consumption of oxygen due to bacterial respiration; this increases with time up to the limits of the available oxygen, but the contraction is counterbalanced by evolution of nitrogen if nitrite and nitrate are present as sources of oxygen.

B. *Factors affecting the duration of the lag phase.*

(*a*) *Effect of time of addition of nitrite.* Six cultures, $H1$–$H6$, were inoculated at the same time, and incubated at 27°. 25 mg. of KNO_2 were added immediately to $H1$ and $H2$, after a lapse of 12 hours to $H3$ and $H4$, and after 24 hours to $H5$ and $H6$. Gas production began almost simultaneously in $H1$, $H2$, $H3$ and $H4$ about 20 hours after inoculation, and in $H5$ and $H6$ about 8 hours later, *i.e.* about 4 hours after addition of the KNO_2. The following inferences were drawn.

(i) The time that elapses between the inoculation of a culture into a nitrite broth and the evolution of gas is due mainly to the lag in the development of a healthy culture. When, as in the above experiment, the period of this lag is about 20 hours, the time of first gas evolution is independent of whether the nitrite is present at the beginning or whether it is added up to 12 hours after inoculation.

(ii) The addition of nitrite to a 24-hour culture (*i.e.* one that would have begun to produce gas if nitrite had been present at the time of inoculation) does not cause immediate gas production, but there is a further delay of 4 or 5 hours. This may be due to a necessary accommodation on the part of the bacteria to its slightly altered nitrite environment, or it may more probably be due to the nitrite-reduction taking place in two stages with the

formation of an intermediate compound. The first stage does not produce nitrogen.

The experiment was repeated under slightly different conditions. Six similar cultures were prepared and inoculated at the same time. They were kept at room temperature for 2½ hours, and then placed in a thermostat at 32°, and the production of gas for 100 hours measured at intervals by the capillary tube method.

KNO_2 was added to one of the cultures 2½ hours after inoculation, and to the others at intervals as shown in Table I. The last column shows the time interval from inoculation when nitrogen was first evolved, and Fig. 6 gives the denitrification curves of the series.

Table I.

Designation of culture	Time in hours after inoculation	
	KNO_2 added	Gas first produced
*HH*1	2½	13
*HH*2	5½	13
*HH*3	8½	13
*HH*4	11½	16
*HH*5	14½	19
*HH*6	18½	23

The curve for *HH*3 was spoiled because the rubber tubing joining the culture tube to the capillary tube was defective. The production of gas appeared to be as great as in the other cultures.

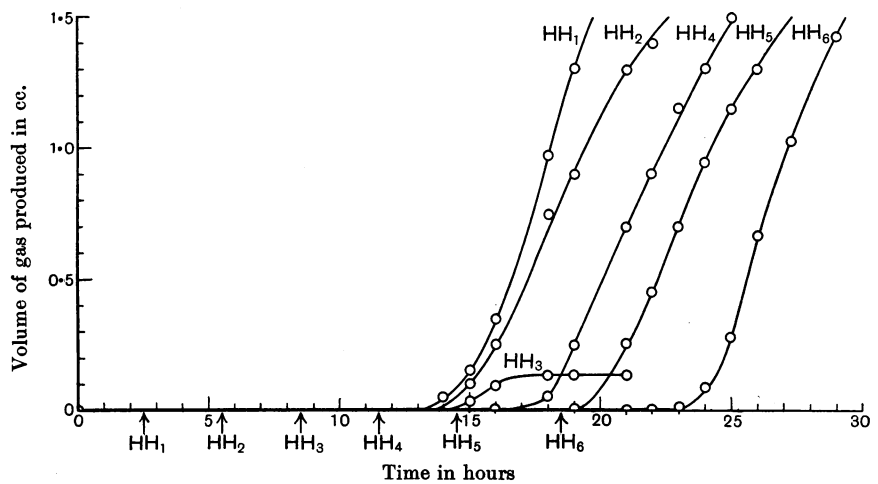

Fig. 6. Curves illustrating the effect of the time of addition of nitrite on the duration of the lag in gas production. The arrows indicate the time of addition of the nitrite to the culture designated.

This experiment confirms the previous *H* series. The same inferences may be drawn, but may be made more precise. *HH*5 and *HH*6 showed very definite growth before the addition of KNO_2; yet there was a lag of 4½ hours in each case between the addition of the KNO_2 and the rapid evolution of gas. This

lag must be attributed to the formation of an intermediate compound. It is very remarkable that this same period of lag occurred in *HH* 3 and *HH* 4. The conclusion can be drawn that in the nitrite-broth culture the normal lag of 13 hours between the time of inoculation and the appearance of gas was made up of a bacterial lag of 8½ hours, and a "chemical lag" of 4½ hours.

(*b*) *Concentration of nitrite or nitrate added.* A series of similar cultures was prepared in equal amounts—about 9 cc.—of Difco broth which was adjusted before sterilisation to a p_H value of 8·5. Varying amounts of KNO_3 were added, and denitrification curves obtained at 32° (Fig. 7). The air above the broth was displaced by nitrogen so that the only source of oxygen was the nitrate.

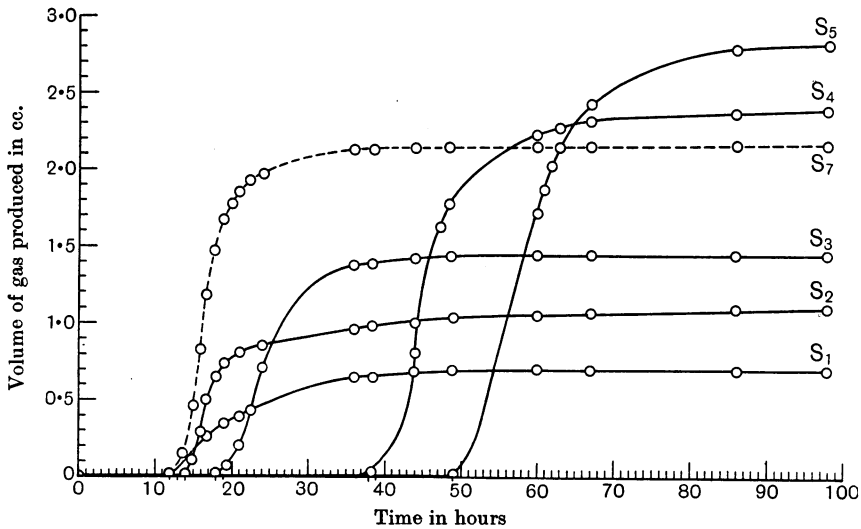

Fig. 7. Curves showing the progressive increase of the lag in gas production with increase in initial concentration of nitrate. The broken curve illustrates the difference when nitrite is used.

Table II shows that the time elapsing before evolution of nitrogen increases with the increase in concentration of the nitrate added. The experimental facts are accounted for by assuming the duration of the lag phase of bacterial growth to be about 8 hours in each culture. The numbers of bacteria then increase rapidly, satisfying their oxygen requirements by reducing the nitrate to nitrite, and then possibly to hyponitrite. No nitrogen is produced until these intermediate reductions have been completed or nearly completed, which first occurs of course in the culture with least initial amount of nitrate. The other cultures begin to evolve nitrogen in the sequence of their nitrate concentrations.

At the same time as these nitrate-broth cultures were experimented with, three nitrite-broth cultures were observed under identical conditions in order to test the validity of the following hypotheses. If nitrate is reduced in three stages, viz. to nitrite, to hyponitrite, and to nitrogen, and these stages

do not occur simultaneously to any great extent so that nitrogen is not produced until the first two stages are nearly over, it was expected that (i) a nitrite-broth culture would show a considerably shorter lag in gas production than a culture containing an equivalent amount of nitrate. If hyponitrite is the intermediate compound formed, and if it is not attacked until all the nitrate and the nitrite is reduced, it was expected that (ii) a nitrite-broth culture would show the same duration of lag in gas production as a culture in a broth containing half the equivalent amount of nitrate, because as is shown in equations for *S*4 and *S*7 below, twice as much oxygen must be taken from nitrate as from an equivalent amount of nitrite to produce the hyponitrite.

The curve *S*7 was obtained from one of three cultures, *S*6, *S*7 and *S*8, which differed from the others in that 20 mg. of potassium nitrite were added and no nitrate. In each of these cultures, 13 hours elapsed before nitrogen began to be evolved. 20 mg. of KNO_2 are equivalent molecularly to 24 mg. of KNO_3, and it will be noted that the quantity of nitrogen produced by *S*7 is only a little less than that produced by *S*4 which contained 24 mg. of KNO_3. Whereas, however, *S*7 produced nitrogen 13 hours after inoculation, *S*4 did not do so until 37 hours had elapsed. The culture *S*2, which contained 12 mg. of KNO_3, and which therefore required the same oxygen consumption to convert the nitrate into hyponitrite as is required in *S*7 to convert the nitrite into hyponitrite, first produced nitrogen 13 hours after inoculation, *i.e.* simultaneously with *S*7.

Thus the two expectations were realised, and quantitative evidence is produced to show that hyponitrite is formed during nitrate-reduction, and that, under the conditions of the above experiments at any rate, no nitrogen is evolved until all the nitrate and nitrite is converted into hyponitrite. The following equations therefore represent the reductions for the two cultures, *S*4 and *S*7, before nitrogen is evolved, if hyponitrite be assumed to be the product.

$$S4 \ \ldots \ 2KNO_3 - 2O_2 = K_2N_2O_2$$
$$S7 \ \ldots \ 2KNO_2 - O_2 = K_2N_2O_2.$$

Twice as much oxygen was consumed in *S*4 as is *S*7 before nitrogen was evolved, and the delay in the production of gas in *S*4 is explained.

The extent of denitrification. Calculations were made to determine what percentage of the total available oxygen of the nitrate was utilised. It is reasonable to suppose that if the concentration of nitrate is very high originally, oxygen will not be a limiting factor in the growth of the culture. Some other factor may operate to stop the growth before the stage in nitrate-reduction that results in liberation of nitrogen is reached. Examples of these may be seen in the cultures *L*5 and *L*6, and to a lesser degree in *L*3, mentioned previously. In the equation for the complete denitrification,

$$4KNO_3 - 5O_2 = 2K_2O + 2N_2,$$

it is seen that 2·5 molecules of oxygen are consumed for each molecule of nitrogen evolved. If the reaction does not go to completion, it would be incorrect to base the oxygen consumption on the nitrogen produced without allowing for the fact that the reaction occurs in stages. This allowance can be made by noting that four of these molecules of oxygen are consumed before nitrogen is evolved at all, thus

$$4KNO_3 - 4O_2 = 2K_2N_2O_2.$$

The ratio of the amount of nitrogen evolved to the total quantity in the original nitrate determines what fraction of the remaining 20 % of oxygen is consumed, because two molecules of nitrogen are evolved in this final stage for each molecule of oxygen consumed, thus

$$2K_2N_2O_2 - O_2 = 2K_2O + 2N_2.$$

The ratio of the oxygen consumed to the total amount available in the nitrate may be stated algebraically in terms of the amount of nitrogen evolved as follows. If the calculated total amount of nitrogen in a given weight of potassium nitrate is x cc., and the amount produced is y cc., then the ratio of the amount of nitrogen evolved to the amount in the salt is y/x, but the proportion of the oxygen consumed to the total amount available is

$$(2x + 0{\cdot}5y)/2{\cdot}5x.$$

Table II shows that in each culture the percentage of the available oxygen consumed, calculated from the volume of nitrogen evolved, was practically 100.

Table II.

Designation of culture	*S*1	*S*2	*S*3	*S*4	*S*5	*S*7
Wt of KNO_3 in mg.	6	12	18	24	30	20 (KNO_2)
Time in hours before nitrogen evolved	12	13	18	37	48	13
Available nitrogen, x (calculated)*	0·7	1·4	2·1	2·8	3·5	2·8
Vol. of nitrogen evolved, y	0·71	1·10	1·47	2·39	2·80	2·20
†Available oxygen (calculated)	1·75	3·5	5·25	7·0	8·75	4·2
‡Calculated vol. of oxygen consumed before nitrogen evolved	1·4	2·8	4·2	5·6	7·0	2·8
§Total oxygen consumed	1·75	3·35	4·93	6·8	8·4	3·9
% of available oxygen consumed	100	96	95	97	96	93

* All vols. of O_2 and N_2 in cc.

† This is the calculated volume at 17° and 760 mm. pressure of five-sixths of the oxygen in the nitrate, or of three-quarters of the oxygen of the nitrite.

‡ Equal to the volume of the nitrogen available in the case of the nitrite, and equal to double this volume for the nitrates.

§ $2x + 0{\cdot}5y$ in the case of the nitrates; $x + 0{\cdot}5y$ in the case of the nitrite.

(*c*) *Effect of the initial acidity of the broth.* Four cultures, *G*1, *G*2, *G*3 and *G*4, were made in nitrite broth buffered with mixtures of phosphate and sodium hydroxide solutions to give initial p_H values of 8·2, 8·0, 6·8 and 6·4 respectively. The thermostat was kept at 27°, and observations of gas production

made regularly over a period of 575 hours. Fig. 8 shows the denitrification curves, and Fig. 9 shows the rate of denitrification curves for these cultures.

The final p_H values after 575 hours were 9·3, 9·2, 8·7 and 8·2 respectively. The phosphate solutions employed as buffers were thus unable to prevent the development of alkalinity which always accompanies denitrification. Qualitative tests for nitrites (Griess-Ilosvay) showed a trace present in *G*3, but gave negative results for the others.

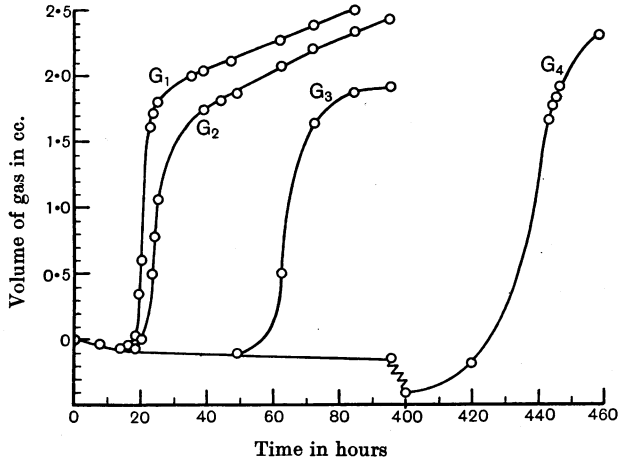

Fig. 8. Curves showing the progressive increase of the lag in gas production with increase in initial acidity of the broth. The time scale is abbreviated by omitting the interval from 96 to 400 hours.

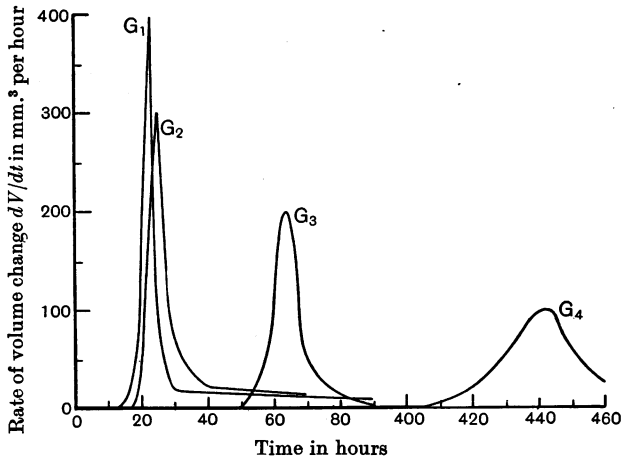

Fig. 9. The rate of denitrification curves of the *G* series of cultures calculated from experimental readings, some of which are shown in Fig. 8. The time scale is abbreviated as in Fig. 8.

The amount of gas produced was 2·8, 2·6, 2·1 and 2·8 cc. respectively. A rough analysis of the gas in the capillary tube at the end of 575 hours was made in each case by passing the gas several times over anhydrous $CaCl_2$

and NaOH. Less than 0·1 cc. was absorbed in each case, indicating the absence of CO_2 in amount exceeding 3 %; this agrees with more accurate analyses [Cranston and Lloyd, 1930] which showed that the gas evolved from this organism is pure nitrogen.

It is seen from the curves that increased acidity causes

(1) increase in the lag in nitrogen production, viz., 14, 18, 51 and 400 hours respectively;

(2) diminution of the rate of evolution of nitrogen when once started; this implies a corresponding diminution in the rate of increase of bacteria;

(3) progressive decrease in the maximum rate of gas production (shown by the peaks of the curves in Fig. 9), which may be taken to indicate that the maximum number of bacteria alive in the medium at any given time decreases with increased acidity.

(*d*) *Effect of temperature.* A series of similar nitrite-broth cultures, *T* 1–*T* 6, was prepared, and the six cultures were incubated at the different temperatures, 18°, 25°, 30°, 35°, 40° and 45° respectively.

A thermostat was regulated to maintain a temperature of 40°, and into this were immersed up to their necks three thermos flasks containing water at 45°, 35° and 30° respectively. The temperature of the interior of a thermos flask was so little different from that of its environment that it remained sufficiently constant throughout the experiment. Thus these three thermos flasks and the thermostat provided constant temperature baths for four of the cultures. A second thermostat, regulated at 25°, provided for a fifth culture, and the sixth was kept at room temperature in the vicinity of the first thermostat where the temperature remained constant at 18°.

The denitrification curves, which are not reproduced here, gave a series similar to those of Fig. 6, and enabled the duration of the lag phase to be determined. The results, which were confirmed by being carried out in duplicate, were as follows.

Designation of culture	*T* 1	*T* 2	*T* 3	*T* 4	*T* 5	*T* 6
Temperature, ° C.	18	25	30	35	40	45
Time in hours when gas was first produced	26	20	17	13	25	None in 50 hours

The optimum temperature for the growth of this organism under the given experimental conditions was thus about 35°. The following were found to be the two most marked effects as this temperature was departed from in either direction.

(i) A marked increase occurred in the lag phase. This was specially shown at unfavourably high temperatures, for at 45° no gas was produced in 50 hours. Incidentally, several days after the conclusion of the experiment, during which time the thermostat had been at room temperature, the culture *T* 6 produced gas in normal amount. Thus the temperature of 45°, whilst inhibiting growth, had not destroyed the organism.

(ii) The rate of denitrification became progressively slower [see also

Cranston and Lloyd, 1930]. This again was more marked at higher temperatures, the rate at 40° being 20 times less than that at 35°. This was probably due either to high mortality among the bacterial cells at this temperature, or to lethargy induced by this temperature. It may well be that at temperatures from 40° upwards, the bacterial cells develop into arthrospores and so are able to survive considerably higher temperatures.

C. *Other phases of bacterial growth.*

It has already been shown that the study by denitrification of the middle phases of bacterial growth can only be done with accuracy by using large quantities of broth culture and by making observations of volume changes at intervals not greater than half an hour.

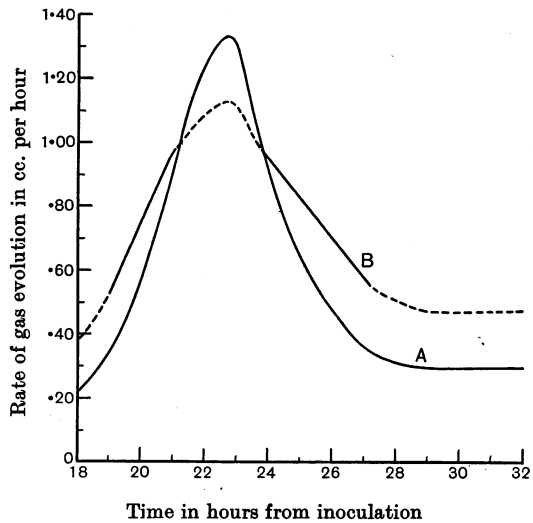

Fig. 10. A rate of denitrification curve *A* showing various phases of gas production which may be correlated with bacterial growth phases. The curve *B* is obtained by plotting the logarithms of these rates against the time. Two portions of this curve are straight, and represent the logarithmic growth phase and the logarithmic death phase respectively.

An experiment was carried out at 20·7° with 90 cc. of a broth culture containing 0·25 % KNO_2. This was contained in a boiling-tube fitted with a rubber stopper through which passed a glass tube connected to the capillary tube. After the broth was inoculated, the air above it was replaced by nitrogen. Gas began to evolve 17 hours later, and the volume was then measured at intervals of about 15 minutes for 12 hours. The average rate of denitrification was then calculated over successive periods of about half an hour and plotted against the mean time from inoculation. The result is shown in curve *A*, Fig. 10. The logarithm of the rate of denitrification was also plotted against the mean time, and the result is shown by the dotted curve *B*, Fig. 10.

The following phases are shown.

Logarithmic growth phase	from time	19	–21 hrs.
Phase of negative growth acceleration ...	,,	21	–22·4
Maximum stationary phase	,,	22·4	–23·0
Phase of accelerated death	,,	23	–25·5
Logarithmic death phase	,,	23·5	–27
Phase of slow decline	,,	27	–29

From time 29 to 54, the rate of evolution of gas was almost constant at 0·3 cc. per hour, and then it deceased gradually until the 115th hour, after which no further gas was evolved.

It will be seen that in general the successive phases are of short duration; this is probably characteristic of growth in a medium containing nitrite because, in addition to the abundance of food supply available initially, oxygen is present in the nitrite in a well-distributed assimilable form. This results in rapid growth until the oxygen is used up, after which the decline is equally rapid. The intermediate phases are thus compressed, and the death phase becomes of the type usually associated with the presence of a germicide, *i.e.* it is logarithmic.

D. *The changes in acidity of the medium during growth.*

In the reduction of nitrates or nitrites to nitrogen two substances are formed which affect the acidity of the medium, viz. CO_2 and K_2O. These, of course, influence the p_H value in opposite directions. In the initial stages of the reduction, no alkali is formed at all, but oxygen is being consumed and presumably is combining with carbon in metabolism. At this stage there is a small increase in the acidity, the minuteness of which indicates that only a small proportion of the oxidised carbon is liberated into the broth as dissolved carbon dioxide; certainly no appreciable amount of CO_2 escapes in the gaseous state. At the later stage of the reduction, when nitrogen is evolved, K_2O is liberated, and forms KOH. The broth then rapidly becomes alkaline.

In pure aqueous solution the reduction of nitrate to nitrite results in considerably increased alkalinity, due to the hydrolysis of the nitrite. In broth, however, owing to its buffer action, the change in p_H for this reduction is found to be only 0·05, which is negligible compared with the other changes.

The following is a summary of many observations that have been taken on the changes in the acidity of a broth during the growth of a culture.

(i) *In absence of nitrites or nitrates.* When a sub-culture of Organism XIV is made into a broth of initial p_H 8·5 and free from nitrates or nitrites, the growth of the culture is accompanied by a slight increase in acidity to a p_H of about 8·2. After the usual phases of growth and decline have been completed, the senescent culture slowly, over a period of a few weeks, restores the medium to a p_H of about 8·6.

(ii) *In presence of* KNO_2. In a nitrite-broth culture of p_H 8·5, there is at first a slight increase in acidity, but when nitrogen is evolved, the p_H rapidly increases to a value of about 9·3. The final value depends on the initial concentration of the nitrite, for the greater the concentration up to 0·25 % the nearer to 9·3 is the final value.

(iii) *In presence of* KNO_3. In a nitrate-broth culture, the p_H first falls to 8·0, and then attains a final value of 8·2 to 8·8 according to the initial concentration of the nitrate. The lower final p_H value attained with nitrates as compared with nitrites is explained by the greater amount of oxygen available in the former (and consequent greater production of carbon dioxide) for the same amount of nitrogen produced.

Summary.

1. The phases in gas production by actively denitrifying cultures have been studied by continuous measurements of the rate of gas production.

2. The conditions under which such rates of gas production can be directly correlated with rates of growth are discussed.

3. Some of the factors determining the duration of the lag phase of growth in organisms are considered.

4. By varying the amount of nitrate or nitrite experimentally, it is demonstrated that these substances are completely converted into some intermediate compound, which it is suggested is hyponitrite, before nitrogen is produced.

5. It is shown that one of the important factors in determining whether gas is produced or not, *i.e.* whether true denitrification occurs or not, is the initial concentration of the nitrate or nitrite.

6. The effect of temperature on growth is investigated.

7. In the interpretation of denitrification curves in terms of bacterial growth, it should be remembered that when all the factors are favourable to growth, the presence of nitrate or nitrite is specially conducive to rapid growth because oxygen is thus furnished throughout the medium in a most accessible manner. The multiplication of bacteria is therefore specially rapid as long as these substances last, after which the decline in the culture may be abnormally rapid, lack of oxygen being the principal limiting factor.

The above work was carried out in part at the Millport laboratory of the Scottish Marine Biological Association. Thanks are due to the Superintendent, Mr R. Elmhirst, for the facilities placed at the disposal of the authors.

REFERENCES.

Ampola (1907). *Ann. R. Staz. Chim. Agrar. Sper. Rome* (ii), **1**, 2.
—— and Ulpiani (1899). *Gazzetta*, **29**, 49.
Baur (1902). Wissenschaftliche Meeresuntersuchungen (Kiel), **6**, 9.
Buchanan and Fulmer (1928). Physiology and biochemistry of bacteria.
Carapelle (1908). *Centr. Bakt. Par.* I, **47**, 545.
Caron (1912). *Centr. Bakt. Par.* II, **33**, 62.
Chesney (1916). *J. Exp. Med.* **24**, 387.
Cranston (1930). *Biochem. J.* **24**, 525.
—— and Lloyd (1930). *J. Roy. Tech. Coll. Glasgow*, **2**, 301.
Drew (1911). *J. Mar. Biol. Assoc.* **9**, 142.
Gran (1901). *Bergens Mus. Aarbok.* No. 10.
Korsakova (1927). *Bull. Acad. Sci. U.S.S.R.* **6**, 1221.
Kunnemann (1898). *Landw. Versuchs-Stat.* **50**, 65.
Lane-Claypon (1909). *J. Hyg.* **9**, 239.
Ledingham and Penfold (1914). *J. Hyg.* **14**, 242.
Lumia (1915). *Ann. Chim. Applic.* **4**, 1.
Penfold (1914). *J. Hyg.* **14**, 215.