THE RELATION BETWEEN OXYGEN CONSUMPTION AND THE UTILIZATION OF AMMONIA FOR GROWTH IN SERRATIA MARCESCENS

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The metabolic differences between assimilating and nonassimilating cells have been the subject of several recent investigations. In this connection a stimulation of glycolysis when ammonia is added to yeast has been shown by several authors (Runnstrom, Brandt, and Marcuse, 1941; Winzler, Burk, and du Vigneaud, 1944). Armstrong and Fisher (1947) have demonstrated a comparable increase in the rate of oxygen consumption by *Escherichia coli* when the assimilation of ammonia is taking place. It follows from these observations that it may be possible to determine the amount of glycolysis or carbon dioxide produced and the amount of oxygen consumed during the assimilation of known quantities of the nitrogen source.

It will be shown in the present work that in the bacterium *Serratia marcescens* the rate of oxygen consumption is also higher during the assimilation of ammonia than it is in the absence of such assimilation. Following the uptake of the last of the ammonia, as noted for *E. coli* by Armstrong and Fisher, the rate at which oxygen is consumed by the bacterium falls sharply to a lower rate, which is typical of resting cells. This lower rate is a definite percentage of the higher one regardless of how much growth has taken place. It is, therefore, permissible to calculate the resting rate which corresponds to each rate observed for the growing cells. Any oxygen consumed in excess of the amount expected for resting cells must then be associated with the assimilation of ammonia. This quantity of oxygen has been measured along with the quantity of ammonia actually assimilated.

MATERIALS AND METHODS

The preparation and maintenance of the bacteria. The organism used in this investigation was the bacterium *Serratia marcescens* (*Bacillus prodigiosus*), American Type Culture Collection no. 990. It was maintained on a synthetic medium, modified from that used by Bunting (1940), having the following composition: glycerol 1.25 g, citric acid 4 g, K_2HPO_4 9 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and NH_4Cl 1 g, adjusted to pH 7 with NaOH, and made up to 1 liter with distilled water. Twenty-five g of agar were added and the medium was autoclaved at 15 pounds' pressure for 15 minutes.

The bacterial suspensions for the respiration experiments were prepared as follows: A slant was inoculated from 1 loopful of bacteria; it was incubated for

17 hours at 30 C (a preliminary experiment showed that this temperature gave better growth than 20 or 37 C); and the growth was then washed off into 0.07 M potassium phosphate buffer at pH 7. The suspension was made up to the desired concentration, about 1×10^9 bacteria per milliliter, by the reflectometer (Libby, 1941).

The measurement of oxygen consumption. The rate of oxygen consumption was measured in a Warburg respirometer (Umbreit, Burris, and Stauffer, 1945) at 30 C, with air being used as the gas phase and with the vessels shaking through an arc of 5 cm approximately 100 times per minute. Under these conditions there was no indication that the concentration of carbon dioxide was a limiting factor. The vessels were prepared with 1.0 ml of the bacterial suspension plus 0.5 ml of solution A (i.e., $MgSO_4 \cdot 7H_2O$ 2.0 g, glycerol 20 g, sodium citrate 12.6 g, adjusted to pH 7 with HCl, and made up to 1 liter with distilled water) in the main space of the vessel, 0.5 ml of distilled water or a solution of ammonium chloride in the onset, and 0.3 ml of 10 per cent potassium hydroxide in the inset with filter paper.

The determination of ammonia. For this analysis the bacteria were separated from the suspending medium by filtration through fritted glass filters (pyrex, no. 36060, 15 UF) under reduced pressure. The filtrate was collected in 1 ml of 50 per cent (by volume) sulfuric acid.

The ammonia in the filtrate was determined by a procedure essentially the same as that described by Peters and Van Slyke (1932) in connection with the determination of urea in urine. To the acid filtrate was added distilled water to a volume of 10 ml and then 5 ml of 5 N KOH. Air, after passage through 5 per cent H_2SO_4, was drawn through the alkaline mixture and thence through 15 ml of 0.02 N HCl, the ammonia being trapped in the latter. The total ammonia thus collected was estimated colorimetrically using a Cenco-Sheard-Sanford photelometer, following the procedure outlined by Snell and Snell (1936), and using Jackson's modification of Nessler's reagent. This procedure can be used provided the quantity of ammonia present is not over about 12×10^{-2} mg. It is reproducible to within about 0.25×10^{-2} mg of ammonia in the sample filtered.

Determination of total (Kjeldahl) nitrogen. The contents of the respirometer vessel were washed into 1 ml of the digestion mixture (1 part saturated K_2SO_4, 1 part concentrated H_2SO_4, and a small amount of selenium powder; cf. Snell and Snell, 1936) in a pyrex test tube. Two glass beads were added and a glass bulb was placed on top. The tube was heated vigorously over a microburner until the water had been boiled off and the contents of the tube had begun to fume, the flame was reduced, and the mixture was allowed to boil gently until it was well charred. When charring had taken place, the tube was cooled for about 30 seconds, and a few drops of 30 per cent H_2O_2 were dropped on the charred material. The mixture usually decolorized at once. It was then reheated, decolorized again if necessary, and finally boiled until it had remained clear for several minutes. This was taken as the end point of the digestion. The nitrogen then present as ammonium sulfate was determined exactly as described above for ammonium chloride.

EXPERIMENTAL RESULTS

In order to establish the actual relationship between the uptake of ammonia and the rate of oxygen consumption, both processes were studied simultaneously. The experiments were conducted as follows: The respirometer vessels were prepared with the bacterial suspension and solution A in the main part of the vessel and with an amount of ammonium chloride (0.19 mg) which would sustain growth for only a few hours in the onset. After being shaken 1 hour in the constant temperature bath with the ammonium chloride in the onset, the bacteria

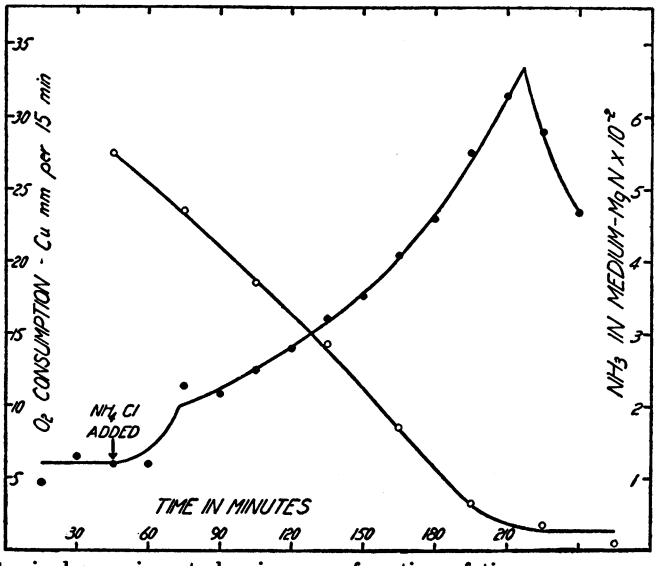

FIG. 1. A typical experiment showing as a function of time:
(1) the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens* (dots) and
(2) the quantity of ammonia present in the medium (circles).
Each point is the average result in two identical vessels. The ammonium chloride was added to the organisms from the onsets of the vessels at the point indicated.

reached a "resting" state, and the rate of oxygen consumption was comparatively steady although decreasing very gradually with time.[1] The ammonium chloride was then added to the bacteria. Measurements of the rate of oxygen consumption were continued, and at intervals the contents of the vessels were analyzed for ammonia, one of the vessels being removed for this purpose immediately following the addition of the ammonium chloride, and others every few minutes thereafter.

The results of a typical experiment are shown in figure 1 in which the solid circles indicate the rate at which oxygen was taken up in the respirometer vessels, whereas the open circles indicate the ammonia remaining in the medium. Before

[1] Any nutritive materials washed off the culture slants with the organisms were apparently in such low concentrations as to be completely metabolized during this initial hour in the respirometer.

the addition of ammonia, the rate of oxygen consumption is relatively constant and there is, of course, no growth. Upon adding ammonia, however, the medium becomes one which will support growth—it is, in fact, the one on which the organism was being maintained. At this point the rate of oxygen consumption rises quite abruptly and after some 20 to 30 minutes reaches a value which is nearly double the initial value. There then ensues a period during which the logarithm of the rate of oxygen consumption is a linear function of time. The curve drawn through the observed points in figure 1 during this phase of the experiment was obtained by calculation presuming that the logarithm of the rate is a linear function of time. It is clearly a good representation of the data. It undoubtedly represents the gradual increase in the quantity of bacterial protoplasm in the respirometer vessel, as others have noted (Greig and Hoogerheide, 1941; Hershey and Bronfenbrenner, 1938). From it the time for the bacterial mass to double, that is, for the logarithm of the rate of oxygen consumption to increase by the logarithm of 2, may be determined. This averaged 72 minutes (standard deviation,[2] 6 minutes) in 10 experiments.

It will be noted in the figure that the amount of ammonia present in the suspending medium decreases steadily throughout the experiment. It does so, of course, because it is taken up by the cells for elaboration into new protoplasm. The curve describing the utilization of the ammonia actually, therefore, represents the time course of the formation of new protoplasm. It is in fact a "growth curve."

As the concentration of ammonia approaches zero, the rate of oxygen consumption quite suddenly falls, just as has been described for *E. coli* (Armstrong and Fisher, 1947), to a relatively steady value. The latter is illustrated in the experiments of longer duration which are shown in figure 4 and which are to be discussed in detail below. In 9 experiments this resting rate was on the average 56.6 per cent of the maximum rate seen in the respirometer (standard deviation, 3.6 per cent). This steady (strictly, slowly declining) rate represents the resting rate which is characteristic of the amount of bacterial protoplasm now present in the respirometer vessel. Since the ammonia has been exhausted, it is evident that no appreciable uptake of ammonia can occur after the rate of oxygen consumption starts to decrease. It follows, then, that these organisms consume oxygen at either of two different rates, just as *E. coli* does, depending upon whether or not assimilation of ammonia is occurring.

This conclusion arises again when the rate of oxygen consumption and the rate of ammonia utilization are compared. As noted above, there is a rapid rise in the rate of oxygen consumption when the ammonia is first added. The rate of ammonia utilization, however, does not show any evidence of a similar initial spurt. It seems again, therefore, that in order to grow under these conditions the cells present must consume oxygen at a rate above that characteristic of a resting phase.

[2] The standard deviation was taken as $\frac{(x - \bar{x})^2}{N - 1}$ where x is the result of one experiment, $\bar{x}$ is the mean of the results, and N is the number of experiments.

It should perhaps be pointed out that it is not possible from the data available to estimate the difference between the activity and resting rates at the beginning of nitrogen assimilation. This results, firstly, from the lack of information about the existence of an initial lag period in the growth curve and, secondly, from the fact that the chemical systems involved here have considerable inertia, as indicated by the observation that, following the exhaustion of the nitrogen source, the rate of oxygen consumption does not decrease instantaneously.

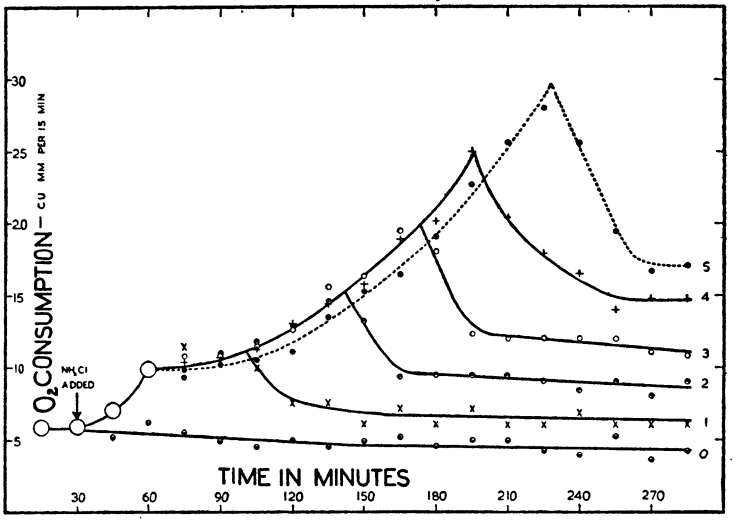

FIG. 2. The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens*. Each point is the average result in two identical vessels. As in figure 1 ammonium chloride was added to the organisms from the onset of the vessel at the point indicated. The numbers appearing at the right-hand end of each curve give, in hundredths of milligrams, the actual quantities of nitrogen added as ammonium chloride to the several vessels.

During the first hour the rates of oxygen consumption in the various vessels are essentially identical. Observations during this interval have, therefore, been indicated in the figure by single circles which have been made large enough to encompass all of the observations made at each time. In this particular experiment, although it was not usually so, the data for the highest concentration of ammonium chloride differed slightly from the curve describing the remainder of the points.[3] To avoid confusion, therefore, the trend of these points is indicated by dashes.

To provide further information about the changes in the rate of oxygen consumption when ammonia is added or exhausted, the consequences of adding different quantities of ammonia were studied. These experiments were made by placing aliquots of bacterial suspension in each of several respirometer vessels, in the onsets of which different amounts of ammonium chloride were placed. As in the experiment described in figure 1, the resting rate of oxygen consumption was determined, and then the ammonia was tipped into all the vessels. Typical observations of the rate of oxygen consumption in an experiment of this kind are given in figure 2.

[3] This would result if, by accident, fewer bacteria had been placed initially in one of these vessels.

In every case, when the ammonium chloride is added there is an initial rapid increase in the rate of oxygen consumption. This is followed by the gradual logarithmic increase already described. After the ammonia is exhausted, the rate of oxygen consumption falls to the lower resting rate.

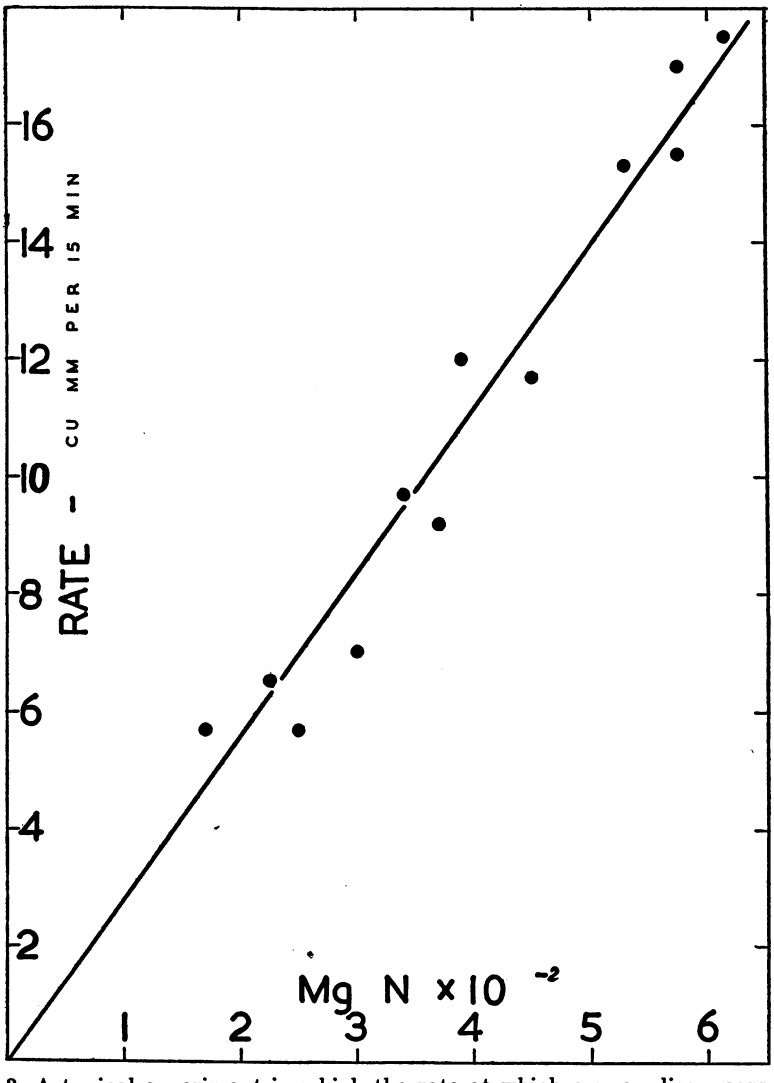

FIG. 3. A typical experiment in which the rate at which oxygen disappears from a respirometer vessel containing resting cells is shown as a function of the quantity of bacterial nitrogen present in the vessel. Each point is the result for one vessel.

It will be noted that the only significant differences between the several curves in figure 2 are the durations of the logarithmic phase and the absolute levels to which the rates fall after exhaustion of the ammonia. As might be expected, the logarithmic phase lasts longer, and the final resting level attained is higher, the

greater the amount of ammonia added. It was found that the resting rates were a constant percentage of the maximum rates reached, the percentage being independent, therefore, of the amount of growth which had taken place. As noted above, the resting levels averaged approximately 56 per cent of the peak rate.

At the termination of the experiments illustrated in figure 2, the contents of the vessels were analyzed for nitrogen. Determinations of the quantity of ammonia and total nitrogen in the suspending medium alone indicated that at this time all of the nitrogen present was in the cells. The analysis on the entire contents of the respirometer vessels thus measures the bacterial nitrogen present at this time. The latter can also, of course, be obtained by adding to the nitrogen present in the original aliquot of bacterial suspension, with which the experiment was begun, the amount of ammonia tipped into the vessel to initiate growth. In any case it is possible to compare the resting rates observed, after assimilation has ceased, with the amount of nitrogen present in the bacteria. This has been done in figure 3, and it is apparent there that the rate at which oxygen disappears in a respirometer vessel, containing resting cells, is directly proportional to the quantity of bacterial nitrogen which is present. It is to be noted that this is true even for the initial aliquots of bacteria, i.e., before any growth occurs in the respirometers. Moreover, the line in figure 3 passes through the origin, indicating that the nitrogen content is an absolute measure of the rate of oxygen consumption (cf. Hershey, 1939; Burris and Wilson, 1940). It is quite definite, therefore, that the several different resting rates recorded in figure 2 indicate the presence of different quantities of bacterial protoplasm. It may be calculated from the data in figure 3 that, on the average, these bacteria consumed oxygen at the rate of 1.12×10^3 cu mm per hour per mg of nitrogen when suspended in solution A.

It is now evident that for any particular rate of oxygen uptake along the logarithmic part of the curve in figure 1, there is a corresponding lower resting rate to which the rate at which oxygen is disappearing would fall if the ammonia were suddenly removed. This lower rate was shown above to be determined solely by the amount of bacterial protoplasm present. Since it forms a constant percentage (approximately 56 per cent) of the activity rate, it is possible to plot on a graph, such as that in figure 1, a line which shows the time course of the resting rate following the addition of ammonia to the cells. During the period of logarithmic growth the resting rate is 56 per cent of the activity rate. To obtain the resting rate during the initial rapid rise in the rate of oxygen consumption, the curve describing the time course of the resting rate during logarithmic growth may be extrapolated backwards. Similarly the resting rate during the fall in the rate of oxygen consumption, following exhaustion of the ammonia, may be obtained by extrapolating backwards the nearly horizontal straight line which at the termination of the experiment describes the resting rate.

A calculated line giving the time course of the resting rate has been plotted along with a set of experimentally determined rates in figure 4. It is evident that the area enclosed by the lines describing, respectively, the observed rate of oxygen consumption and that indicating the time course of the resting rate rep-

resents the volume of oxygen consumed by the growing cells in excess of that required by resting cells. It is an accompaniment of the growth process. More specifically, it is the amount of oxygen consumed during the assimilation of a known quantity of nitrogen in the form of ammonia. The number of oxygen atoms consumed during the assimilation of each nitrogen atom given in the form of ammonia may, therefore, be calculated. The average value found in 10 experiments was 2.19, the standard deviation of the individual values about this mean being 0.14. This value was observed to be independent of the quantity of ammonia assimilated for quantities varying from 0.012 to 0.06 mg.

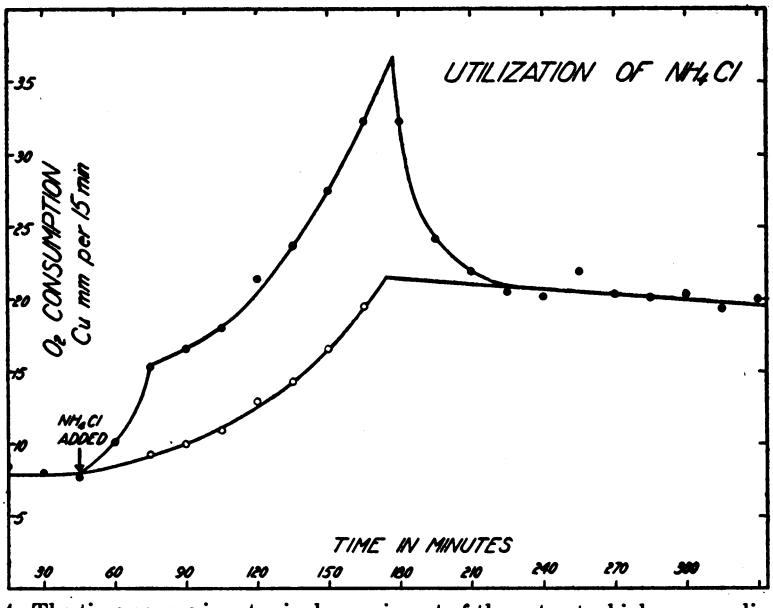

FIG. 4. The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of *S. marcescens* (dots). Each point is the average of three identical vessels. Ammonium chloride was added at the point indicated. The calculated time course of the resting rate is indicated by circles.

SUMMARY

The oxygen consumption of the bacterium *Serratia marcescens* was studied in both growing and resting cells, and the rate of oxygen consumption per milligram of bacterial nitrogen was found to be higher when the assimilation of ammonia was taking place.

The extra oxygen used during the assimilation of the ammonia was determined. It was found that 2.2 oxygen atoms were taken up for each nitrogen atom assimilated.

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