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*SIMULTANEOUS USE OF MOLECULAR NITROGEN AND AMMONIA BY CLOSTRIDIUM PASTEURIANUM**

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It has been shown that *Azotobacter* grown on N_2 and then supplied ammonia utilizes the ammonia to the exclusion of molecular nitrogen.[1] During investigations on the comparative biochemistry of biological nitrogen fixation, it was discovered that the nitrogen fixing anaerobe *Clostridium pasteurianum* excreted nitrogenous compounds during its active phase of growth.[2] Ammonia with extremely high N^{15} concentration was isolated from the supernatant of these cells when they were supplied N_2^{15} for a short time during the period of logarithmic growth.[3] This isolation provides direct evidence that ammonia is the key intermediate in biological nitrogen fixation.

Further studies revealed that *C. pasteurianum* cells that had been fixing N_2, rapidly utilized added ammonia; excess N^{15} immediately appeared in organic compounds in the cells when the cells were supplied N^{15}-ammonia and normal N_2. These results were similar to those that had been obtained earlier with *Azotobacter*,[4] and indicated that this nitrogen-fixing *Clostridium* had enzymes for utilizing ammonia without a period of adaptation. It would be anticipated that no enzymatic adaptation would be required for utilization of a compound which is a normal intermediate in N_2 fixation. A check on the N^{15} concentration of the supernatant ammonia in these experiments showed that the atom per cent N^{15} of the supernatant decreased rapidly. This unexpected result prompted further investigations, which provide evidence that this organism once it has been fixing N_2 continues to fix N_2 even in the presence of a large excess of ammonia. This behavior helps to explain how *Clostridium* can discard newly formed ammonia while actively fixing N_2.

Experimental.—A 4-liter culture of *Clostridium pasteurianum*, strain W5, was grown anaerobically under N_2 by the methods described by Rosenblum

and Wilson.[2] When the culture was in the log phase of growth and nitrogen fixation, it was divided into 3 equal parts. At zero time, 20 ppm. of N as $N^{15}H_4^+$ containing 34 atom per cent N^{15} excess was added to each flask. N_2 was bubbled vigorously through one, H_2 through the second, and He through the third. At intervals thereafter for 2 hours, 65 ml. samples were withdrawn from each flask and pipetted into 35 ml. of N sulfuric acid. The cells then were heated rapidly to boiling to insure quick inactivation. The cells were centrifuged, washed with 30 ml. of a solution of ammonium sulfate containing 80 ppm. of N to remove or exchange with any occluded

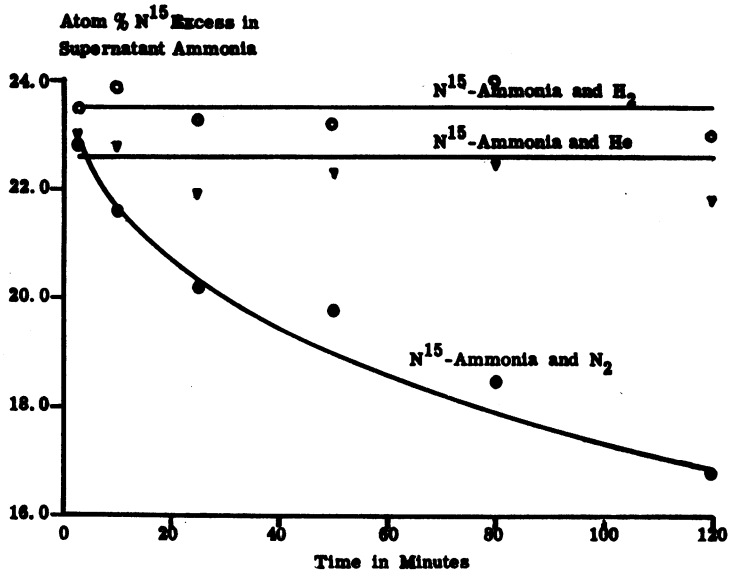

FIGURE 1

Decrease in atom per cent N^{15} of added N^{15}-ammonia by *Clostridium* also supplied N_2. Nitrogen fixing cells in the log phase of growth were given the gas indicated and 20 ppm. of N as N^{15}-ammonia.

ammonia, and then the ammonia was distilled. The supernatant medium was made alkaline, and its ammonia was distilled and recovered. The residual supernatant contained the non-ammonia supernatant nitrogen. All analyses for N^{15} were made in duplicate with a Consolidated-Nier isotope-ratio mass spectrometer, and the average of the determinations is shown in the figures.

Analyses of supernatant ammonia are plotted in Figure 1. Normal ammonia, present in the supernatant and soluble portion of the cells, diluted the ammonia added to an initial concentration of about 23 atom per cent N^{15} excess. In the presence of H_2 or He the N^{15} atom per cent

excess was constant. The He control indicated that H_2 had no specific effect on the phenomenon. In the presence of N_2, the N^{15} concentration fell from 23 to 17 atom per cent excess in 120 minutes.

Figure 2 represents the change in the ammonia-N fraction during the course of the same experiment. The supernatant ammonia-N remained constant within the error of analysis (calculation by isotope dilution indicates that about 2.5 mg. ammonia-N per liter disappeared) when either He or H_2 was bubbled through the medium containing $N^{15}H_4^+$. However, in the flask in which N_2 was bubbled through the culture, there was a marked increase in ammonia-N. This increase in ammonia-N accounted for the decrease in N^{15} concentration of the supernatant ammonia within 2%. Apparently, in spite of the addition of ammonia, nitrogen fixation

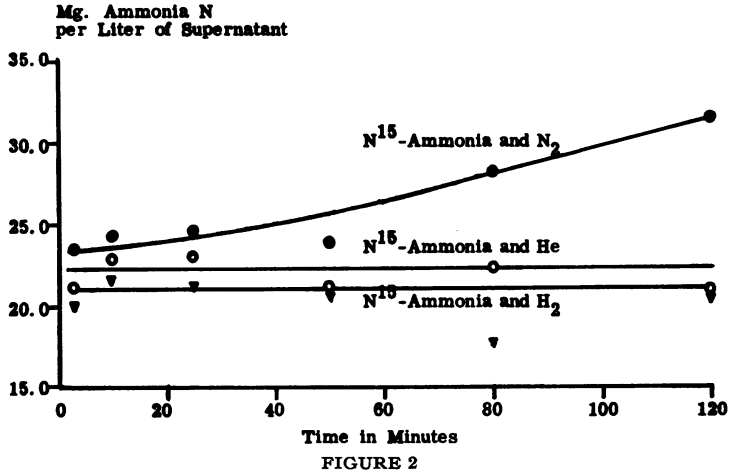

FIGURE 2

Increase in total ammonia-N in a culture of *Clostridium pasteurianum* in the presence of added ammonia and N_2.

still was taking place and was rapidly producing non-labeled ammonia. The rest of our data confirm this conclusion.

Figure 3 shows the concentration of N^{15} in the cells. The rate of assimilation of N^{15} was much more rapid in the presence of He and H_2 than in the presence of N_2. This would be expected if the added $N^{15}H_4^+$ were competing with the normal ammonia formed in the cells by nitrogen fixation in the culture supplied N_2.

Figure 4 illustrates the increase of N^{15} in the non-ammonia supernatant nitrogen. The higher content of the labeled nitrogen in this fraction in the presence of H_2 or He provides additional evidence that utilization of ammonia and fixation of N_2 are occurring concomitantly in the culture given N_2.

As a further test of the hypothesis that nitrogen fixation can take place in these cells in the presence of excess ammonia, two 1-liter cultures of *C. pasteurianum* were grown side by side. To one was added 200 ppm. of N as ammonium sulfate, more than the cells could assimilate during their growth, but no additional ammonia was added to the second. N_2 was bubbled through both flasks in series. When both cultures were in the log phase of growth, the gas was removed and replaced by 0.2 atmosphere of

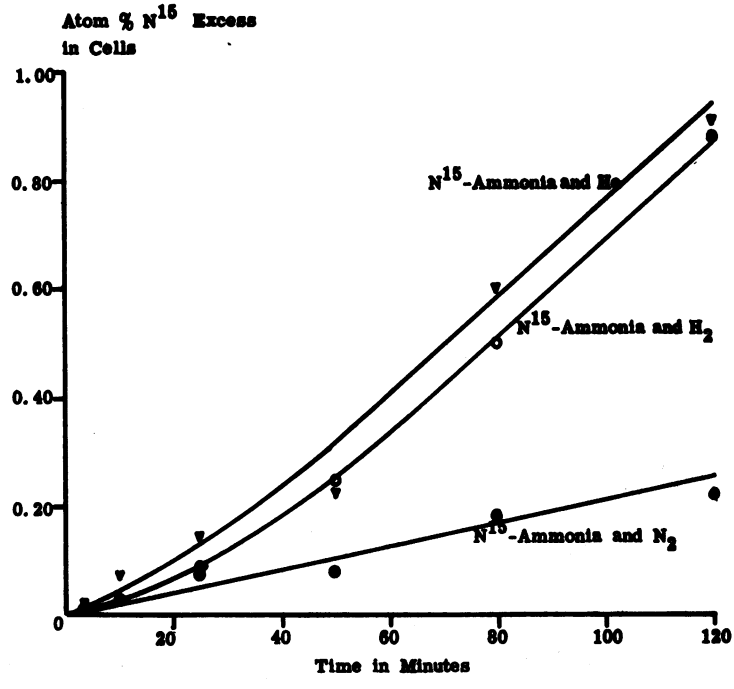

FIGURE 3

Decrease in rate of assimilation of N^{15} from N^{15}-ammonia by *Clostridium pasteurianum* supplied N_2 compared with cultures supplied He or H_2.

N^{15}-enriched N_2 (the N_2 contained 32 atom per cent N^{15} excess) and 0.8 atmosphere of He. At the same time, 20 ppm. of N as normal ammonium sulfate was added to the culture grown without added ammonia to insure an excess. The gases were bubbled vigorously through both flasks in series with a Vanton pump,[5] then samples were withdrawn from each flask at intervals and analyzed for their N^{15} concentration.

Figure 5 shows the results of this experiment. Only the cells that had been fixing N_2 continued to fix N_2 in the presence of an excess of added ammonia. Much of the nitrogen fixed was excreted rapidly, but 20 ppm. of

normal ammonia-N had been added, hence the figures for N^{15} content shown in curve *A* have been lowered by dilution. This is consistent with our earlier observation that excreted ammonia had a phenomenally high N^{15} concentration.[3]

Discussion.—There now appears to be little doubt that ammonia is a key intermediate in biological nitrogen fixation, both aerobic and anaerobic, but unlike *Azotobacter*, *Clostridium* can fix N_2 in the presence of an excess of ammonia. This fact serves to explain how *Clostridium* can excrete am-

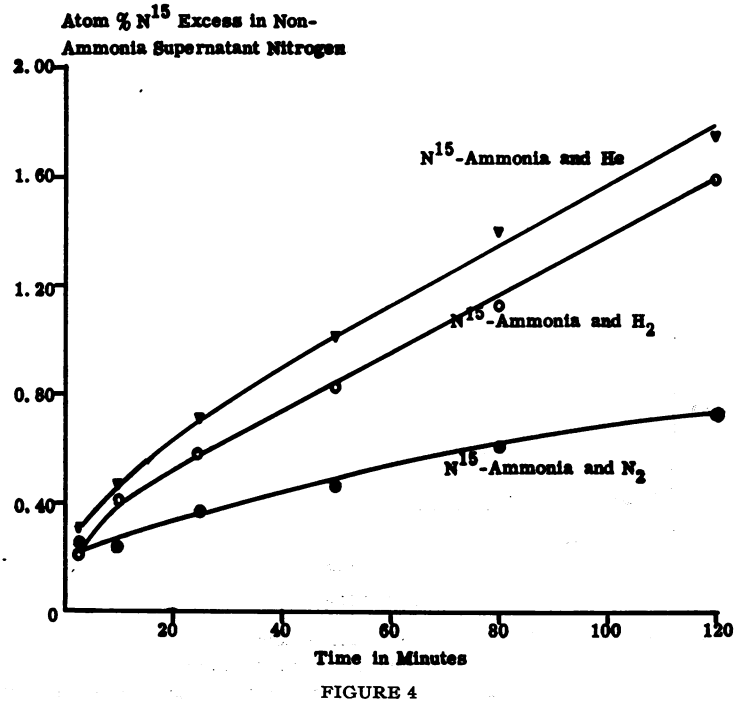

FIGURE 4

Decrease in rate of assimilation of N^{15} from N^{15}-ammonia into the non-ammonia supernatant N of a culture of *Clostridium pasteurianum* supplied N_2 as compared with cultures supplied He or H_2.

monia during active fixation of N_2. Evidently the process of N_2 fixation in *Clostridium* does not limit the rate of over-all growth, which may be limited by such factors as the quantity of ammonia acceptors produced by the organism. *Azotobacter*, however, with its vigorous aerobic respiratory system can utilize ammonia as rapidly as it is formed by the nitrogen fixing process, and there is no appreciable excretion of ammonia during active N_2 fixation.

Summary.—*Clostridium pasteurianum* cells that are fixing N_2, immedi-

ately accept ammonia as a source of nitrogen without a period of adaptation. This indicates that enzymes required for the utilization of ammonia are present in nitrogen fixing cells, and that ammonia is a normal intermediate in nitrogen fixation. When such cells are provided $N^{15}H_4^+$ and normal N_2, a substantial conversion of N_2 to ammonia occurs.

Clostridium cells that have been fixing N_2 continue to fix N_2 in the presence of added ammonia. Cells that have been growing on ammonia lack the ability to utilize N_2, and this is suggestive that nitrogen fixation itself

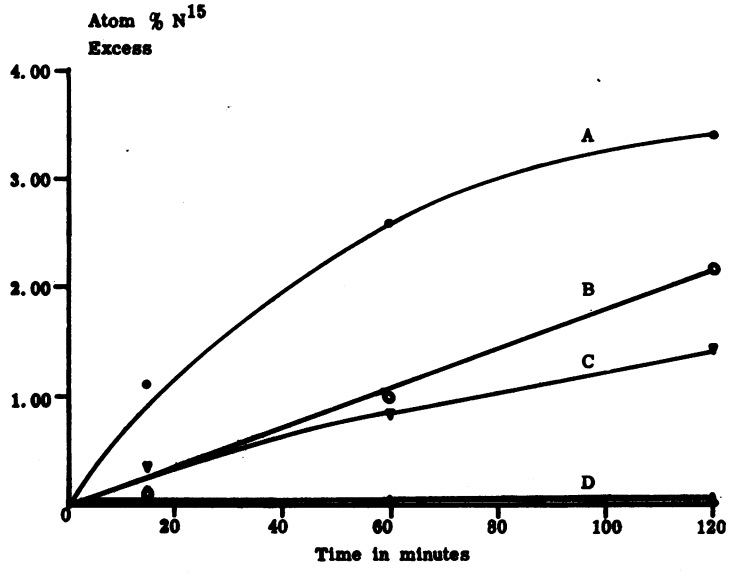

FIGURE 5

Fixation of N_2^{15} by *Clostridium* in the presence of excess added ammonia. Curve *A*—Supernatant ammonia from cells grown without added ammonia to which 20 ppm. of ammonia-N was added when N_2^{15} was supplied at zero time. Curve *B*—Cells from same experiment as curve *A*. Curve *C*—Non-ammonia supernatant nitrogen from same experiment as curve *A*. Curve *D*—Cells previously grown with 200 ppm. of N as ammonia, and N_2^{15} supplied at zero time. Curve was the same for supernatant ammonia, cells, and non-ammonia supernatant nitrogen.

is an adaptive phenomenon. These findings have been demonstrated with $N^{15}H_4^+$ and normal N_2 and with normal ammonia and N_2^{15}. The data reported here and the observation that *Clostridium* can excrete nitrogen while growing actively, help explain how it was possible to isolate ammonia with a phenomenally high N^{15} concentration in the supernatant of actively growing *C. pasteurianum*.

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[1] Wilson, P. W., Hull, J. F., Burris, R. H., PROC. NATL. ACAD. SCI., **29**, 289–294 (1943).

[2] Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **59**, 83–91 (1950).

[3] Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., *J. Biol. Chem.*, **191**, 295–298 (1951).

[4] Burris, R. H., and Wilson, P. W., *J. Bact.*, **52**, 505–512 (1946).

[5] Manufactured by Vanton Pump Corp., Empire State Building, New York, N. Y.

THE VIRUS OF VACCINIA IN CHICK EMBRYO MEMBRANE

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In any broad study of the way viruses grow within infected cells, the poxes are important as a group of closely related agents which can attack the same or related tissues. Several of them can be grown on chick-embryo membranes. In an earlier paper[1] it has been shown that the individual virus particles of fowl pox can readily be seen and recognized using the electron microscope. The present note is a record of preliminary studies made to determine how easy it is to observe the elementary particles of vaccinia in these membranes and to see if these observations give an obvious clue to the way this virus proliferates.

As in the preceding experiments with fowl pox a dilution of bacteria-free virus was placed on a dropped chorioallantoic membrane of a ten-day embryo, the shell opening was closed with scotch tape and the incubation continued. Vaccinia grows so freely on these membranes that best preparations have been obtained from embryos harvested 48 and 72 hours after inoculation. Areas of obvious pox development on the excised membranes have been chosen, fixed by immersion for not more than one hour in neutral 4% formalin-saline, dehydrated by passage through alcohols and into methacrylate monomer. In this they were embedded by polymerization in a 40°C. oven. Sections cut by the thermal expansion method[2] were mounted on formvar-covered grids, the methacrylate was removed by immersion in amyl acetate and the resulting tissue lightly shadowed with palladium.