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Investigation of nitrogen fixation by three species of lichen

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INVESTIGATION OF NITROGEN FIXATION BY THREE SPECIES OF LICHEN

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INTRODUCTION

Since the appearance of the classic work of Schwendener in 1867, lichens have been studied with great interest. The aspects most often studied have been taxonomy, morphology, and anatomy. However, during the last four decades lichen physiology has also been studied in considerable depth. Now texts on lichen chemistry and physiology are available, whereas before 1940 the majority of the lichen texts dealt mainly with taxonomy and morphology.

Another area of lichenology which has also been extensively studied is lichen ecology. Lichens are accepted by botanists as being of major importance as pioneer plants, capable of growing on bare rock. They are known to secret polyhydroxypolycarboxylates, which are the most powerful, naturally occurring, chelating agents known (11).

Algal physiology has been studied in some detail and a few species of one group, the Cyanophyceae, have been shown to fix atmospheric nitrogen (7, 8, 12, 16). Although these species of Cyanophyceae have been shown to fix nitrogen in the free state, it is not known whether these species carry on this process while functioning as phycobionts (term proposed by Scott (14) which means the algal components of a lichen). Some of the lichens with a blue-green algal component, e.g., Nostoc, have been shown to fix nitrogen when in the lichen association. (10, 12, 13). However, with the exception of Lecidea crystalefera and Parmelia conspera, for which marginal fixation has been shown (10), green algae are not known to fix nitrogen in either the free or lichenized

forms. This being the case, one wonders why Collema wyomingensis with nitrogen-fixing Nostoc as its phycobiont, and Caloplaca elegans with Trebouxia (Chlorophyceae) as its phycobiont, both live on rocks. Proof that lichen phycobionts can fix nitrogen would help explain how these lichen plants can grow on rock, thereby enhancing their role as pioneer plants.

Nitrogen fixation studies have been greatly facilitated since Burris and Miller (4) published their techniques for using heavy nitrogen to detect fixation. Scott and Bond (12) performed the first experiments on nitrogen fixation by lichens, using the techniques of Burris and Miller. In 1966 Rogers, et al., (10) used the method of Scott and Bond on eleven different species. Scott and Bond (12) and Scott (13) and Rogers, et al., (10) together investigated a total of fifteen lichen species to determine their relative abilities to fix atmospheric nitrogen.

After a review of the literature and after having received personal communication from four leading lichenologists (2, 5, 8, 15), the author concluded that very little experimental work has been done on lichen fixation of atmospheric nitrogen. Because fifteen lichen species from nine genera was not considered to be an adequate sampling of the 66 recognized lichen genera (1), and in view of the problem stated above concerning nitrogen fixation by free-living versus lichenized algae, the author proposed that additional lichens be tested for their ability to fix atmospheric nitrogen.

MATERIALS AND METHODS

The materials and methods used in this study were patterned after those developed by Burris and Miller (4) and Scott and Bond (12) and Scott (13). The research was divided into three major areas of study as follows: (1) collecting and identifying the lichen species to be used in this research, (2) preparing and attaching the lichen specimen onto a capillary manifold system for introduction of N^{15}, and (3) analyzing the lichen specimen by a modified micro-Kjeldahl procedure and mass spectrometry for detection of nitrogen fixation.

Collection and Identification of Species

Single species of lichens were selected for this study from each of three different families, (Collema wyomingensis , Caloplaca elegans (Link)T. Fries, and Parmelia molliuscula Ach.) These species were selected so that both green and blue-green phycobionts were represented, as well as non-rock and rock-inhabiting forms. The lichen Collema wyomingensis was selected because its phycobiont is Nostoc (9), a blue-green alga which is a known nitrogen fixer (16). Data obtained from Collema samples was used to help validate the techniques employed in this study. The lichen Parmelia molliuscula was chosen because its phycobiont is Trebouxia, a green alga, and because it is abundant in Utah (6). Parmelia molliuscula Ach. is identical with P. chlorochroa Tuck. (6). Currently many lichenologists think that this is only a vagrant form of P. conspersa which has become detached from rocks and

blown about by the wind. Previous work (10) indicated that P. conspersa gave marginal fixation of nitrogen and if P. conspersa and P. molliuscula are the same they should give the same results. Caloplaca elegans was also chosen because it has a green algal component, Trebouxia, and because it is abundant in Utah (6). Furthermore, these species were chosen for study because C. wyomingensis and C. elegans are rock-inhabitors and P. molliuscula is a non-rock inhabitor.

Samples of C. wyomingensis were collected on February 25, 1967, at Johnston's Pass, Tooele Co., Utah at an elevation of 6,400 feet. The thalli, which were greenish to blackish in color, were scraped from pitted, granite cliffs. The thalli were lobed and varied in shape from round to irregular and were attached by rhizoids.

Collections of P. molliuscula were made March 5, 1967, from the desert soils in Antelope Valley, Millard Co., Utah at an elevation of 6,200 feet. Thalli were 1-1.5 inches long, procumbent, subfruticose, straw-colored to light green, and the lobes were narrow and repeatedly branched and convex with recurved margins. The undersides were brownish and contained rhizoids.

On March 5, 1967, C. elegans was also collected in Antelope Valley. Rocks with thalli on them were brought to the laboratory. The thalli were subfoliose, orange to yellowish-red colored above, white below, and the margins were stellately lobed, the lobes were long and branched. All collections were transferred to the Cluff Plant Science Laboratory (CPSL) at Brigham Young University. P. molliuscula and C. wyomingensis were kept in unsealed plastic bags and C. elegans was left on the rocks in an open box. They were not watered until March 23, 1967.

Preparation of Lichens and The Gas Manifold System

Preparation of Lichens

On March 23 C. elegans was scraped from the rocks and all three species were washed thoroughly with distilled water to remove surface contamination of dust and bacteria.

On March 23 the lichens were removed from the CPS laboratory and placed in a warm, moist room of the greenhouse on paper towels. Periodically, these towels were soaked with water in order to subject the lichen thalli to alternating periods of wet and dry. In this manner the lichens were kept in their composite form without the algal or fungal component overgrowing the other partner (1).

On April 17 seven 1.0-g samples of each species were weighed out. Glass wool (about 5 ml) was placed in each of 21 glass tubes of 20-ml capacity. The tubes, transfer spatula, and glass wool had been previously autoclaved to insure freedom from contamination. The glass wool was then saturated with 0.5 M nitrogen-free Hoaglands nutrient solution which, according to Scott (15), was satisfactory for the normal growth of lichens. One lichen sample was placed in each tube and the tubes were ready to be sealed onto the gas manifold system.

Gas Manifold System

A flow chart of the manifold system used for this study is shown in Fig. 1. The manifold system was an adaptation of the one used by Scott and Bond (12) and Scott (13) and was constructed to allow for evacuation of the specimen tubes and rapid introduction of an N^{15}-

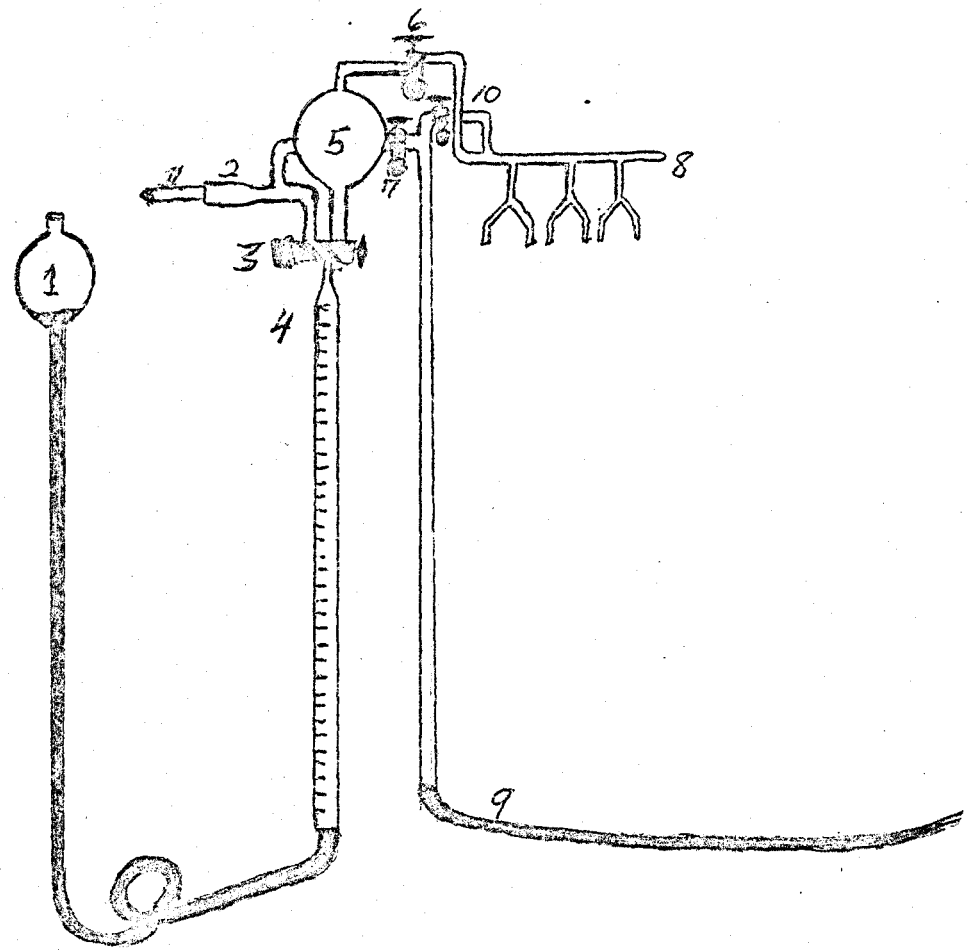

Fig. 1. Gas Manifold System. 1) leveling bulb, 2) inlet tube, 3) three-way stopcock, 4) 50-ml buret, 5) mixing bulb, 6) two-way stopcock, 7) two-way stopcock, 8) capillary manifold, 9) hose to pump, 10) two-way stopcock, 11) 4-inch filter tube.

enriched atmosphere into the tubes.

The apparatus was all glass except for some Tygon rubber tubing. The leveling bulb (#1) was 500 ml and contained 15 lbs. of mercury. The buret (#4) was a standard 50-ml buret with thick-walled tubing connecting it to the leveling bulb. The capacity of the mixing bulb (#5) was 302 ml. The extension arms of the manifold were constructed of 2-mm capillary tubing and the connecting arm of the manifold was 3-mm capillary tubing (#8). The connections from points 2 to 3, 3 to 5, 5 to 6, 2 to 7, 7 to 10, and 7 to 9 were standard glass tubing. The inlet socket (#2) had a 10/30 $\overset{T}{S}$ joint and had a 4-inch tube (#11) which fitted into it. This 4-inch tube was packed with glass wool and served as a filter. Stopcock 3 was a 3-way stopcock stopcocks 6, 7, and 10 were 2-way stopcocks.

The CO_2, oxygen, and nitrogen went into the buret more easily and safely if the mercury level was allowed to drop a little faster than it would have if the gas pressure alone was forcing it down.

Preparation of N^{15}-enriched Atmosphere

In order to maintain the volume and pressure needed to fill three sets of six tubes each, 408 ml of gas at 650 mm Hg pressure had to be forced from the buret into the mixing bulb. The mixture desired was the following: 70% nitrogen with 22 atoms % N^{15}, 25% oxygen, and 5% carbon dioxide. It was obtained in this manner:

Step 1- The mixing bulb was evacuated by opening stopcocks 6 and 10 while operating the mechanical pump connected at 9. Stopcock 6 was closed.

Step 2- A small amount of crushed Dry Ice was placed in a 25-ml

vacuum filter flask, stoppered, with its side arm connected to the inlet tube with a rubber hose. Stopcock 7 was closed. Stopcock 3 was opened and CO_2 went into the buret. Stopcock 3 was closed and the CO_2 flask was removed. Stopcock 3 was reopened and the air in the buret was forced out by raising the leveling bulb. When the mercury reached the stopcock it was quickly closed. This procedure was repeated four times in order to flush the air out of the buret and insure the purity of the CO_2 entering the mixing bulb. The fifth time 21 ml of CO_2 at 650 mm Hg pressure was forced to flow into the mixing bulb by turning the stopcock 3 in the opposite direction and allowing the mercury level to rise up to the stopcock. Stopcock 3 was closed.

Step 3- With the mercury level up to stopcock 3, the pressure gauge on the oxygen tank was set at 3 lbs./sq. in. and the oxygen line was connected to the inlet tube via the filter tube. With stopcocks 3, 6, and 10 closed and stopcock 7 open, the inlet and filter tubes were evacuated; stopcock 7 was closed. The oxygen valve was opened; stopcock 3 was opened. The amount of oxygen let into the buret was 54 ml at 650 mm Hg pressure. Stopcock 3 was closed. The oxygen valve was turned off and stopcock 3 was turned to allow the first portion of oxygen to pass into the mixing bulb. The mercury level was raised up to stopcock 3 to empty the oxygen from the buret into the mixing bulb. The above procedure was repeated, exclusive of evacuating the inlet and filter tubes, to provide a second portion of 49 ml of oxygen at 650 mm Hg pressure.

This portion of oxygen was also forced into the mixing bulb, making a total of 103 ml of oxygen measured at 650 mm Hg pressure.

Step 4- With the mercury level up to stopcock 3 the nitrogen line was connected to the filter tube with the pressure on the tank set at 3 lbs./sq. in.. With stopcocks 3, 6, and 10 closed and stopcock 7 open, the inlet and filter tubes were evacuated. Stopcock 7 was closed, the nitrogen valve was opened and then stopcock 3 was opened. There was 54 ml of nitrogen at 650 mm Hg pressure in the buret when stopcock 3 was closed. The nitrogen valve was closed. Stopcock 3 was turned to allow this first portion of nitrogen to go into the mixing bulb. The mercury level was raised up to stopcock 3 to empty the nitrogen from the buret into the mixing bulb. This procedure was repeated four more times, exclusive of evacuating the inlet and filter tubes. Portions of nitrogen equaling 54, 54, 54, and 18, ml respectively were forced into the mixing bulb. This made a total of 234 ml of nitrogen measured at 650 mm Hg pressure.

Step 5- The N^{15} used was 95 atoms % pure. There was 50 ml in a break-seal ampule at 698 mm Hg pressure. The ampule was connected to the inlet tube (the ampule had a 10/30 $\overline{\text{S}}$ joint) and the inlet tube was evacuated. The break-seal was broken with a metal slug and a magnet. The magnet on the outside caused the slug in the neck of the ampule to slam against the seal. Because the N^{15} had been bottled at 698 mm Hg and there was no way to force it out, the N^{15} had to be "pulled" out by

opening stopcock 3 and dropping the mercury down to the bottom of the buret. The mercury was then used to push the N^{15} into the mixing bulb. This procedure was followed nine times in order to get as much out of the ampule as possible.

By following the above steps 408 ml of gas measured at 650 mm Hg pressure was transferred from the buret to the 302-ml mixing bulb, resulting in a gas pressure of 887 mm Hg pressure.

Introduction of N^{15} Atmosphere

With the gas mixture ready, six specimen tubes were fused by heat onto the manifold. With stopcocks 3, 6, and 7 closed and stopcock 10 open, the manifold and the specimen tubes were evacuated to less than 5 mm Hg pressure. Stopcock 10 was closed and stopcock 6 was opened. The gases were allowed to equilibrate in the mixing bulb-manifold area for 15 minutes. Stopcock 6 was closed and the tubes were sealed off and removed. The second set of six tubes was sealed on by heat and evacuated to less than 5 mm Hg pressure. With stopcocks 6 and 10 closed and stopcock 3 open, the leveling bulb was raised along the meter stick until 82 ml of mercury had entered the mixing bulb. Stopcock 3 was closed and stopcock 6 was opened. After 15 minutes stopcock 6 was closed and the tubes were sealed off and removed.

The final six tubes were sealed on and evacuated. Stopcocks 3 and 6 were opened after stopcock 10 was closed. An additional 201 ml of mercury was forced into the mixing bulb (the bulb contained a total of 283 ml of mercury). Stopcock 3 was closed. Stopcock 6 was closed and the tubes were sealed off and removed.

All 18 tubes, plus one control tube for each species, were placed

in a Percival E 57 Environator growth chamber set for alternate twelve hour periods of light and dark. During the light hours the light intensity at the level of the specimen tubes was 1000 foot candles and the temperature was 25 degrees C. During the dark period the temperature was 18 degrees C. According to Ahmadjian (1) most lichens grow best within this temperature range. The incubation period began at 6 o'clock p.m. April 17, and ended at 12:30 p.m. April 24. According to Scott (13) this length of time is adequate to detect fixation and any longer period might have allowed the tissue to deteriorate.

At the termination of the incubation period the tubes were stacked in a box with Dry Ice and placed in a deep freeze in order to quickly stop all chemical activity and preserve the tissue. They were kept there until they were removed for analysis.

Analysis

A modification of the micro-Kjeldahl apparatus described by Steyermark (17) was used to digest the samples and convert the tissue nitrogen to ammonium sulfate. Figure 2 shows a diagram of the apparatus used. This arrangement was designed by the author because of the unavailability of a functional micro-Kjeldahl rack. The titration portion of the Kjeldahl analysis was not carried out because the determination of the quantity of nitrogen per sample was not the objective.

The samples were dropped into 100-ml Kjeldajl flasks, each of which contained a pinch of mercuric oxide and 1-2 g of potassium sulfate (reagent grade). Approximately 7 ml of concentrated sulfuric acid was added to each flask and the flasks were attached to the ringstand. Each ringstand held four flasks and was placed in a hood. Regular bunsen

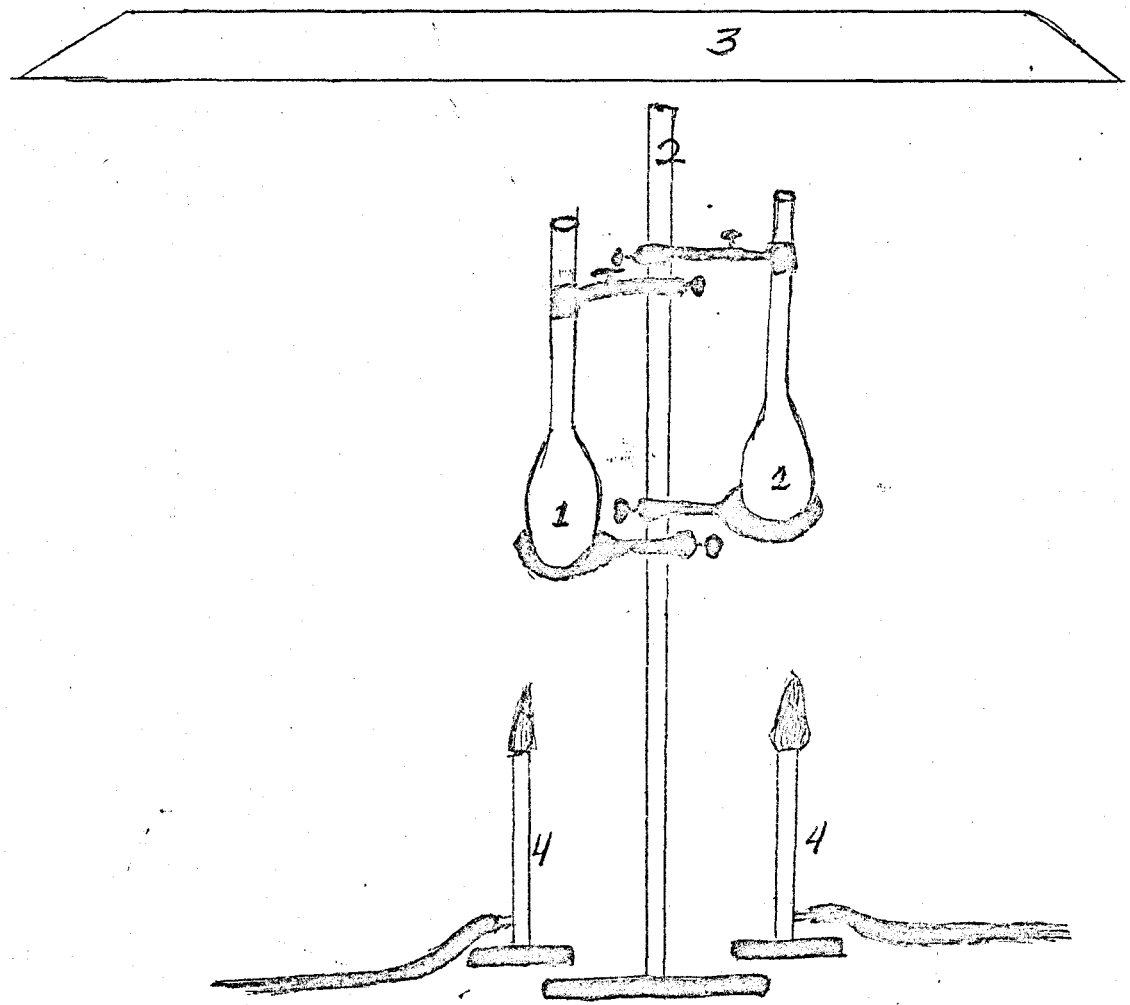

Fig. 2. Modified Micro-Kjeldahl Apparatus. 1) 100-ml Kjeldahl flasks, 2) ringstand, 3) hood, 4) bunsen burners.

burners with low flames were used. To facilitate the clearing of the digest, drops of superoxol (30% H_2O_2) were added slowly to each flask. This caused rapid combustion of the charred carbohydrate material in the digest mixture, making it easier to tell when digestion was completed.

When a digestion mixture turned creamy-clear that flask was removed, stoppered, and placed in a refrigerator set at 2 degrees C. All flasks were stored until June 19, 1967.

The author, with the help of John Wing (19), designed the apparatus shown in Fig. 3. This apparatus was used to convert ammonium sulfate to ammonia and from ammonia to free nitrogen. The free nitrogen was collected in evacuated collection bulbs. Eight 15-ml bulbs with a capillary stopcock and a 12/30 $\overline{S}$ joint (#6) had been made by the glassblower at Brigham Young University. The portion of the quartz tube in the furnace (#3) was packed with cupric oxide (#4) and the rest was filled with phosphorus pentoxide (#5). A Y-tube connected the quartz tube to the collection bulb hose and to the aspirator trap.

The following procedure was used:

Step 1- The system was attached to the aspirator and evacuated as much as possible. After 10 ml of distilled water was added to a digest flask, a 4-ml capsule of 50% NaOh was dropped into the mixture and the flask was IMMEDIATELY stoppered with the free stopper (#2). The screw clamps were closed off (the aspirator was still running) and the capillary stopcock was opened to the collection bulb. The ammonium sulfate reacted with the 50% NaOH to free the ammonia.

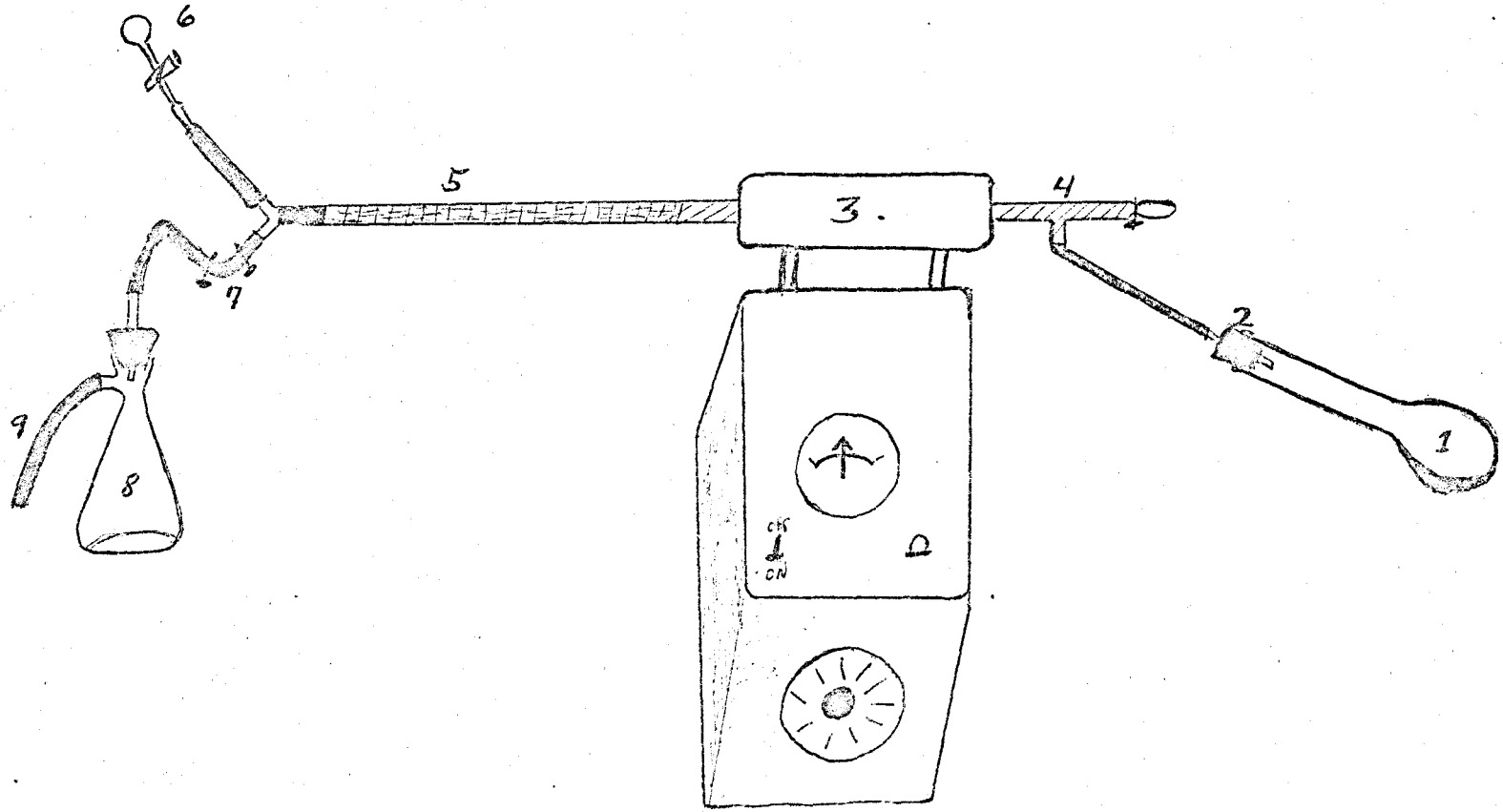

Fig. 3. Ammonium Sulfate Conversion Apparatus. 1) 100-ml digest flask, 2) free stopper, 3) heating unit of the micro combustion furnace, 4) cupric oxide end of the quartz tube, 5) phosphorus pentoxide end of the quartz tube, 6) collection bulb, 7) screw clamps, 8) aspirator trap, 9) hose to the aspirator.

Step 2- The ammonia passed into the quartz tube and through the section of red-hot cupric oxide (482 degrees C.). At this point the hydrogen atoms were stripped from the nitrogen.

Step 3- The free nitrogen flowed through the phosphorus pentoxide and up into the collection bulb. The stopcock was closed and the bulb was removed.

Step 4- Another evacuated bulb was attached and the screw clamps were opened so the system could be evacuated. Water was added to another flask and the procedure as described in steps 1, 2, and 3 above was followed. Processing time for each sample was approximately 10 minutes.

The mass spectrometric analyses were conducted at the University of Utah. The instrument is the property of the Chemistry Department and is under the direction of Dr. Austin L. Wahrhaftig. It is a model CEC 21-110 double-focusing, high resolution mass spectrometer. The manufacturer is Consolidated Electrodynamics Corporation of Pasadena, California.

RESULTS

The results of the mass spectrometric analyses are given in Table 1. Two peaks for N^{14} and two for N^{15} were selected from the graph of each sample. The height of each peak was measured in millimeters and the average of the two N^{14} peaks was multiplied by the attenuation factor. The product was divided into the average of the N^{15} peaks to obtain a ratio of N^{15} to N^{14}. The quotient obtained was multiplied by 100 to convert the ratio into per cent.

Table 1. Results of Mass Spectrometric Analyses.

Sample	N^{14} peak in mm x atten. factor*	N^{15} peak in mm x atten. factor* of 1	ratio N^{15}/N^{14}	%
C. wyomingensis				
1 control	71 x 100	58 x 1	0.0081	0.81
2	87 x 30	20 x 1	0.0076	0.76
3	65 x 30	15 x 1	0.0077	0.77
4	83 x 30	20 x 1	0.0080	0.80
5	111 x 100	89 x 1	0.0080	0.80
6	93 x 100	67 x 1	0.0072	0.72
C. elegans				
1 control	95 x 100	74 x 1	0.0077	0.77
2	75 x 100	57 x 1	0.0076	0.76
3	61 x 100	44 x 1	0.0072	0.72

Table 1. (continued)

Sample	N^{14} peak in mm x atten. factor*	N^{15} peak in mm x atten. factor* of 1	ratio N^{15}/N^{14}	%
4	60 x 100	43 x 1	0.0072	0.72
5	77 x 100	55 x 1	0.0072	0.72
6	50 x 100	39 x 1	0.0078	0.78
P. molliuscula				
1 control	91 x 100	74 x 1	0.0081	0.81
2	73 x 30	20 x 1	0.0091	0.91
3	91 x 100	69 x 1	0.0075	0.75
4	101 x 100	71 x 1	0.0070	0.70
5	94 x 100	71 x 1	0.0076	0.76
6	82 x 100	62 x 1	0.0076	0.76
tank nitrogen	100 x 100	78 x 1	0.0078	0.78
air	101 x 100	55 x 1	0.0074	0.74
"	79 x 100	55 x 1	0.0070	0.70
"	93 x 100	83 x 1	0.0080	0.80

*The attenuation factor is the number of times the true height of a peak is decreased, or attenuated, in order to fit on the graph paper.

For a comparison of the oxygen content, four samples were scanned for O_2^{16}. The ratio of the oxygen peak to the N^{14} peak in "air" samples was 7900 mm/ 1800 mm or 22%. The ratio of the oxygen peak to the N^{14} peak in C. elegans #3 and #6 was 6000 mm/ 1100 mm and 5000 mm/ 1100 mm or 22% and 18%, respectively.

DISCUSSION

An interpretation of the results leads to the conclusion that there was very little or no N^{15} fixation by any of the three species of lichen used in this study. The range of % N^{15} in the sample was from 0.91 to 0.70 which is not sufficient to prove fixation since the controls ranged from 0.70 to 0.81. These results were not unexpected for C. elegans and P. molliuscula because their phycobiont is a green alga, Trebouxia, but lack of evidence for fixation by C. wyomingensis with Nostoc as its phycobiont was unexpected because fixation has been shown for other species of Collema (13) and for nonlichenized Nostoc (16).

Lichens are very slow growing plants and would be expected to fix only a small amount of atmospheric nitrogen in one week. There was no reason to suspect that the growth rates of the three lichens used in this study were any different than the growth rates of lichens in general. Scott (13) claims that five to seven days of exposure is long enough to detect N^{15} fixation. Therefore, it was assumed that the three species tested in this study were very slow growing and would fix only small amounts of N^{15} during the seven days of exposure to the N^{15}-enriched atmosphere, but the amount of N^{15} fixed should have been enough to detect.

With the knowledge that N^{15} fixation would have been small there are two possible explanations for the results obtained. First, the three lichens tested did not fix atmospheric nitrogen either because they were not genetically capable of doing so, or because the experi-

mental conditions damaged them so that no N^{15} fixation occurred. Second, the amount of N^{15} fixed by any of the species was so small that the equipment and techniques used in this study were not adequate to detect the fixation.

The time of year the lichens were collected could be one reason why no fixation was detected. The peak of the growing season for lichens is late spring and early summer (1). The collections were made late February and early March. Due to complications with the gas manifold system the lichens were not exposed to the enriched atmosphere immediately, but were kept at room temperature until April 17, which would be about when they would normally begin their most active growth. Similar studies in the future might yield better results if collections could be made in the late spring and exposed to an enriched atmosphere within three or four days. Any increase in growth rate the lichens might have had due to the warmer temperatures in the laboratory and greenhouse may have been offset by the length of time they were subjected to artificial conditions, even though efforts were made to simulate field conditions.

The glass wool in the specimen tubes was saturated with nitrogen-free nutrient solution. This was the technique used by Scott and Bond (12) and Scott (13). After the tubes were sealed and placed in the growth chamber the relative humidity was very high. During the light hours droplets of moisture accumulated on the inside walls of the tubes. The natural habitats of these lichens are much drier than this. Perhaps the large increase in humidity in the tubes over that to which the lichens were accustomed affected their growth and the fungal-algal relationship. A relative humidity nearer to the humidity level of the natural habitats might be necessary before fixation can take place.

The light intensity in the growth chamber at the level of the specimen tubes was approximately 1000 foot candles. This is about one-tenth of the brightness of full sunlight. More light might be necessary for normal growth of lichens, but 1000 foot candles is sufficient for normal growth of higher plants. It is doubtful that this level of light intensity had any adverse affect on the lichens.

The gas manifold used by Scott and Bond (12) and Scott (13) was modified for use in this study by having the glassblower seal the specimen tubes on by heat. Scott connected the specimen tubes to the capillary manifold with rubber stoppers. The author was aware that the rubber stoppers allowed for a greater possibility of loss of N^{15} by diffusion. Therefore, the decision was made to seal the tubes onto the manifold and to seal them closed for the incubation period in order to eliminate the possibility of N^{15} leakage. Warm temperatures are unavoidable when the tubes are fused on with a flame. The warm temperatures within the sealed tubes took a few minutes to drop to room temperature. During this short period of time the lichen thalli may have been damaged.

Burris and Miller (4) assumed that there was no exchange of fixed N^{15} for N^{14} in the atmosphere and reported that their results supported this assumption. However, Broadbent (3) said there was the possibility that exchange could have taken place after the tissue nitrogen was converted to the inorganic ammonium sulfate form. The possibility that some N^{15} could have been lost because of the N^{15} exchanging with atmospheric N^{14} might be given credence except that during the two month waiting period (April 24 to June 19) the ammonium sulfate was kept at 2 degrees C., which would significantly reduce the possibility of exchange of N^{15} for N^{14}.

Although a micro-Kjeldahl procedure was used, the 100-ml flasks used in this study allowed for more than 85 ml of air space above the samples. The conversion system was originally designed so that the flask could be evacuated before the 50% NaOH was dropped into the digest mixture from a separatory funnel. Because the ammonia was escaping up through the funnel it was decided to add the 50% NaOH in a capsule. Due to the volume of the flask and the length of the quartz tube there was not sufficient time to evacuate the flask before the capsule reached the digestion mixture. A comparison of the amount of N^{14} to oxygen in the collection bulbs with the amount of N^{14} and oxygen in the "air" samples suggests that air got into the collection bulbs. Since the quartz tube had been evacuated, the air must have come from the flask. The ammonium sulfate conversion apparatus was constructed with the only quartz tube available and it may have been too long for all the ammonia to get out of the 100-ml flask and all the way to the evacuated 15-ml collection bulb before the bulb was filled with air from the digestion flask.

According to Flowers (6), lichen thalli are approximately 17% nitrogen. Since 1.0 g samples were used, there was 0.17 g of nitrogen per sample. There was approximately 16 ml of gas in each specimen tube at 22 atoms % N_2^{15}, which means there was 0.05 g of nitrogen gas in each tube. Of the total amount of nitrogen in the tubes there was 0.031 g N^{15}, or 14% of the nitrogen in the tube. There was 0.5 ml or 0.0023 g N^{15} in the 85 ml of air in the digestion flask. Assuming there was no exchange of N^{15} for N^{14} between the ammonium sulfate and the air, there would have been 13.5 times as much N^{15} in the digest as there was in the 85 ml of air if fixation had been 100%. At 40% fixation the ratio of N^{15} in the digest to the N^{15} in the 85 ml of air would have been 6.75:1.

At 10% fixation the ratio would have dropped to 1.35:1, or almost 1:1.

Considering the above figures, at any amount of fixation greater than 10%, the increase in N^{15} could have been detected despite the dilution by the 85 ml of air in the flask. If the amount of fixation was less than 10% the increase could not have been detected because there would have been more N^{15} in the 85 ml of air than there was in the sample.

The best way to eliminate this dilution of the fixed N^{15} in future studies would be to replace the 85 ml of air in the digest flask with an inert atmosphere. Replacement of the air with helium would allow for the detection of N^{15} from the sample only. Using this technique, fixation of less than 10% of the N^{15} should be detected.

CONCLUSIONS

Using the materials and methods described in this study, no atmospheric nitrogen fixation was detected for *Collema wyomingensis*, *Caloplaca elegans*, or *Parmelia molliuscula*. Future research involving atmospheric nitrogen fixation by these or other lichens is desirable since the possibility exists that fixation did occur, but was not of sufficient magnitude to be detected with the methods used in this study.

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ABSTRACT

One species of lichen from each of three different families (Collema wyomingensis , Caloplaca elegans (Link)T. Fries, and Parmelia molliuscula Ach.) was studied to determine whether or not these lichens can fix atmospheric nitrogen. Samples of these three lichens were collected in the early spring of 1967. Samples of each species were placed in 20-ml glass tubes which contained 5 ml of glass wool saturated with 0.5 M nitrogen-free Hoaglands nutrient solution. These tubes were sealed by heat onto a gas manifold system and evacuated. An N^{15}-enriched atmosphere (22 atoms % N^{15}) was introduced into the tubes from the gas manifold system. The tubes were sealed off with heat and placed in a growth chamber for seven days with alternate twelve hour periods of light and dark with temperatures of 25 degrees C. and 18 degrees C., respectively. At the end of the incubation period the lichen samples were subjected to a modified micro-Kjeldahl analysis in order to convert the tissue nitrogen to ammonium sulfate. The ammonium sulfate was treated with strong base to release the ammonia, which in turn was dehydrogenated over hot cupric oxide. The freed nitrogen was collected in 15-ml collection bulbs and the contents were scanned in a mass spectrometer for the presence of an increased percentage of N^{15}.

The results showed no fixation of atmospheric nitrogen by any of the three species of lichen. Further research on atmospheric nitrogen fixation by these and other lichen species is desirable because the possibility exists that fixation did occur, but in such small amounts that the fixation was not detected.