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# Aspects of Symbiotic Nitrogen Fixation in Non-leguminous Plants.

Thesis presented by

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for the degree of

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in the

University of Glasgow.

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# C O N T E N T S.

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## General Introduction.

The importance of the fixation of atmospheric nitrogen by biological means in the maintenance of a continuous supply of combined nitrogen for plant life and indeed for life in general, is very great. A balance sheet for the agricultural lands of the United States for 1936, quoted by Fogg (1955) shows an estimated annual loss of 24 million tons of soil nitrogen. This nitrogen is lost in various ways, mainly due to the removal of crops, the leaching of soluble nitrogen compounds from the soil, soil erosion, and the action of denitrifying bacteria. Partially offsetting this annual loss was a gain of 16 million tons, of which 10 were ascribed to fixation by biological agencies, compared with only 3 added as fertiliser or manure. Although these figures still show a net loss of 8 million tons, were it not for the biological fixation, the combined nitrogen resources of the soil would swiftly run down.

The organisms capable of fixing nitrogen are of two main types, free-living ones and those living symbiotically. The first category comprises soil bacteria such as Azotobacter and Clostridium, some photosynthetic bacteria, for example, Rhodospirillum, Chromatium, and Chlorobium, some

blue-green algae, and probably some soil fungi.

Symbiotic nitrogen fixation is immediately associated with the root nodules of leguminous plants. Their importance in agriculture has been realised for centuries. In these nodules there exists a symbiotic union between the plant and the bacterium Rhizobium, the bacterium providing nitrogen for the plant while in turn receiving from it a supply of carbohydrate.

It is much less generally known, however, that certain other Angiospermous plants quite apart from the Leguminosae also bear root nodules. These genera together with their systematic position according to Engler & Diels (1936) are as follows:-

| Genus | Family |
|---|---|
| Myrica | Myricaceae |
| Alnus | Betulaceae |
| Hippophaë | Elaeagnaceae |
| Elaeagnus | |
| Shepherdia | |
| Casuarina | Casuarinaceae |
| Coriaria | Coriariaceae |
| Ceanothus | Rhamnaceae. |

Since, however, these are all woody genera with no immediately obvious agricultural or economic importance, they have been regarded rather as curiosities.

That these nodules also were caused by the invasion of the root cells by a soil micro-organism was soon realised, but, despite much study, the nature of the endophyte has not yet been satisfactorily elucidated. This is mainly due to the fact that an organism, capable of re-infecting the appropriate plant, has not so far been isolated in pure culture. Attempts at identification have thus rested on the appearance of the organism in sections of the nodules. It has been suggested by some workers to be an Actinomycete, others have regarded it as a member of the Plasmodiophorales, a bacterium, or a Hyphomycete. A good review of this literature is given by Hawker and Fraymouth (1951). Today, however, although the majority of workers favour the actinomycetal nature of the endophyte, it still remains a subject of controversy.

Other symbiotic associations proved or claimed to be nitrogen fixing are to be found in the root nodules of some Gymnosperms, the leaf nodules of the Rubiaceae, and certain lichens and liverworts. These associations will not be dealt with here.

Although the existence of nodules on the roots of the above Angiosperms has been recorded since the nineteenth century and much work has been done in restricted circles on their cytology, it is not until very recently that the

physiological significance of these non-leguminous nodules has been rigorously investigated.

In the Botany Department at the University of Glasgow, however, much information has been obtained particularly on the native nodule-bearing species, namely, Alnus glutinosa (L.) Gaertn., Myrica gale L., and Hippophaë rhamnoides L., together with Casuarina, a well known tree species of tropical and sub-tropical regions. By means of growth experiments in water culture, free of combined nitrogen (Bond, Fletcher & Ferguson, 1954; Quispel, 1954; Bond, MacConnell & McCallum, 1956; and Bond, 1957a) and of isotopic tracer experiments involving whole plants and later detached nodules (Bond, 1955; 1957b), the property of fixation of atmospheric nitrogen has been conclusively demonstrated for nodulated plants of the above species. That the nodule was the site of fixation was also shown by the above means. The nitrogen nutrition of these plants has also been extensively studied and it is now clear that these nodules have a similar function to legume nodules.

Evidence is accumulating that fixation in non-legume nodules has an important ecological and biogeochemical role. Many of these plants are widespread in their occurrence although in some countries their distribution has now been severely affected by agriculture. Fossil pollen records show them to have been especially prominent in a

post-glacial era, when Alnus glutinosa is believed to have covered large areas of Britain (Tansley, 1939), Hippophaë likewise in Europe (Walker, 1955), while evidence provided by Good (1947) suggested that at an even earlier time Coriaria was prominent in many regions of the world. Even in the present day with so much land devoted to agriculture these genera are to be found to quite an appreciable extent, typically in poor soils. Indeed alder and bog myrtle grow in acid conditions in which legumes can seldom thrive. Accodding to figures quoted by the Forestry Commission, 24,500 acres of Britain at the very least are covered by pure alder stands, while Myrica gale covers large areas of bogland. Observations by Crocker & Major (1955) at Glacier Bay, Alaska, indicate that under alder thicket, the development of which is a regular stage in the colonisation of the bare areas left by the recession of the glaciers, a considerable build up of soil nitrogen occurs amounting to 55 lb. per acre annually. This, no doubt, facilitates the next stage in succession, the replacement of the alder by Sitka spruce.

Of recent years the soil enriching prppertiés of some of these non-legumes have been exploited. An interesting letter has been received in Glasgow concerning such a scheme by the Pacific Northwest Forest and Range Experiment Station, United States Department of Agriculture.

Their recent studies, in Oregon, have indicated that interplanted Alnus rubra increased the growth of associated Douglas-fir. Over 27 years a 60% increase in soil nitrogen was found for the interplanted stands compared with those having fir alone. A similarly significant increase in foliar nitrogen was also obtained.

It is thus clear that these non-legumes are important in ecology and forestry. Further experimental studies on this group are clearly desirable and Section I of this thesis relates to plant culture studies in which the development and function of the root nodules in Shepherdia, Elaeagnus, Myrica (M. cerifera) and Ceanothus are investigated

A second reason for the importance of these non-legume genera and their nodules lies in the provision of new material for studying the chemical pathway of nitrogen fixation. Isotopic studies in the University of Wisconsin, by Aprison & Burris (1952) using isotopic nitrogen, $^{15}N$, have shown excised legume nodules to be capable of continuing active fixation for several hours after detachment from the plant. Such excised nodules provide a much simpler and more convenient medium than whole plants in which to study the mechanisms of symbiotic fixation. The information obtained by further isotopic study of these detached legume nodules at Wisconsin will be reviewed later in the thesis.

The observations of Bond (1955, 1957b) showed that fixation persists to a notably greater degree in non-legume nodules after detachment from the plant, than in legume nodules. Hence not only do these nodules present yet another nitrogen fixing system for biochemical study, they also provide more favourable material than previously studied. Experiments to be described in Section II of the thesis were designed to take advantage of the opportunity thus presented.

# SECTION I.

## PLANT CULTURE STUDIES.

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## General Cultural Methods.

The same basic techniques of plant culture were employed throughout with the various genera to be considered in this Section. The plants were all grown from seed in the Botany Department greenhouses. The seed was sown either in washed sand moistened with diluted nitrogen-free culture solution, or in horticultural peat moistened with tap water. Experience has shown that the nodule endophytes in these non-legume genera, unlike the legumes, are rarely seed-borne. Thus surface sterilisation of the seed is unnecessary in order to gain control over the subsequent incidence of nodule development.

### Water culture Techniques.

The seedlings, either when the cotyledons had expanded or at the two-leaf stage (*Myrica*), were transplanted into water culture of non-aseptic type. Water culture has previously been proved an ideal medium for the growth of those non-legume nodule plants already studied in Glasgow. The pH and nitrogen level of such a medium are more easily controlled than in sand culture, and the root systems readily inspected.

A nitrogen-free modification of Crone's solution was the basic solution employed and was used at half its normal

strength. The work reported later on Myrica suggested this dilution of the medium was advantageous to growth. The solution was thus prepared as follows:-

| | |
|---|---|
| KCl | 3.75 gm. |
| $CaSO_4.2H_2O$ | 2.50 " |
| $MgSO_4.7H_2O$ | 2.50 " |
| $Ca_3(PO_4)_2$ | 1.25 " |
| $Fe_3(PO_4)_2.8H_2O$ | 1.25 " |
| Distilled water | 10 litres. |

5 ml. of a minor element mixture, based on Hoagland's A to Z solution (Templeman, 1941) with molybdenum added was supplied to each 10 litre batch of solution prepared. This solution has a pH of 6.7. As containers, glazed earthenware or glass vessels of 2 or $2\frac{1}{2}$ litre capacity respectively were used. They were covered by waxed teak tops bored with 6 or 7 holes. Sections of thick walled rubber tubing were fitted into each hole, the seedlings being supported in the centre of these by small rubber wedges. All jars were heat sterilised and tops freshly waxed before each experiment.

With Elaeagnus, Shepherdia and Ceanothus seedlings were transplanted as the cotyledons expanded, and for the first few weeks of growth in solution they were supplied with combined nitrogen, reasons for this procedure being

given later. 20 mgm. nitrogen per litre of solution was given in the form of ammonium sulphate. When plants grow vigorously in such a solution, the pH falls rapidly. The pH level of the solutions was thus checked frequently and adjusted when necessary by the addition of the calculated amount of sodium hydroxide. Seedlings of Myrica were transplanted straight into the solution in which they were to be inoculated.

Inoculation of Plants.

Two days before inoculation the plants were transferred to nitrogen-free solution. Since the non-legume endophytes have not yet been obtained in pure culture, for nodulation purposes an inoculum was prepared by grinding nodules from the appropriate plant in a mortar with sand and water and then filtering through muslin. This filtrate containing a suspension of the nodule organism was then applied to the roots with a brush and one ml. added to each jar. Since this inoculum may also contain some combined nitrogen or toxic substances, 2 ml. of an autoclaved sample was added to each jar of plants which were to serve as non-nodulated controls.

After inoculation the plants were grown on through the summer, the pH of the solution being checked regularly and any drift corrected. The entire culture solution was

renewed every four or five weeks.

Harvest of Plants.

On harvesting the plants, the nodules were removed and treated separately from the roots and shoots. Dry weights were obtained for these tissues by heating in an oven overnight at 95°C. Nitrogen estimations were obtained by individual Kjeldahl or micro-Kjeldahl determinations carried out on material from each plant.

# STUDIES ON SHEPHERDIA.

## Introduction.

Shepherdia and Elaeagnus together with Hippophaë comprise the Elaeagnaceae, members of which are distributed throughout the world. The genus Shepherdia consists of three species (S.canadensis, S.argentea, and the rarer S.rotundifolia) which are limited to North America. According to Macoun (1883) and Servettaz (1909) and information received from Professor Walton, the first two species are of frequent occurrence, growing typically in the vicinity of water courses and lakes between the latitudes 35° and 65°N., but sometimes extending north of the Aretic Circle.

The first record of nodules for Shepherdia and indeed for all three genera of the Elaeagnaceae appears to have been given by Warming in 1876. Since then some work on their cytology has been attempted but little attention given to their physiological significance until recently. Nobbe and Hiltner (1904), however, grew a few nodulated and non-nodulated plants of S.canadensis for four years in sand culture free of combined nitrogen. At the end of this period the two control plants had reached a mean height of 25 cm., while the other five bearing nodules grew more strongly and attained a final mean height of 68 cm. In view of the

height attained by the control plants it would appear that the growth medium had not been entirely free from combined nitrogen. However, even taking this into consideration, the authors would still seem to have some justification for their tentative suggestion of nitrogen fixation by the nodulated Shepherdia plants.

As no further investigations have been made into this problem, confirmation and extension would seem necessary.

Recently the physiology of the root nodules of Hippophaë rhamnoides was examined. Bond, Fletcher & Ferguson (1954) by growth experiments in nitrogen-free water culture obtained substantial evidence of nitrogen fixation by these nodules and Bond (1955) confirmed this point using isotopic techniques. The effect on nodulation of varying the pH and the addition of combined nitrogen to the medium was subsequently investigated by Bond, MacConnell & McCallum (1956). Nodulation occurred satisfactorily within the pH range 7.0 to 5.4 but not at lower levels. The presence of combined nitrogen in the rooting medium greatly reduced nodule formation, no nodules developing in the presence of 50 or 20 mgm. ammonium nitrogen per litre of culture solution and only sparse nodulation being effected with 5 mgm. ammonium nitrogen. These results were in contrast to those of Allison & Ludwig (1934) and others for legume nodules and to some other

non-legume nodulated plants, e.g., Alnus and Myrica.

By similar experiments confirmation of nitrogen fixation in the root nodules of Shepherdia has now been sought and related aspects investigated. Some of the following data obtained for Shepherdia have already been published (Gardner & Bond, 1957).

## Methods.

Fruits of Shepherdia canadensis, Nutt., were obtained from Churchill, Manitoba, through Professor N.W. Radforth. Germination studies on seeds from these fruits by Bond in 1955 had shown that the pretreatment of the seeds in moist sand at +2°C for five weeks prior to sowing gave better results than similar storage either at room temperature or at -8°C. In, 1956, therefore, the seed was pretreated at +2°C for several weeks before sowing in trays of sand moistened with Crone's solution free of combined nitrogen. The first seedlings appeared after 6 days and 65% germination was obtained. When the cotyledons were beginning to spread the seedlings were transplanted from the sand and thereafter grown in water culture, as described.

Preliminary nodulation studies had been carried out in the spring of 1955 by Bond, when an inoculum was prepared from nodules of a plant of S.argentea, growing in Edinburgh Royal Botanic Gardens. No nodulation was obtained in this experiment, the reason not being obvious. However, Bond later found satisfactory nodulation to occur when Shepherdia roots were brushed with a suspension prepared from Hippophaë nodules. Roberg (1933) had already shown that Hippophaë and Elaeagnus were cross inoculable and hence it would now appear that the endophytes of the three genera are identical.

For the main 1956 experiments attempts were made to obtain Shepherdia nodules from Canada for inoculation purposes. Since, however, this proved impracticable, Hippophaë rhamnoides nodules from St.Andrews, Fife, were used in the proportion 3.5 gm. nodules to 100 ml. water. A small number of plants were inoculated when they had reached the two-leaf stage but for the major experiment inoculation was not performed until the four-leaf stage.

This major experiment was designed to investigate not only the possible nitrogen fixing properties of the plants but also the effect of the pH (5.0, 6.0 and 7.0) of the culture solution and the presence of small amounts of combined nitrogen (5 and 20 mgm. nitrogen per litre) on nodulation. Some 120 plants were used in this experiment.

Once nodules had appeared on the roots, counts were made every second or third day on each plant for 6 weeks. About this time when nodulation had become well established continuous forced aeration of the culture solution was provided, the bubbling tubes consisting of fine bore glass capillary with obliquely ground ends.

## Experimental Results.

In the 1956 experiment, in which the plants were inoculated at the four-leaf stage, nodules were found to develop satisfactorily on the roots immersed in solutions of pH 6.0 and 7.0, but failed completely to develop on those at pH 5.0. No nodules were found on the roots of uninoculated control plants at any of the three pH levels. It was also shown that Shepherdia plants were capable of growth at pH 5.0 if provided with combined nitrogen. Hence the failure of nodulation at this pH was due to the effect on the nodule organism rather than on the host plant.

Nodulation also failed in the presence of 20 and of 5 mgm. ammonium nitrogen per litre of culture solution. Data on the effect of pH and the presence of combined nitrogen on nodulation are given in Table 1.

The plants supplied with combined nitrogen, although without nodules, grew more strongly in the early stages than those with nodules in nitrogen free solution, as shown in Plate 1. The former plants had the advantage of available nitrogen during the period before nodules had formed and become functional on the latter. However, after about two months the roots of the combined nitrogen plants ceased growth and assumed an unhealthy appearance suggesting that cultural conditions were in some way unsuitable. The pH of

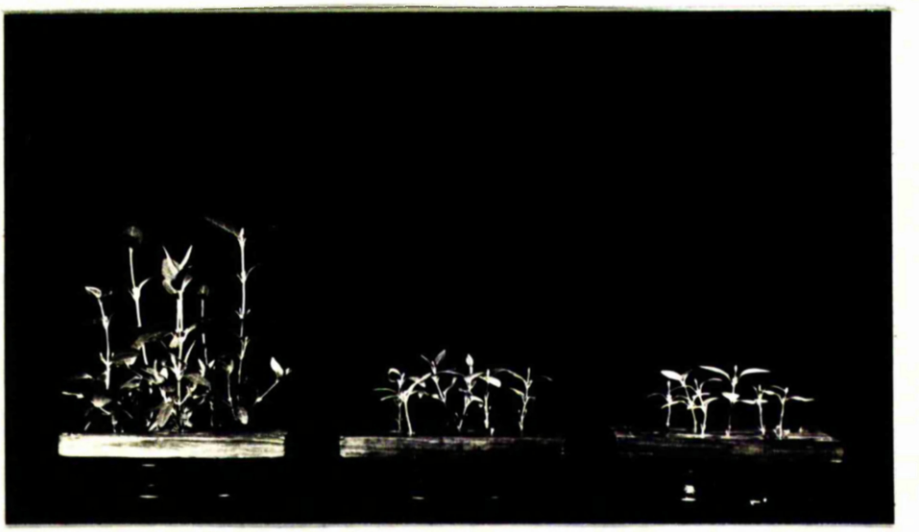
Table 1.

Effect of pH and the Presence of Combined Nitrogen on Nodulation in Shepherdia canadensis.

| pH of culture solution | Mgm. $NH_4$-N per litre of culture solution | Total number of plants set up | Number of plants nodulated 5 weeks after inoculation |
|---|---|---|---|
| 5.0 | 0 | 24 | 0 |
| 6.0 | 0 | 30 | 29 |
| 7.0 | 0 | 24 | 23 |
| 7.0 | 5* | 12 | 0 |
| 7.0 | 20* | 12 | 0 |

* The ammonium content of the culture solution was determined periodically and further amounts added as required to compensate for uptake by the plants.

Plate 1.



Plants of Shepherdia canadensis 5 weeks after inoculation.

Left to right: Plants supplied with 20 mgm. ammonium-nitrogen per litre of culture solution; nodulated plants in nitrogen-free solution; control plants in similar solution.

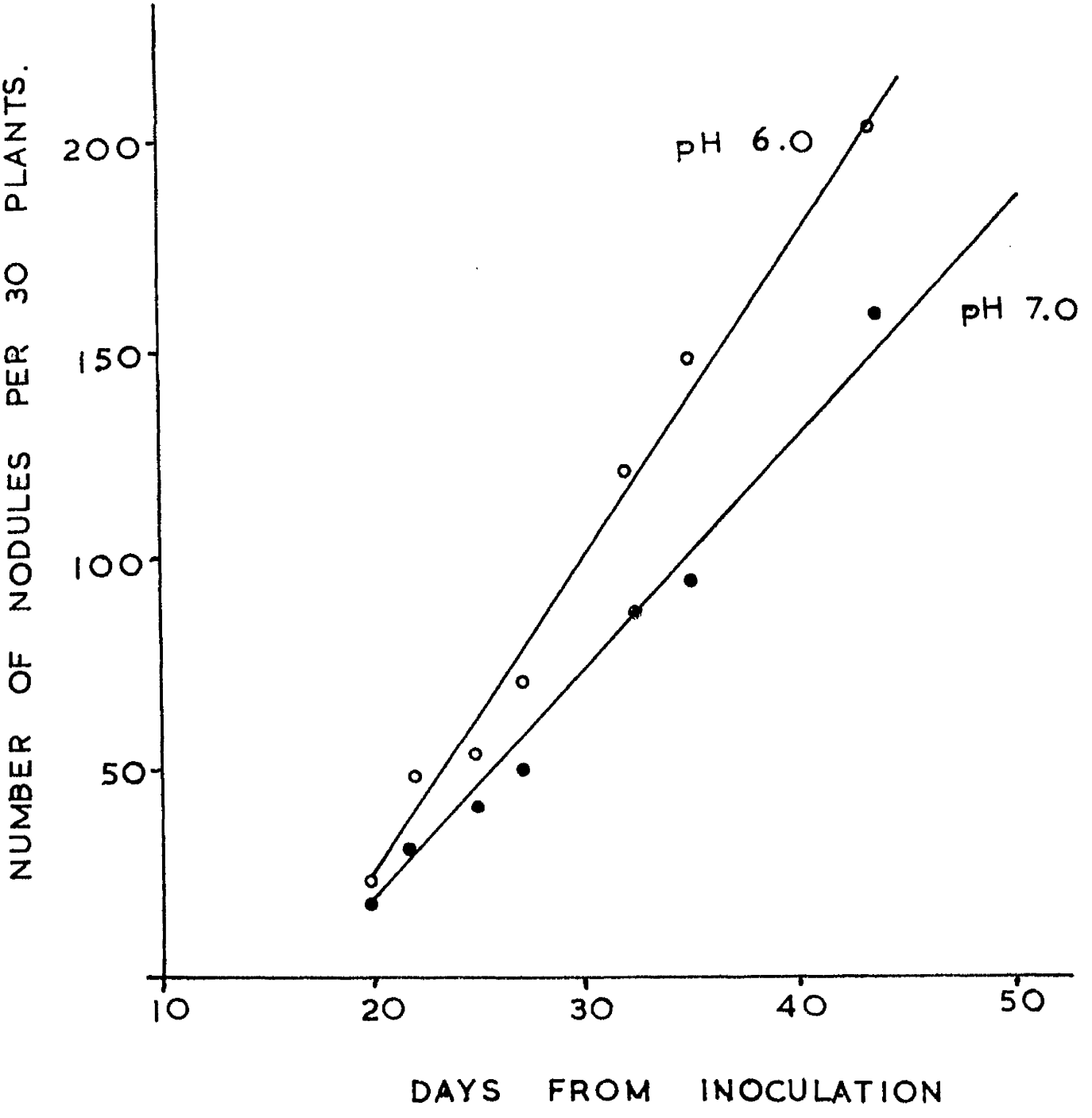
(X 1/5).

these solutions was watched carefully and certainly never fell below 6.0. Aeration of these solutions was found to have no beneficial effects on the roots. The symptoms were however confined to these particular plants and were investigated later.

In nitrogen-free solution at pH 6.0 and 7.0 the first nodules appeared 18 days after inoculation and practically all plants were nodulated in 4-5 weeks. Figure 1 shows that the total number of nodules was greater at pH 6.0 than at pH 7.0 and that in both cases the number was still increasing when regular counting was terminated after six weeks. 17 weeks after inoculation, when some of the plants were harvested, the number of nodules per plant ranged up to 56, the larger nodules then showing a branched formation as in other non-legume root nodules. Plate 2 illustrates a typical nodulated root system at this stage.

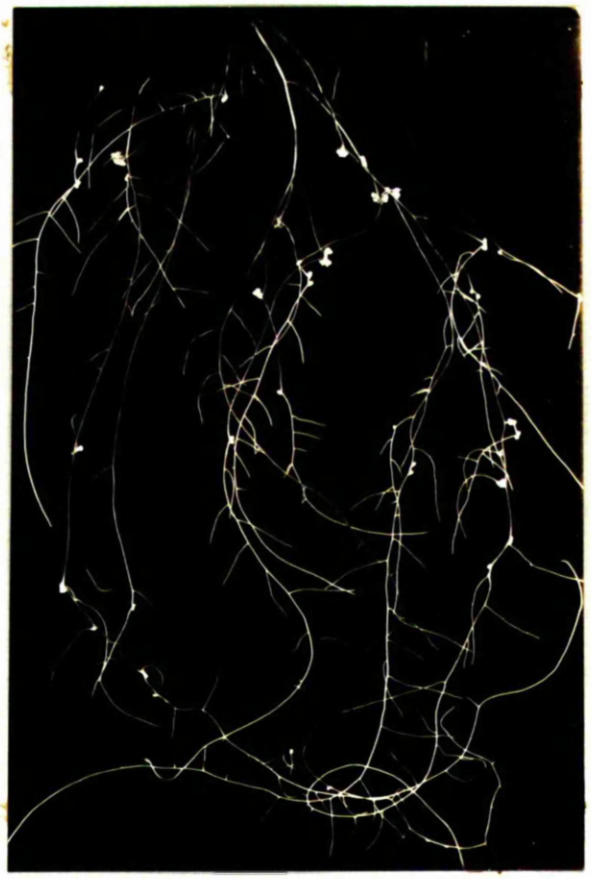
Although for the main 1956 experiment the plants were not inoculated until they had attained the four-leaf stage, 12 plants had also been inoculated when only two leaves had developed. After 6 weeks one plant showed two nodules, another one, and the remainder were un-nodulated. Bond, MacConnell & McCallum (1956) had noted a similar phenomenon in the related genus Hippbphaë where only 6 out of 36 plants inoculated at the cotyledon stage eventually formed nodules.

# FIGURE 1.




Progress of nodule formation on Shepherdia canadensis plants growing in culture solution of different pH. The data for pH 7.0 were calculated from counts actually made on 24 plants.

Plate 2.



Nodulated root system of *Shepherdia canadensis*, from water culture, 17 weeks after inoculation. (X 1/2).

It is well known that in legumes an important factor in nodulation is the level of the carbohydrate/nitrogen ratio. The hypothesis, propounded by Wilson (1940), is that a high carbohydrate/nitrogen ratio favours nodulation and a low one inhibits it. In view of the above findings this factor might also be operative in the case of Hippophaë and Shepherdia. Experiments were thus set up to test this supposition. Hippophaë rhamnoides seedlings were transplanted into nitrogen free solution at the cotyledon stage and grown thus for the duration of the experiment. Different groups of these plants were inoculated at successively later dates. The nitrogen content of similar plants in similar conditions was determined at each inoculation date. The results presented in Table 2 clearly show that, as the carbohydrate/nitrogen ratio rose, the infectibility of the plants increased rapidly. In the last set of plants to be inoculated nodules appeared after 11 days, which is the shortest time yet experienced for any non-legume.

Similar investigations were made with Shepherdia canadensis in 1957. Although trouble was experienced with browning of the roots, so that plants were not maintained after nodulation, the results in Table 3 do indicate a similar picture to those of Hippophaë.

After 17 weeks' growth from the date of inoculation in

Table 2.

Effect of Nitrogen Status of Plant on Infection in Hippophaë rhamnoides.

| Time of inoculation (days from transplanting)* | % N content of dry matter of similar plants | Number of plants inoculated | Number of plants nodulated after 6 weeks | Time of pods. appearing after inoculation (days) | Total number of nodule |
|---|---|---|---|---|---|
| 2 | 9.8 | 11 | 2 | 46 | 2 |
| 15 | 5.1 | 10 | 4 | 37 | 5 |
| 36 | 2.8 | 12 | 10 | 11 | 53 |

* The seedlings were transplanted at the cotyledon stage into nitrogen-free solution and remained in such a solution throughout. The % nitrogen (dry wt. basis) of kernels of seeds = 7.4.

Table 3.

Effect of Nitrogen Status of Plant on Infection in *Shepherdia Canadensis*.

| Time of inoculation (days from transplanting)* | % N content of dry matter of similar plants | No. of plants inoculated | No. of plants nodulated | Time of nodulation (days from inoculation) | Total no. of nodules |
|---|---|---|---|---|---|
| 9 | 5.1 | 12 | 3 | 30 | 4 |
| 23 | 2.9 | 12 | 2 | 20 | 2 |
| 47 | 2.0 | 16 | 14 | 15 | 60 |

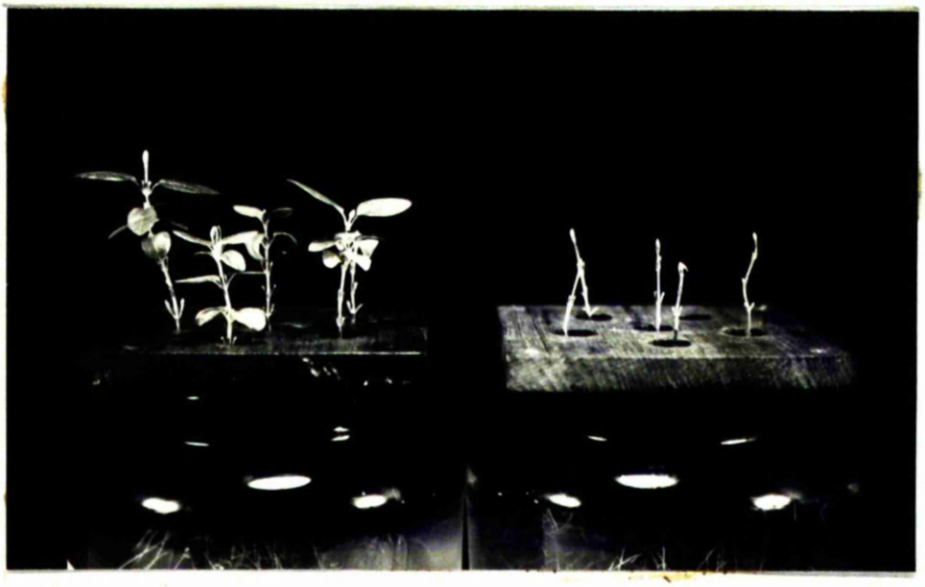
* Seedlings were transplanted at the cotyledon stage into nitrogen-free solution and remained in such a solution throughout. The % nitrogen (dry weight basis) of kernels of seeds = 8.2.

the main Shepherdia experiment 5 nodulated and 5 non-nodulated control plants were harvested. These plants are shown in Plate 3 and their harvest data presented in Table 4. The most vigorous nodulated plants were selected to assess maximum benefit of nodulation. The control plants which had remained without nodules ~~new~~ showed little further growth. They had developed increasing signs of nitrogen deficiency with premature yellowing and loss of leaves. The nodulated plants were now obviously superior to the control ones in shoot height, root length, dry weight and nitrogen content, although there had been a tendency to lose leaves before nodulation was fully established.

Calculations from the data in Table 4 show that the mean percentage nitrogen content of the roots and shoots together from nodulated plants was 2.87 compared with 4.36 for nodules alone, much variation being shown in the nodule figure for individual plants. Nodule dry weight as a percentage of total dry weight amounted to 5.9. The percentage nitrogen for the control plants was 1.56.

The remaining plants were subsequently maintained over the winter months. Solutions were continuously aerated throughout this period and the solutions completely renewed from time to time. In spite of this, however, both roots and nodules became brown and soft and it appeared that the

Plate 3.



Plants of Shepherdia canadensis at time of harvest after growing for 17 weeks from date of inoculation in nitrogen-free solution.

Left: Nodulated plants.

Right: Non-nodulated control plants which had shed all their leaves as a result of nitrogen deficiency. (X 1/3).

## Table 4.

Harvest Data of Nodulated and Non-nodulated Plants of *Shepherdia canadensis* Grown in Solution Free of Combined Nitrogen*.

| Type of plant | Plant No. | Height of shoot Cm. | Maximum root length Cm. | No. of nodules+ | Dry weight. Mgm. | | | Total nitrogen. Mgm.+ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Shoot and root | Nodules | Total | Shoot and root | Nodules | Total |
| Nodulated | 1 | 8.5 | 38 | 51 | 285 | 11 | 296 | 7.7 | 0.81 | 8.51 |
| | 2 | 6.0 | 35 | 27 | 183 | 11 | 194 | 5.0 | 0.42 | 5.42 |
| | 3 | 7.5 | 38 | 44 | 193 | 14 | 207 | 5.4 | 0.61 | 6.01 |
| | 4 | 9.0 | 37 | 56 | 268 | 20 | 288 | 8.2 | 0.67 | 8.87 |
| | 5 | 7.0 | 35 | 55 | 237 | 17 | 254 | 7.1 | 0.67 | 7.77 |
| Non-nodulated | 6 | 2.0 | 23 | 0 | 41 | - | 41 | 0.7 | - | 0.7 |
| | 7 | 3.5 | 30 | 0 | 68 | - | 68 | 1.1 | - | 1.1 |
| | 8 | 4.5 | 21 | 0 | 67 | - | 67 | 1.1 | - | 1.1 |
| | 9 | 5.0 | 22 | 0 | 87 | - | 87 | 1.2 | - | 1.2 |
| | 10 | 3.0 | 18 | 0 | 56 | - | 56 | 0.9 | - | 0.9 |

* Calendar of the experiment:- seed sown 27.3.56; seedlings transplanted into water culture 17.4.56; inoculation 25.5.56; harvest 18.9.56.

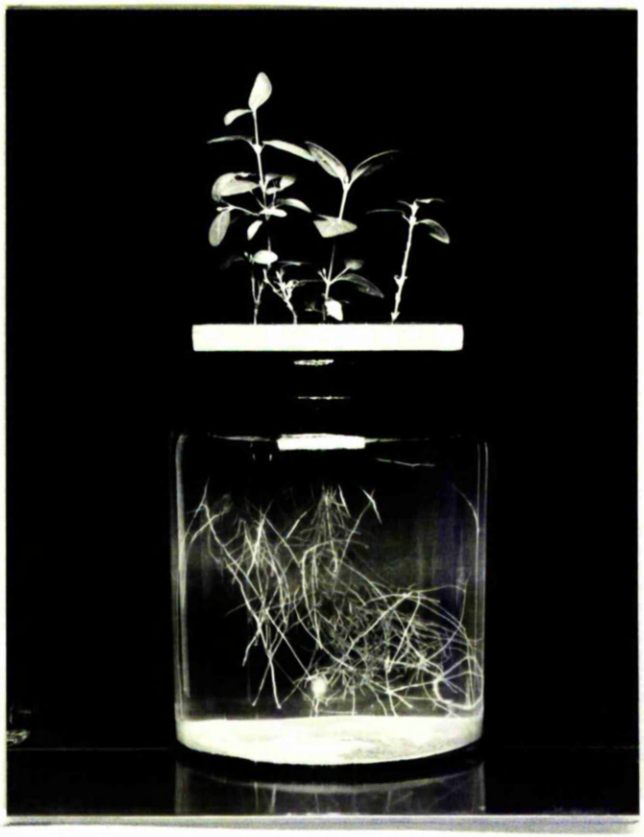
+ The larger nodules had become branched, compound structures, but were still counted as single nodules.

* Individual Kjeldahl or micro-Kjeldahl determinations were carried out on the material of each plant.

plants were not thriving in culture solution. It thus became necessary to discard the plants.

The growth of non-nodulated Shepherdia plants in the presence of combined nitrogen was further studied in 1957. If, in the presence of ammonium nitrogen, the pH of the solution was maintained at 5.4, growth was satisfactory. Plate 4 shows plants so treated. At this pH the roots remained healthy, in contrast to the findings of the previous summer, when the pH was kept at 7.0.

Plate 4.



Non-nodulated Shepherdia canadensis plants in Crone's solution pH 5.4 with 20 mgm. ammonium nitrogen per litre. (X 1/4).

## Discussion.

The conditions under which nodules are formed on the roots of *Shepherdia canadensis* are very similar to those already obtained by Bond, MacConnell & McCallum (1956) for *Hippophaë rhamnoides*, although they differ appreciably from corresponding data for *Myrica* and *Alnus* as given by Bond, Fletcher & Ferguson (1954). In particular, the *Shepherdia* nodule organism does not show such a great acid tolerance as do those of *Myrica* and *Alnus* since a pH of 5.0 inhibits nodulation. Nodulation has also been shown in *Shepherdia* to be much more sensitive to the presence of combined nitrogen in the culture medium than is the case in *Alnus* and *Myrica*.

The views expressed by Nobbe & Hiltner (1904), that the fixation of atmospheric nitrogen may be associated with nodulation in *Shepherdia* are confirmed by the growth experiment data provided in Table 4. These data show definitely that nodulated *Shepherdia* plants are able to grow and increase their nitrogen content in a solution free of combined nitrogen. The high percentage nitrogen content of the nodules compared with the rest of the plant indicates that it is indeed within the nodule that the nitrogen is initially fixed. Additional evidence of fixation and of the nodular function of the fixation has recently been supplied by Bond

(1957b) by a short term isotopic test. After exposing the root systems of nodulated Shepherdia plants to an excess of free $^{15}N$ for 72 hours the greatest accumulation of the isotope fixed was subsequently found in the nodules.

From such results it is clear that in the Shepherdia nodule there exists a symbiotic relationship between endophyte and host plant similar to that of the legumes in which the products of nodular fixation are made available for use by the plant as a whole.

If, as has been mentioned earlier, it is true that nodules of all three genera of the Elaeagnaceae harbour the same organism, then the use of Hippophaë material for inoculation purposes need not detract from the ecological significance of the above experiments. Admittedly evidence of fixation has only so far been obtained for Shepherdia plants in water culture, but there appears to be no reason to doubt, however, that nodulated plants under field conditions will be active in fixation. Support for this idea is given by Moss (1953) who holds, what may well be a widespread view, that the presence of S.canadensis is an indication of poor soil conditions. Raup (1941) found Shepherdia to occur very frequently in the arctic and sub-arctic regions of North America and speculated that it might possess special soil-enriching powers. Crocker & Major (1955) also

report that Shepherdia is prominent in the early stage of plant succession on recently deglaciated areas at Glacier Bay. Unfortunately definite confirmation of nodulation of the plants in such situations is lacking. The reports do however suggest that the Shepherdia plant in the field can grow in very poor soils and being able to obtain nitrogen from the atmosphere, thrive. On decaying it will then enrich the soil with nitrogen and thus aid the growth of other plants on the same area.

Dr. J. Terasmae, of the Geographical Survey of Canada has informed us that his unpublished pollen studies show Shepherdia to have been of frequent occurrence in early post glacial times in Canada. Thus both in the present and in the past Shepherdia would appear to be the New World counterpart of Hippophaë.

# STUDIES ON ELAEAGNUS.

## Introduction.

The genus Elaeagnus comprises 30 species which occur widely in Asia, Europe and North America. Many of these species can vegetate in similar conditions to Shepherdia and Hippophaë, being found along the edges of water courses, in dried up water courses or on screes. Certain species however (E.pungens and E.Henryi) have xerophytic characters with leathery leaves and spiny shoots and grow in soils not too rich in calcium. E.hortensis grows on the shores of the Mediterranean. As in Hippophaë and Shepherdia, so in Elaeagnus, the third genus of the Elaeagnaceae, nodulation appears to be of general occurrence and has been recorded since 1876.

Nobbe & Hiltner (1904) grew two plants, one nodulated and the other non-nodulated, of E.angustifolia in sand free of combined nitrogen. After four years the nodulated plant had attained a height of 120 cm. compared with 24 cm. for the non-nodulated control. A tentative suggestion of nitrogen fixation by the nodules was hence put forward. Some years later, in the course of experiments which demonstrated the cross-inoculation between Elaeagnus and Hippophaë, Roberg (1933) reported signs of the fixation of atmospheric

nitrogen in both genera. Panosjan (1945), whose paper is available only in summary form, also stated that he had evidence that nodulated plants of Elaeagnus were independent of the presence of combined nitrogen.

It was with the aim of putting these rather vague conjectures as to the function of the nodules present on the roots of Elaeagnus on to a firmer basis, that the following work was undertaken. Part of this work has recently been published (Gardner, 1958).

## Methods.

No previous information on the germination of Elaeagnus seed could be found. E.umbellata seed was obtained from Thompson & Morgan, seed merchants, Ipswich in 1955. After treatment at 42°C for several weeks, which is the usual pretreatment for Hippophaë and Shepherdia seed, the seeds were sown in sand in March 1956 but no germination resulted. Seed of E.angustifolia was then obtained from Vilmorin-Andrieux, S.A. Paris, via Edinburgh Royal Botanic Gardens, in February 1956, and sown in sand without any previous treatment. A 40% germination resulted. This species was thus used throughout the following studies. Since germination was not at all uniform, being spread over 6 weeks, the seedlings available for inoculation purposes were not all at the same stage of development at a given time. This necessitated rather long term growth experiments to obtain significant differences.

The tap root of these seedlings elongated very rapidly after germination, hence transplanting of the seedlings to water culture was begun even before the cotyledons had expanded.

Two to three weeks after transplanting and while still in medium containing combined nitrogen the roots became rather stunted. This was thought to be due to lack of iron,

and hence the pH of the solution was lowered to 5.5 and ferric citrate added. This treatment appeared to be effective and normal growth continued.

Inoculation was effected by applying to the roots a suspension of crushed nodules obtained (by the kindness of the Curator) from a plant of E.pungens growing in Glasgow Botanic Gardens. The inoculum was used at a strength of 2 gms. of nodules in 20 ml. tap water. After 4 weeks no nodules had appeared on any of the plants, and it was concluded that the inoculum had been inactive. As noted already, according to Roberg (1933) the two genera Elaeagnus and Hippophaë are cross inoculable. A further inoculum was therefore prepared from Hippophaë rhamnoides nodules growing in the greenhouse.

The plants were subsequently grown through the summer and over the following winter with periodic changing of the culture solutions and frequent checking of their pH. The plants were eventually harvested 15 months after inoculation.

## Results.

As the first signs of nodule formation were noted only five days after the inoculation with Hippophaë material, it is not absolutely certain to which inoculation the formation of these first nodules is to be ascribed, though it is more probable that the Hippophaë inoculum was responsible.

By ten days from the date of the Hippophaë inoculation, however, numerous nodules were present on the roots of the plants inoculated in nitrogen free solution at pH 6.0. No nodules appeared on the correasponding plants at pH 5.0 or on the uninoculated controls. Once nodulation had been established the plants began to make good growth in nitrogen-free culture solution. During the first seven weeks after inoculation the control plants which were without nodules showed only feeble development and began to shed their leaves, whereas the nodulated plants made satisfactory growth. Plants seven weeks after inoculation are shown in Plate 5, while Plate 6 illustrates a typical root system from one of these nodulated plants. The nodules were white in colour and beginning to show a lobed structure due to branching.

Since nodules were not formed until late in July, the plants were not harvested at the end of their first

# Plate 5.

Plants of *Elaeagnus angustifolia* 7 weeks after inoculation.

Left:- nodulated plants.

Right:- non-nodulated control plants (X 1/4).

Plate 6.

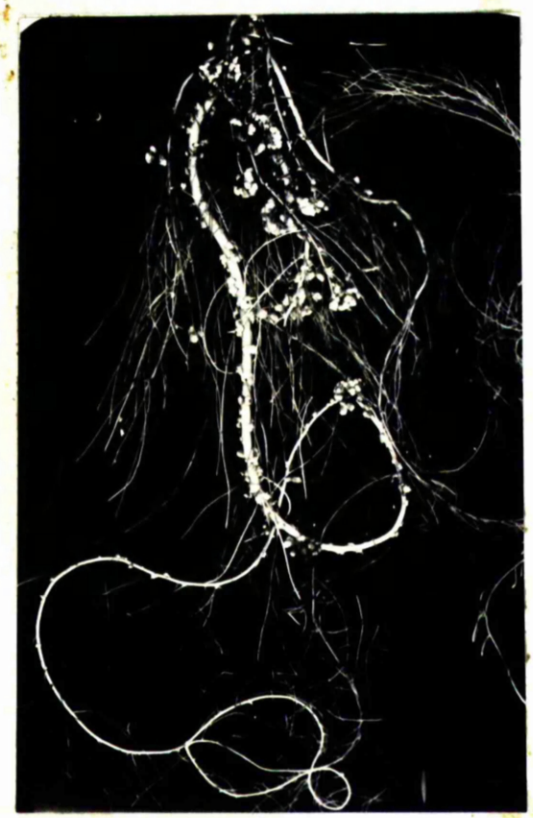
Nodulated root system of Elaeagnus angustifolia from water culture, 7 weeks after inoculation.

(X 2/5)

season's growth but allowed to grow on into a second. In their second season the nodulated plants were spaced out, one per jar, some in 2½ and others in 5 litre containers. The solution in certain jars was now also aerated in a manner similar to that already described for Shepherdia. Aeration appeared to suit the Elaeagnus plant. By the end of the second season's growth the nodules had become much larger compound branched structures. A typical root system is shown in Plate 7.

After 15 months' growth the plants were harvested. Typical plants are shown in Plate 8 and the harvest data presented in Table 5. Nodulated plants are seen to be vastly superior in every way, namely in size, dry weight, and nitrogen content, to the control plants. From these figures the percentage nitrogen (on a dry weight basis) can be calculated for the various parts of the plant. Within the nodulated plant the highest percentage nitrogen is to be found in the nodules, the appropriate mean figures being 3.5% for the nodules, 1.8% for roots and 1.8% for the shoots. The control plants showed a mean % nitrogen of 1.5.

Plate 7.



Nodulated root system of Elaeagnus angustifolia after two seasons' growth in water culture. (X 2/5).

Plate 8.

Plants of Elaeagnus angustifolia after two seasons' growth in culture solution free of combined nitrogen, the plant on the left bearing root nodules. (X 1/8).

Table 5.

Harvest Data for Elaeagnus angustifolia.

| Type | Plant No. | Shoot Height (cm.) | Root Length (cm.) | Dry wt. (gm.) | | Total nitrogen (mgm.) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Root + Shoot | Nodules | Root + Shoot | Nodules | Whole Plant |
| Nodulated | 1 | 45 | 93 | 2.220 | 0.110 | 23.45 | 3.56 | 27.01 |
| | 2 | 62 | 120 | 6.585 | 0.210 | 140.60 | 8.22 | 148.82 |
| | 3 | 56 | 89 | 5.840 | 0.187 | 122.00 | 6.00 | 128.00 |
| | 4 | 74 | - | 9.64 | 0.397 | 173.00 | 13.40 | 186.40 |
| Non-nodulated | 5 | 10 | 29 | 0.154 | - | 2.058 | - | 2.058 |
| | 6 | 8 | 26 | 0.156 | - | 2.186 | - | 2.186 |
| | 7 | 4 | 15 | 0.028 | - | 0.532 | - | 0.532 |
| | 8 | 6 | 20 | 0.099 | - | 1.346 | - | 1.346 |

Seeds sown 27.3.56

Inoculation 1.5.56

Harvest Plants 1, 2, 3, 5, 6 and 7 - 7.8.57
4 and 8 - 1.10.57.

## Discussion.

It would seem from the work of Roberg (1933) and that reported in the previous sub-section on Shepherdia that the nodules of all the Elaeagnaceae harbour the same endophyte. If this is indeed true then the use of Hippophaë nodules as a source of inoculum does not detract from the ecological significance of these experiments.

The conditions for nodulation of Elaeagnus in water culture are, as far as they have been studied, similar to those already reported for Shepherdia, nodulation being inhibited by acid conditions, namely, a pH of 5.0.

That control plants of E.angustifolia without nodules showed very feeble development whereas nodulated plants grew satisfactorily and accumulated substantial amounts of nitrogen when grown for 2 seasons in solution free of combined nitrogen, can be seen from Table 5 and Plates 5 and 7. It is obvious that fixation of atmospheric nitrogen was associated with the nodulated plants, and there are good reasons for concluding that the nodules are the site of fixation. In the case of Elaeagnus the only direct evidence of this so far obtained is that the percentage nitrogen content of the nodule dry matter was twice that of the rest of the plant. However, since there is every indication that the nodule function is similar in all these

non-legumes, it is permissible to refer to evidence based upon more fully studied genera which proves that the function is nodular. Thus, as noted by Gardner & Bond (1957), if the nodules are removed from a plant, indications of nitrogen deficiency promptly appear in the plant; tests with $^{15}N$ show that such plants have no powers of fixation, although the detached nodules do possess such powers. Again, when the root systems of nodulated plants are exposed to excess free $^{15}N$ for a short period, the highest enrichment is unfailingly shown by the nodules. By analogy it therefore seems reasonable to conclude that in Elaeagnus the nodules are responsible for the fixation.

Because of the difficulty that pure cultures of the endophytes are not available, the nodulated plants of Elaeagnus (and of the other genera considered in the Thesis) were not grown under aseptic conditions. It cannot thus at once be assumed that the nodule endophyte is the agent of the fixation, as is generally believed for legumes. Various organisms may have been introduced in the inoculum, and it might be argued that the fixation was due to a contaminating organism associated with the nodules. It must however be concluded that the nitrogen fixing organism is in symbiotic relationship with the higher plant, for it is obvious that the bulk of the nitrogen fixed quickly becomes available to the higher plant, while the latter is the only

source from which the organism can gain carbonaceous materials, large amounts of which must be utilised in the achievement of the observed fixation. Thus the fixation must be intra-nodular, and since most cytological observers have seen only one organism within the nodule, there is at present no reason to doubt that the nodule organism is responsible for the fixation associated with these non-legume nodules. Thus it may be concluded that as in other non-legumes investigated, so in Elaeagnus the nodules have the same function as those of the legumes.

Although nitrogen fixation data have been obtained for only one species of Elaeagnus, there seems no reason why other nodulated species should not likewise fix nitrogen. The same argument holds true for Shepherdia, and since from a study of the literature it would appear that nodulation is widespread in both genera, it may now be said that the record for nitrogen fixation in the Elaeagnaceae is now complete.

A measure of the nitrogen fixed by a plant may be derived by subtracting a mean figure for the nitrogen content of control plants from that of the nodulated ones. Maximum figures were obtained for Shepherdia and Elaeagnus from Tables 4 and 5. These are recorded in Table 6 together with data for Hippophaë. Fixation by Elaeagnus over two seasons compares satisfactorily with the value for Hippophaë

Table 6.

Data for Nitrogen fixed by Members of the Elaeagnaceae.

| Plant | Fixation per Plant (mgm. nitrogen) | |
|---|---|---|
| | 1st season | 2nd season |
| *Shepherdia canadensis* | 7.9 | - |
| *Elaeagnus angustifolia* | - | 182 |
| *Hippophaë rhamnoides* | 26* | 200 |

* Data from Bond, MacConnell & McCallum (1956).

obtained by the author. Both these genera were found to benefit from aeration of the culture solution. A typical Hippophaë plant so reared by the author is shown in Plate 8A As a whole, however, the Elaeagnaceae would appear to be somewhat slower nitrogen fixers than other non-legume plants. A fixation of 1200 mgm. nitrogen by the end of the second year is reported in the following pages for Myrica gale. Fixation in Shepherdia particularly appears poor. It has already been pointed out that cultural arrangements which have proved satisfactory for other nodulated plants appeared unsuited to Shepherdia.

Andersson (1953) has reported the occurrence of Elaeagnus pollen in early post glacial deposits in Canada, while Major (1957) in a personal letter reports that Elaeagnus occurs on recently de-glaciated areas of Alaska where it grows vigorously on the nitrogen deficient soil and he concludes that it may function as does the alder as a soil enricher. From such data it would indeed appear that Elaeagnus may have had in the past and still has in the present a part to play in the biogeochemistry of soil nitrogen.

Plate 8A

Nodulated plant of *Hippophaë rhamnoides* grown for two seasons in nitrogen-free water culture.

(X 1/8)

## STUDIES ON MYRICA.

### (a) Myrica gale. L.

As already mentioned in the General Introduction, much work has been carried out in Glasgow on the physiological function of the root nodules of Myrica gale. Conclusive evidence of nitrogen fixation by these nodules has been obtained by Bond (1951, 1955). As noted by MacConnell & Bond (1957a) a source of inconvenience in all these previous experiments with M.gale has been that when seedlings are transplanted from peat trays to water culture with Crone's solution and their roots treated with crushed-nodule inoculum, a considerable mortality occurred in the young plants, prior to or during the early stages of nodule formation. The present author has conducted trials with the object of finding the reason for this loss of plants, which contrasts with the almost 100 percent establishment of nodulated plants usually obtained with Alnus, Hippophaë, Casuarina and other genera.

The following were considered as possible reasons for the unsatisfactory establishment:-

(1) Crone's solution as usually employed may be unsuited to M.gale, and in particular may be too concentrated;

(2) the nitrogenous reserves of the seeds may be too small to maintain the young plants until the nodules begin to

supply fixed nitrogen;

(3) the crushed-nodule inoculum might contain substances (e.g., tannins) injurious to roots, or alternatively the inoculum in the strength previously employed might contain too few endophytic cells to give maximum nodule formation.

An experiment was set up to provide information on the first two and last possibilities. Seeds germinated in peat after the usual pre-treatment at +2°C for several weeks were transferred to water culture at the two-leaf stage in July 1955. Three culture solutions were employed, namely, Crone's nitrogen-free solution at full strength and also at quarter strength, and a Hoagland nitrogen-free solution (Hoagland & Arnon, 1950) which, at one-quarter of its normal strength, was favourably reported on by Quispel (1954) as a medium for the culture of young plants of Alnus.* Data for the gross composition of these solutions are as follows:-

---

| | | |
|---|---|---|
| * Hoagland's | KCl | 0.93 gm. |
| N-free | $CaCl_2$ | 1.39 " |
| soln. | $MgSO_4$ | 1.20 " |
| (diluted form) | $KH_2PO_4$ | 0.34 " |
| | Distilled water | 10 litres |
| | + 10 ml. A - Z soln. | |
| | + 10 ml. Fe tartrate (sat.). | |

| | Dissolved matter, gm. per litre of solution | Undissolved matte gm. per litre of solution |
|---|---|---|
| Crone's, full strength | 1.64 | 0.61 |
| Crone's, quarter strength | 0.42 | 0.14 |
| Hoagland's, quarter strength | 0.39 | 0 |

All solutions were adjusted to and maintained at pH 5.0 during the duration of the experiment. Twenty plants, divided between four jars, were set up for each solution and with each solution small amounts of combined nitrogen in the form of ammonium sulphate were added to certain jars.

Inocula were prepared from nodules of field plants, both at the normal strength of 3.5 gm. nodules suspended in 100 ml. water and at twice that strength. The inocula were applied to the roots in the usual way.

The progress of the experiment during the two months following inoculation is indicated in Table 7. As in previous experiments, there was a considerable loss of plants in full strength Crone's solution, which the provision of small amounts of combined nitrogen did not prevent. The loss was greater when the stronger inoculum was employed. The dead plants showed no or very sparse nodulation, probably because the plants were already unhealthy and enfeebled at time when nodule formation should have been active. The data show that in the diluted Crone's and Hoagland's solutions the extent of

Table 7.

Nodulation and Establishment Data for *Myrica gale*.

| Type of culture solution | Combined N added mgm./litre | Inoculum strength | % of plants forming nodules after 4 weeks | Average No. of nodules per plant after 4 weeks | % No. of plants dead after 8 weeks |
|---|---|---|---|---|---|
| Crone's F.S.* | 0 | normal | 86 | 4 | 25 |
| " | 5 | normal | 71 | 3 | 25 |
| " | 0 | double | 76 | 3 | 40 |
| Crone's ¼ S. | 0 | normal | 95 | 7 | 10 |
| " | 5 | normal | 100 | 8 | 0 |
| " | 0 | double | 85 | 7 | 10 |
| Hoagland ¼ S. | 0 | normal | 89 | 8 | 10 |
| " | 5 | normal | 100 | 8 | 5 |

* F.S. - full strength

¼ S. - quarter strength.

nodulation and the establishment of the plants were much improved. The presence of the low level of combined nitrogen provided in some jars appears to have been advantageous.

These results indicate that the difficulties previously encountered in establishing M.gale plants in water culture were due to Crone's solution as usually employed being too concentrated for these particular plants. Establishment is satisfactory in quarter strength Crone's solution or in a diluted Hoagland solution. Of the two latter solutions, Crone's, particularly when combined nitrogen is present, is preferable because of its superior buffering power, as demonstrated by MacConnell (1956).

The comparison of growth in the different solutions was discontinued in October, 1955, and the plants which were required for subsequent use in biochemical studies, were all transferred to quarter strength Crone's solution and overwintered. In the following season, the solution was gradually increased to half-strength and the plants showed most prolific growth, entirely on nodule nitrogen, and as a group their rapidity of growth greatly exceeded the average shown by previous populations of M.gale plants grown in full strength Crone's solution. Plate 9 shows the abundance of the nodulation early in the second season, while Plate 10

Plate 9.

Nodulated root system of Myrica gale showing abundant nodulation and the upwardly growing nodule roots, early in the second season.
(natural size)

indicates the shoot growth by the beginning of August of the second season. A typical plant harvested in September, 1956 after 14 months' growth, for 7 of which it had been in the leafless condition, was found to contain 486 mgm. nitrogen. The maximum nitrogen content obtained by the previous workers after a full season's growth (7 months) was 78 mgm. Even allowing for the small amounts of combined nitrogen added in 1955 there is a marked improvement in growth. Full harvest data are given in Table 8.

The above findings provide valuable evidence of the activity of the nodules of M.gale in the fixation of atmospheric nitrogen, and of the ability of the nodulated plant to grow luxuriantly and perfectly normally in a culture solution free of combined nitrogen. In 1956 a second crop of M.gale plants was started, again in the diluted Crone's solution. Two typical plants harvested at the end of their second season's growth attained a total nitrogen content of 1233 and 1266 mgm. respectively, indicating that these plants also have shown luxuriant growth.

Plate 10.

Three typical nodulated plants of *Myrica gale* in early August after 14 months' growth in water culture. (X 1/6)

Table 8.

Harvest Data for *Myrica gale* Plants.

| Plant No. | Dry weight (gm.) | | | | Nitrogen content (mgm.) | | |
|---|---|---|---|---|---|---|---|
| | Shoot | Root | Nodules | Total | Shoot + Root | Nodules | Total |
| 1* | 18.1 | 5.3 | 1.4 | 24.8 | 440 | 46 | 486 |
| 2 | 45.0 | 8.6 | 2.2 | 55.8 | 1156 | 77 | 1233 |
| 3 | 46.3 | 6.0 | 2.3 | 54.6 | 1043 | 112 | 1266 |

* Plant 1. Seed sown 24.6.55, inoculated 1.8.55, harvested 20.9.56.

Plants 2 & 3. Seed sown 27.3.56, inoculated 29.4.56, harvested 24.9.57.

(b) Myrica cerifera L.

The genus Myrica consists of 45 species distributed throughout the temperate and sub-tropical regions of the world. It appears from the literature that nodulation is typical for the genus. However, M.gale L. is the only species in which the physiological functions of the nodules have been investigated to any significant extent (see General Introduction).

M.cerifera L., the "wax-myrtle" is native only to North America, where it has a more southerly distribution than M.gale. Van Dersal (1938) shows it as occurring in the South-eastern regions of the United States. In habit it varies from a small shrub to a 12 metre tree and is to be found in sandy swamps or wet woods being confined almost entirely to coastal regions (Youngken, 1919).

Harshberger (1903) would seem to have been the first to record the presence of nodules on the roots of M.cerifera, although their presence on M.gale and M.rubra had already been noted by Brunchorst (1886) and Shibata (1902) respectively. Early workers were entirely concerned with the morphology and cytology of the nodule. Youngken (1919) claimed the isolation of an Actinomycete, from M.cerifera nodules, which when reinoculated onto the roots formed incipient nodules. However, the control plants also became nodulated, and Fletcher (1955) criticised Youngken's claims.

A few conjectures as to whether the function of these nodules was analogous to that of the legume nodule were made from time to time, but no attempts were made to confirm this experimentally.

Seed of M.cerifera was obtained from Thompson & Morgan, Ipswich. Trials by Bond (1951) showed that the best germination of M.gale seed was obtained after 4 weeks pre-treatment in moist peat at +2°C. No such data were available for M.cerifera, but Barton (1932), working with M.carolinensis, a closely related species to M.cerifera, reported the highest percentage germination after three months' storage at +2°C. In 1956 a germination of 27% was obtained for M.cerifera after four weeks' storage at +2°C. After six months' storage in the above manner in 1957 the seed began to germinate in the refrigerator.

Since only a small quantity of seed was available, the culture conditions best suited for M.gale (see previous sub-section) were employed. The seedlings were thus transplanted at the two-leaf stage into ¼ strength N-free Crone's solution of pH 5.0. To some jars 10 mgm. nitrogen (as ammonium sulphate) per litre were added.

For inoculation purposes, since material of M.cerifera itself was not available, nodules from M.gale plants growing in the greenhouse were used in the proportion 7 gm. nodules to 100 ml. water.

In both years in which nodulation was studied, the formation of nodules on M.cerifera was slow, 4 weeks or even 5 being required before the first signs of nodules appeared. No nodules appeared on the uninoculated control plants or on inoculated plants which had been given 10 mgm. nitrogen per litre of culture solution. Nodulation was in all cases very sparse and was certainly not comparable with that obtained in M.gale under similar conditions. One possibility was that the cultural conditions were not suitable, but against this was the finding that the plants grew vigorously when supplied with combined nitrogen. Another possibility was that there might be a species difference in the nodule organism. Shibata & Tahara (1917) claimed from cytological studies that the endophyte present in the nodules of M.rubra, M.cerifera L., and M.adenophora Hance. was actinomycetal in nature while that in Gale gale, C.K. Schn. (Myrica gale, L.) was entirely different. The present author's experience does not bear this out, but it may well be that the M.gale endophyte is only partly adapted to symbiosis with M.cerifera.

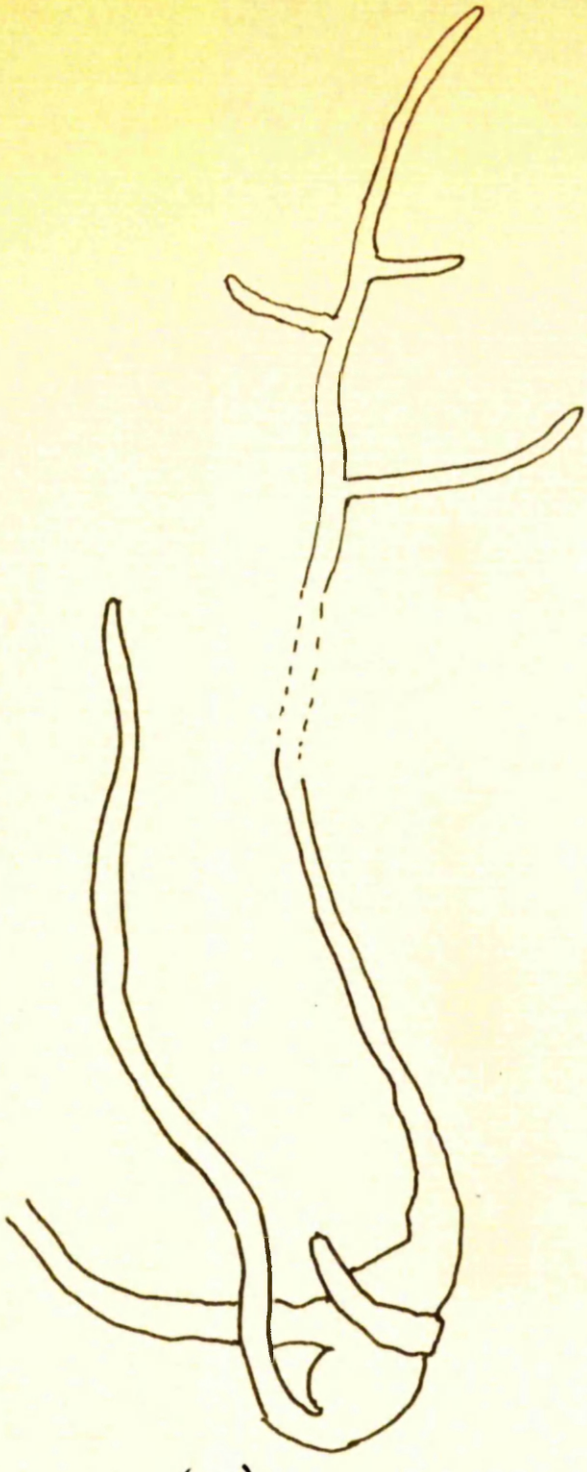
The nodules when first formed often showed a pink colour as observed by Bond (1951) for M.gale. As a result of branching the young nodules developed a lobed structure and, again as in M.gale, the tips of the lobes grew out forming

upwardly growing "nodule-roots". These nodule-roots were at first unbranched but later they frequently produced lateral roots. This occurrence of laterals is rarely seen in M.gale. In one instance the formation of a nodule on a nodule-root was noted in M.cerifera. These upwardly growing roots are thought to have a similar significance to those of M.gale which are considered to aid ventilation of the nodule which is otherwise covered in a layer of cork tissue. Since there has been no opportunity to examine field material of M.cerifera nodules, it is impossible to know whether, as is the case in M.gale, this upward growth of the nodule-roots also occurs in nature. Arzberger (1910) while studying field material noted that a peculiarity of M.cerifera nodules was that "their tips grow out into a narrow thread-like structure often attaining 1.5-3 cm. in length. This again sends out lateral branches which may be found entwined among the roots and grass blades." However, no definite mention can be found as to their orientation in the field. The nodule roots bear at their tips normal root hairs, whereas the root hairs of the main roots in the region of nodule formation were always found to be distorted as in M.gale (Fletcher, 1955). Drawings and photographs illustrating these nodule features in M.cerifera are shown in Plates 11 and 12.

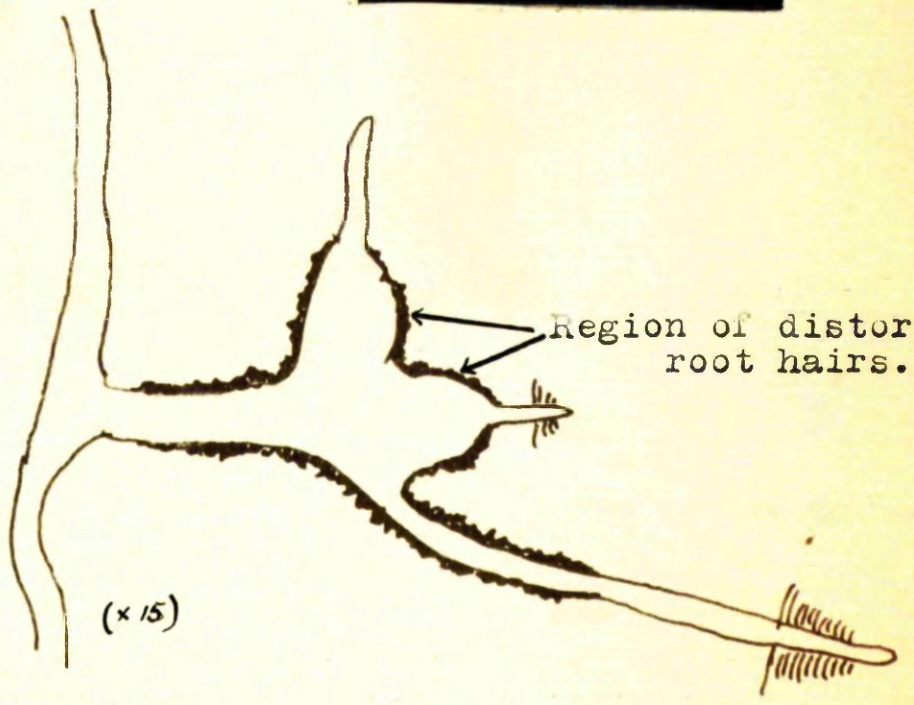
Plate 11.

Root systems of first year Myrica cerifera plants showing sparse nodulation and the upwardly growing nodule roots. (X 1/2)

Plate 12.



(× 10)




(× 15)

Top right:- Part of a root system of Myrica cerifera showing lateral roots arising on the upwardly growing nodule roots.

Drawings show formation and branching of the nodule roots.

The growth of the nodules in culture solution was however very slow, and although by the autumn the nodulated plants showed a superiority over the non-nodulated ones, this was not considered great enough to provide conclusive evidence of fixation by growth measurements alone. A test with isotopic nitrogen, $^{15}N$, was therefore carried out.

Two first year plants, one with and the other without nodules, were exposed in September 1957 to an atmosphere containing excess free $^{15}N$. The root system of each plant was sealed into a special bottle containing culture solution and a 30 ml. gas space which was then charged with the isotopic gas mixture as will be described in Experimental Methods, Section II for "attached" nodules. By this procedure the root systems were exposed to a gas mixture initially consisting of 20% oxygen, 15% nitrogen with 36 atom per cent. $^{15}N$, and 65% argon. The plants were left sealed in this manner for 3½ days under the prevailing greenhouse conditions. No signs of deterioration in the plants were observed as a result of this enclosure. The plants were then submitted to Kjeldahl analysis, the resulting distillates being evaporated down to small volume and subsequently assayed for $^{15}N$ in a Mass Spectrometer. The increase in $^{15}N$ content, as atom per cent. of total nitrogen over the normal value is given in Table 9 for each

plant. A considerable increase in $^{15}N$ content is shown by the nodulated plant. There is no significant increase, however, in the $^{15}N$ content of the plant without nodules, since the apparent increase falls short of the significant difference required, namely 0.008.

The data presented clearly show that the fixation of free nitrogen was associated with the root system of the nodulated plant but not with that of the non-nodulated plant. By analogy with previous work on non-legume systems, it can be deduced that these nodules of M. cerifera possess powers of nitrogen fixation. Thus the natural pioneering of sandy shores by M. cerifera is in keeping with the possession of nitrogen fixing nodules by means of which the fertility of the sand is gradually raised and made more suitable for the growth of other plants.

Table 9.

Harvest and Isotopic Data for *Myrica cerifera*.

| Type of Plant | Dry weight (mgm.) | Nitrogen content (mgm.) | Atom % $^{15}N$ excess |
|---|---|---|---|
| Nodulated | 34.7 | 0.672 | 0.373 |
| Non-nodulated | 21.3 | 0.182 | 0.007 |

Plants had grown for 3 months in nitrogen-free culture solution before exposure of their root systems to excess free $^{15}N$ for $3\frac{1}{2}$ days.

# STUDIES ON CEANOTHUS.

## Introduction.

This substantial genus comprising some 40 species is the only genus of the family Rhamnaceae to bear root nodules. The genus is native only to North America though it has been introduced into other countries for ornamental planting in gardens and shrubberies. In such situations, in Britain at least, it does not form nodules, the nodule organism presumably being absent from these soils. Ceanothus species are often prominent members of the native flora of North America particularly in regions in which forests have been felled or burned. There they grow rapidly and form dense thickets. Van Dersal (1938) gives distribution maps for 38 species of Ceanothus in the United States and it is clear that the genus has a very wide distribution. Some of the best known species are C.americanus (Jersey-tea), C.velutinus (Snowbrush), C.integerrimus (Deerbrush) and C.ovatus (Redroot).

Attention was first drawn to nodules on the roots of Ceanothus when Beal (1890) exhibited "tubercles" on the roots of C.americanus to the American Association for the Advancement of Science. Bottomley (1915) claimed the isolation of Bacillus radicicola from Ceanothus nodules, and

from limited experiments this organism was reputed to assimilate small amounts of atmospheric nitrogen. Since he made no attempt to reinoculate plants with this organism and thus prove its identity with the endophyte, little attention can be paid to this claim.

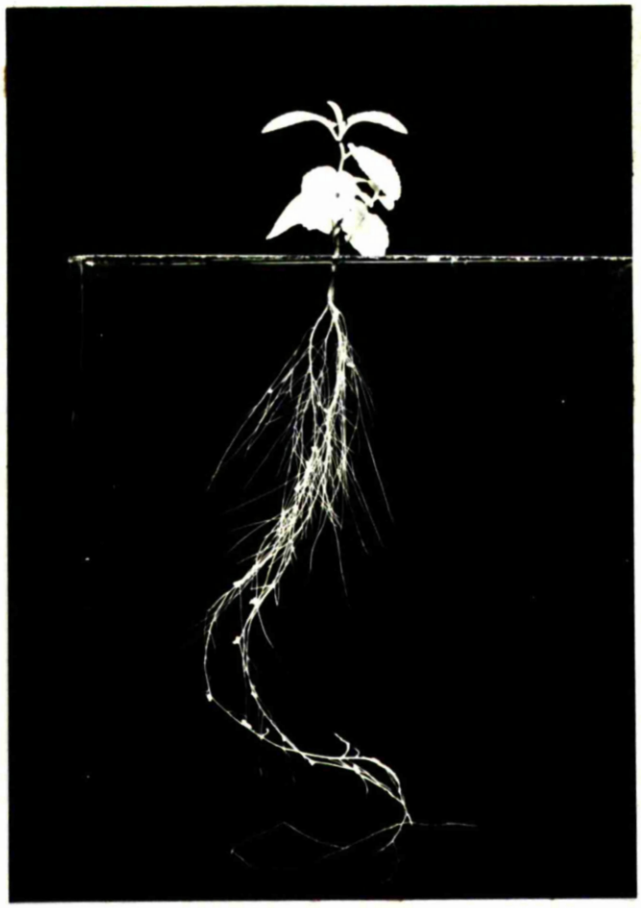
Wahlenberg (1930) referred to the rapid regeneration of Ceanothus, especially C.velutinus, after forest fires, and pointed out that the nodules, if they were nitrogen-fixing, might play a valuable part in restoring fertility after the loss of soil nitrogen by fire. Quick (1944), reviewing work on plant succession in the Sugar Pine Forests of California, discussed the importance of C.cordulatus as a pioneer in such areas and the possibility of nitrogen fixation by the nodules. It is tentatively suggested that the genus, because of its widespread abundance, may assist soil fertility and be an important factor in the development of high quality sites for the growth of timber. The data presented on the growth of seedlings in pot culture appeared to indicate such a soil improving effect for Ceanothus although no definite conclusions as to nitrogen fixation were actually drawn.

Bond (1957b) raised two seedlings of Ceanothus azureus and secured nodulation by the application of a crushed-nodule inoculum prepared from the combined nodular material of C.velutinus and C.integerrimus sent by air-mail from California. These two plants showed clear ability

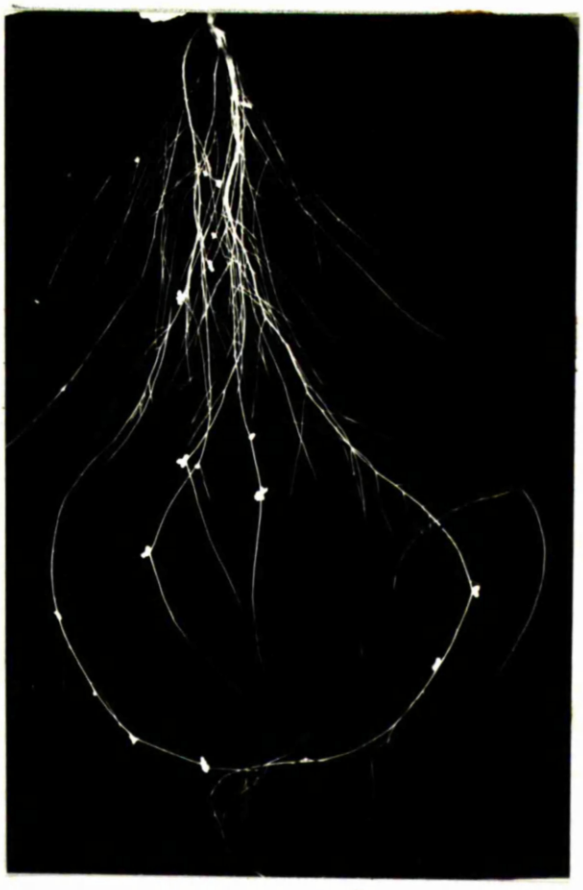
to grow in water culture free of combined nitrogen. By exposure of the root system of one of these plants to excess free $^{15}N$ quite clear evidence of the occurrence of fixation in the root system, and by analogy with other genera, in the nodules in particular, was obtained. Plate 13 shows one of these plants. So far as is known these data provide the only published evidence of fixation in Ceanothus nodules.

Recently, however, access has been obtained to an unpublished thesis presented at the Michigan State University of Agriculture & Applied Sciences, by Petry (1925) in which there is a valuable study of Ceanothus with special reference to nodule formation. Petry secured nodule formation in C.americanus by germinating seed in habitat soil and then compared the growth of nodulated and non-nodulated plants in the greenhouse in a sandy soil fairly low in nitrogen and also in a fertile greenhouse compost. The data shown in Table 10 were obtained after eight months' growth. The improvement of growth attending the presence of nodules is clearly marked particularly in the sandy soil. Since this soil was by no means free of combined nitrogen, Petry felt unable to reach a firm conclusion that nodules were nitrogen-fixing and referred to an alternative possibility that the presence of the nodules might in some unknown way have stimulated the roots to absorb more combined nitrogen from

Plate 13.



(x 2/5)



(x 4/5)

Nodulated plant and root system of Ceanothus azureus grown in nitrogen-free culture solution by Bond in 1955.

the soil. Petry also showed that there was a correlation between vigour of the plants in the field and the number of nodules borne and that the removal of nodules led to a loss of vigour.

In view of the possible importance of Ceanothus ecologically and in connection with forestry the present author planned a series of experiments for the further study of the development and function of these nodules. The experiments of Bond (1957b) in Glasgow described above, suggested that no particular difficulty attended the cultivation of nodulated Ceanothus plants in water apart from breaking the strong dormancy of the seed. Information on this last aspect was subsequently obtained.

Table 10.

Comparison of growth of nodulated and non-nodulated plants of Ceanothus americanus in two soils (from Petry, 1925).

| Soil Type | Non-nodulated Plants | | Nodulated Plants | |
|---|---|---|---|---|
| | Mean air-dry weight per plant (mgm.) | Mean N content per plant (mgm.) | Mean air-dry weight per plant (mgm.) | Mean N content per plant (mgm.) |
| Sandy, low N | 70 | 2 | 430 | 11 |
| Greenhouse compost | 440 | 9 | 840 | 17 |

## Methods and Results.

As already implied, Bond in his 1955 experiments obtained poor germination of Ceanothus seed. On closer study of the literature relating to this genus, however, it became clear that the seed is apt to exhibit persistent dormancy unless drastically treated. Petry (1925) broke this dormancy either by treatment with concentrated sulphuric acid or hand scarification with sand paper, Van Dersal (1938) by heat treatment, and Quick (1944) by cold treatment followed by heat. It is of interest in this connection that the rapid appearance of Ceanothus seedlings after forest fires is thought to be partly due to the breaking of the seed dormancy by the heat of the fire.

The present author achieved satisfactory germination ranging up to 75% with several species of Ceanothus. The seeds were either scarified with sand paper or immersed in water, heated to 80°C and left thus for five minutes. As the quantity of seed of any one species was limited, comparisons of treated or untreated seed were not attempted. However, where possible different treatments were compared for a particular species, and it would seem that different species require specific treatments. On scarification with sand paper C.foliosus gave 40% germination although no germination was obtained when heat treatment was applied.

Some species gave no germination despite differing pre-treatments; probably the seed was non-viable.

In 1956, 50 young plants of C.rigidus, C.rigidus Var. albus, and C."Topaz", raised from seed obtained from the Royal Botanic Garden, Edinburgh, were set up in the usual manner in water culture at pH 5 or 6 and given low levels of combined nitrogen pending the arrival of the nodules for inoculation. Growth of the plants was satisfactory and very long white roots were formed.

Nodules from C.integerrimus were received early in June by air-mail from Dr. W.W. Wagener, of the California Forest & Range Experiment Station, U.S. Department of Agriculture. Owing to the lateness of the spring thaw, however, the nodules had proved difficult to obtain and only a small quantity was sent. An inoculum was prepared by grinding 0.3 gm. nodules with 3 ml. water and applied to the roots in the usual way. No nodules resulted from this inoculation, although the plants remained reasonably healthy (considering they were in nitrogen free solution) and were kept under observation for a period of four months after inoculation.

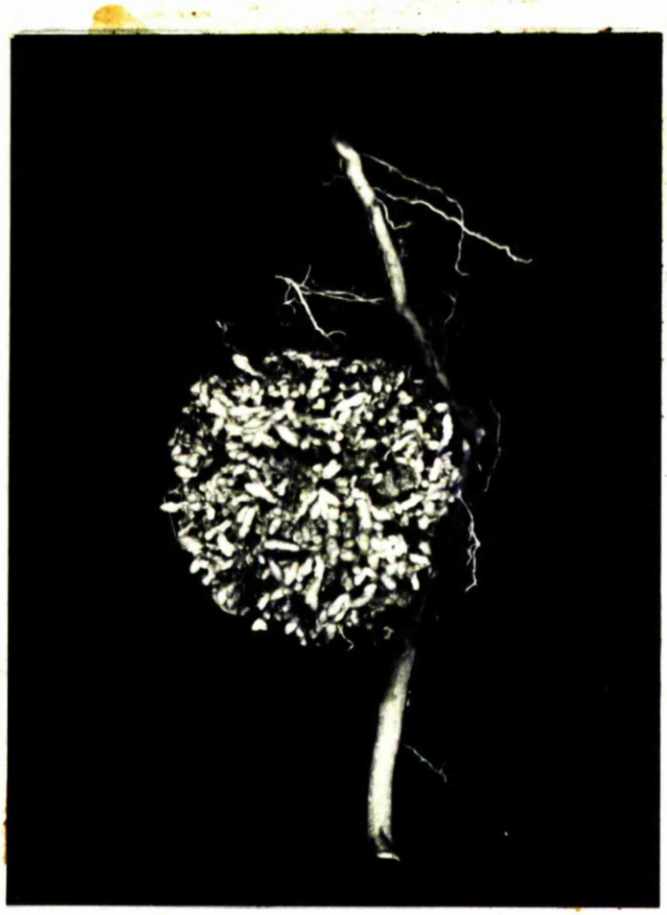
Since the treatment of these plants differed in no material way from Bond's tests of the previous year, this failure to obtain nodulation was rather surprising. Admittedly the plants grown did not include Bond's species

(C.azureus) since seed was unprocurable, while the nodules used for inoculation were from C.integerrimus rather than from that species and C.velutinus, but at the time these differences were not thought important. The failure of nodulation was thus ascribed to the small quantity of inoculum available.

As, in the spring of 1957 an offer of nodules was received from the Director of the Forest & Range Experiment Station in Portland, Oregon where large quantities of easily accessible material were available, further sowings of seed were made and 150 seedlingsset up in water culture. Some 200 seeds of C.azureus were obtained from Thompson & Morgan, Ipswich, but despite various pre-treatments none germinated. The seedlings used were chiefly of C.thyrsiflorus and C.foliosus with smaller numbers of C.rigidus and C.parryi.

Three generous samples of nodules were received by air-mail from Roseburg near Oregon at the beginning of May, June and July respectively, arriving 6 days after collection apparently in good condition. Some very large nodule clusters were included as shown in Plate 14. The first two nodule consignments were of C.velutinus, var laevigatus, while the third contained in addition nodules of C.integerrimus. On receipt of each batch of nodule material, inocula were immediately prepared by grinding 10 gm. nodules in 100 ml. water and applied to the roots in the usual way.

Plate 14.



Large nodule cluster of Ceanothus velutinus, var. laevigatus collected from Roseburg, Oregon.

(X 2/5)

Despite these repeated inoculations no significant nodulation was obtained. Only in September 1957, 9 weeks after the final inoculation, did one to two nodules appear on the roots of each of three plants. As in the previous year, the plants remained in healthy condition, apart from the expected symptoms of nitrogen deficiency during the summer.

## Discussion.

The establishment of a symbiosis under artificial conditions is liable to prove difficult at any time when little previous information is available, but it is especially so when there is the added complication of obtaining endophyte material from abroad.

Nodulation was, however, achieved by Bond in 1955 (see above) and in light of this much thought was given to the experimental procedure which in this case has proved unsuccessful. The only obvious substantial difference was that the Ceanothus species grown did not include that in which nodulation had preciously been established and although a mixed inoculum from C.velutinus and C.integerrimus was used in the final inoculation, previous material was from C.velutinus alone. It is possible that in this large genus (as in the Leguminosae) there is a species specificity, in that not all species are cross-inoculable, the few nodules which were obtained in the present author's experiments being due to the presence of the correct endophyte in the soil adhering to inoculum material. However, this aspect would be difficult to investigate unless easier access to seed and nodules of a range of species was available. Attempts to secure nodules of the species of seed available were made, but proved unsuccessful.

A second possibility is that infection from crushed-nodule inocula is unreliable in Ceanothus and that Bond happened to be fortunate. This, it may be noted, seems to be true in Coriaria according to Glasgow experience and that of two other investigators (Bond, personal communication). It appears that the organism in the nodules of this genus is not capable of further infection and infection can only occur by means of cells of the endophyte existing in the soil.

## SUMMARY OF SECTION I.

1. Nodulation of Plant.

Successful methods of obtaining nodule formation on the roots of Shepherdia canadensis, Nutt., Elaeagnus angustifolia L., and Myrica cerifera L., growing in nitrogen-free culture solution have been found. Attempts to obtain nodulation in various species of Ceanothus have so far proved unsuccessful. An improved method of culturing nodulated plants of Myrica gale L. has been developed.

The effect of variation of the pH of the culture solution on nodule formation in Shepherdia and Elaeagnus has been studied and results very similar to those obtained in previous investigations in this Department for Hippophaë are reported.

The presence of combined nitrogen in the culture medium also produced an effect on the nodulation of Shepherdia similar to that on Hippophaë. The addition of small amounts - 5 mgm. nitrogen per litre of culture solution - inhibited the formation of nodules.

2. Fixation of Atmospheric Nitrogen.

By means of long-term growth experiments it has been demonstrated that the root nodules of Shepherdia and Elaeagnus are capable of fixing atmospheric nitrogen. These findings

together with the previous work on Hippophaë complete the record of nitrogen fixation for the Elaeagnaceae.

A short term isotopic experiment employing $^{15}N$ has shown that nitrogen fixation is associated with the nodules of Myrica cerifera.

3. Ecological Significance.

It has been pointed out that these non-leguminous nodule plants are widely distributed at the present time, occurring typically in poor soils such as sandy shores, recently deglaciated areas or land devastated by fire. By the possession of special facilities for utilising atmospheric nitrogen these plants would be able to enrich these soils and thus allow them to be colonised by other plants. Fossil pollen evidence indicates that Shepherdia and Elaeagnus occurred in post-glacial times and it has been suggested that these non-legume plants may also have been important biogeochemically in the past.

# SECTION II

# BIOCHEMICAL STUDIES

## SECTION II.

## INTRODUCTION.

Within the last twenty years many biochemical problems hitherto virtually insoluble have been elucidated by the use of isotopic tracer methods together with the application of the more recently developed chromatographic techniques. Our knowledge of nitrogen metabolism in particular both in plants and animals has advanced greatly since the stable isotope of nitrogen, $^{15}N$, became generally available and the techniques of separating homologous amino acids established. Recent biochemical studies on the pathway of biological nitrogen fixation have involved both these processes.

A review of the chemical mechanism of nitrogen fixation by Wilson (1940) revealed two main theories as to the nature of the key intermediate between molecular nitrogen and the first organic nitrogen compound assimilated. Those were the classical "ammonia" theory and the more recent "hydroxylamine" theory.

By analogy with the artificial industrial fixation of atmospheric nitrogen and the nitrogen metabolism of ordinary plants, many workers suggested, without any experimental basis, that ammonia was an intermediate in biological nitrogen fixation. Kostytschew & Ryskaltschuk (1925) showed that 25% of the nitrogen excreted by a young culture

of Azotobacter consisted of ammonia, the remainder being amino nitrogen. This excreted nitrogen was taken as representing intermediates of fixation rather than decomposition products, and Kostytschew thus claimed ammonia as the first product of fixation followed by the amino acids.

the first product of fixation followed by the amino acids.

This work was criticised on the grounds that the cultures may not have been pure and that the compounds excreted need not have been initial in formation. Winogradsky (1936) claimed that ammonia was also the key intermediate in symbiotic fixation by legume nodules but his claim was not upheld by any reliable experimental evidence.

The more popular hydroxylamine theory had been proposed by Blom (1931) on purely hypothetical grounds. However, the finding that aspartic acid was the initial product excreted by young legume nodules led Virtanen & Laine (1939) to propose the following mechanism:-

$N_2$ ⟶ hydroxylamine + oxalacetic acid ⟶ oxime-succinic acid ⟶ aspartic acid

The subsequent finding of oxalacetic acid in legume plants together with the detection of traces of the oxime in excreted products was regarded as further proof that hydroxylamine was the key intermediate of fixation. A similar excretion of oxime had been reported earlier by Andres

(1934) from Azotobacter where it had been regarded as a by-product of metabolism. Virtanen (1939) rejected the ammonia hypothesis on the grounds that only once had a trace of glutamic acid been found excreted and in contrast to hydroxylamine, ammonia reacts equally well with oxalacetic acid and $\alpha$-ketoglutaric acid. Hence, if ammonia is an intermediate both aspartic and glutamic acids should be formed and excreted. The formation of aspartic acid from ammonia and fumaric acid was also ruled out as no aspartase had been detected in the nodules (Virtanen & Laine, 1936).

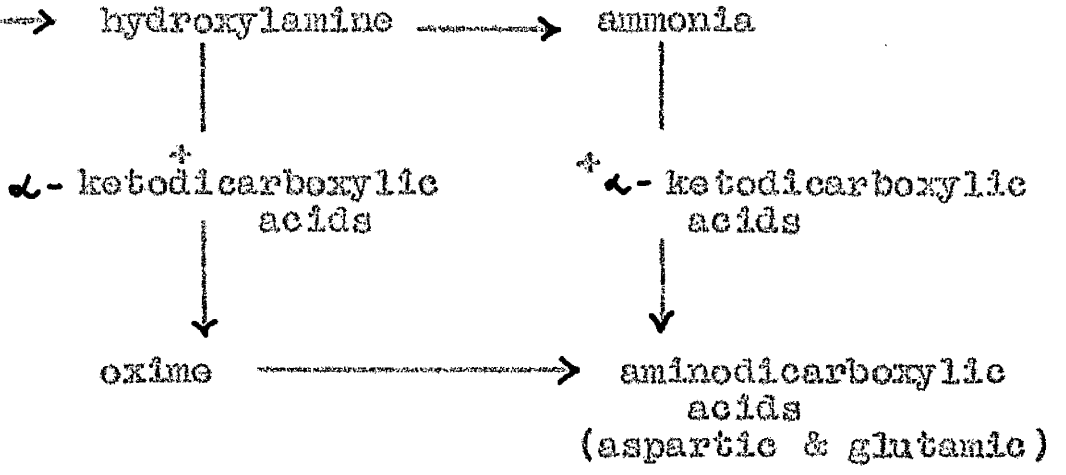
Thus in 1940 the intermediate role of hydroxylamine in nitrogen fixation appeared the more attractive theory. Later, however, several workers attempted to repeat the work of Virtanen on legume nodules and to investigate further the products excreted. These workers failed to obtain any substantial excretion of nitrogen from the nodules and it was impossible to gain confirmation of Virtanen's results. Virtanen's work was also criticised as there was no evidence that the compounds excreted bore any relation to the initial compounds of fixation.

In the early nineteen forties the Wisconsin group associated with P.W. Wilson and R.H. Burris, introduced the use of the stable isotope of nitrogen $^{15}N$ as a tracer in the study of the pathway of nitrogen fixation. Burris (1942) studied the distribution of fixed $^{15}N$ in Azotobacter which

had been supplied with excess free $^{15}N$ for short periods. Although hampered by the incomplete separation of the amino acids, Burris showed that the greatest percentage excess $^{15}N$ was in the glutamic and aspartic fractions and indeed that the glutamic showed the greater enrichment. This high level of $^{15}N$ in glutamic acid was regarded as evidence in favour of ammonia rather than hydroxylamine as the intermediate of fixation.

Further data pointing to the significance of ammonia in fixation by Azotobacter was provided by Burris & Wilson (1946). A culture grown on molecular nitrogen was supplied with ammonia labelled with $^{15}N$. The cells immediately stopped fixing nitrogen and used the ammonia as their exclusive source of nitrogen. When $^{15}N$-labelled nitrate was supplied, however, no $^{15}N$ was found in the cells for 30 minutes, in contrast to its detection in one minute when $^{15}N$ ammonia was supplied. It was thus concluded that cells grown on nitrogen have preformed within them all the enzymes necessary for the utilisation of ammonia, hence when ammonia is supplied from an external source it is incorporated immediately into the cells. This also suggested that in the fixation of nitrogen ammonia was an intermediate. The Azotobacter, on the other hand, must adapt itself to the utilisation of nitrate.

Mainly in view of this new isotopic evidence Virtanen (1947), amended his postulated scheme for the mechanism of fixation. The fact that at this time no enzymes were known which would catalyse the reaction between hydroxylamine and the α-ketodicarboxylic acids whereas that forming glutamic acid from α-ketoglutaric acid and ammonia was well known in plants, also influenced Virtanen. However, the isotopic results do not exclude the possibility of hydroxylamine as a precursor of ammonia in fixation and thus Virtanen suggested that possibly hydroxylamine was chiefly reduced to ammonia and the formation of the oxime was a non enzymatic side reaction, thus:-




Rosenblum & Wilson (1950) established that Clostridium pasteurianum excreted into the medium a considerable portion of the nitrogen fixed. As up to 50% of the total nitrogen fixed may be excreted by this anaerobe and as excretion begins in the early stages of growth, it was thought

that such excreted nitrogen might arise as an intermediate in fixation rather than a product of metabolic decomposition. Zelitch, Rosenblum, Burris & Wilson (1951a) supplied actively fixing Clostridium cells with $^{15}N_2$ for a short time. Free ammonia with an extremely high $^{15}N$ concentration was isolated from the medium. Somewhat lower concentrations, but still considerably in excess of the average either of intact cells or supernatant medium, were found in the amide fraction. These data with the anaerobe provide direct support for the view that ammonia is a key intermediate in biological nitrogen fixation.

The chromatographic separation of amino acids developed by Moore & Stein (1948) first on starch and later on ion exchange columns offered a means of studying the chemical pathway of nitrogen fixation and was employed by Zelitch, Rosenblum, Burris & Wilson (1951b) to study the distribution of fixed $^{15}N$ in Clostridium. After exposure to free nitrogen or ammonia enriched with $^{15}N$, the cells were hydrolysed with 6N HCl. The amino acids of the hydrolysate were separated on a starch column. Regardless of how the $^{15}N$ was supplied to the cells the $^{15}N$ was found in highest concentration in the glutamic acid. Ammonia, alanine and aspartic acid had an appreciable enrichment while the basic amino acids were low in $^{15}N$. If ammonia

is an intermediate in nitrogen fixation and is used to form glutamic acid, the $^{15}N$ concentration of the free ammonia should be as high as or higher than that of the glutamic acid. It was argued, however, that the hydrolysis of the cells would liberate older unlabelled ammonia from amides and thus dilute the $^{15}N$ of the free ammonia. These results together with the previous findings of Zelitch et al. (1951b) that Clostridium excreted extremely highly labelled ammonia into the medium in a very short time were taken as indicative of the role of ammonia in fixation in Clostridium.

On the principle that information concerning different types of nitrogen fixing agents is inherently interesting and useful in examining the comparative biochemistry of fixation, other such organisms were examined in the above manner. Wall, Wagenknecht, Newton & Burris (1952) investigated the distribution of fixed $^{15}N$ in the photosynthetic bacteria, Rhodospirillum, Chromatium and Chlorobacterium, while Magee & Burris (1954) studied the blue-green alga Nostoc. In each organism an isotope distribution similar to that found in Clostridium was obtained whether $^{15}N$ supplied as nitrogen or ammonia.

The Wisconsin group have also carried out similar experiments with legumes, but greater technical problems arose here. Large numbers of plants were required to yield sufficient nodule material for isotopic assay after

fractionation, and to have wholly enclosed the plants to expose their nodules to $^{15}N$ would have been very extravagant in isotope. To expose the root systems alone was more economical but involved the use of rather dubious sealing devices at the base of the shoots while it was also known that the labelled free nitrogen tended to escape through the shoot tissues to the external atmosphere. With either procedure part of the labelled products of fixation would be lost from the nodules into the plant during the period of exposure.

There would be many advantages if nodules could be detached prior to exposure to the $^{15}N$. However, ever since nodules were realised to be the site of fixation, unsuccessful attempts were made to demonstrate that these nodules could continue active fixation after detachment. It had been hoped that the use of the highly sensitive isotopic method would detect fixation. Burris, Eppling, Wahlin & Wilson (1943) however, found only occasional positive evidence for fixation in detached legume nodules using $^{15}N$. Machata, Burris & Wilson (1947) and Tove, Niss & Wilson (1950) tried to stimulate fixation by adding various substrates to the nodules, but in both cases results were still inconclusive. Therefore in the first fractionation experiment Zelitch, Wilson & Burris (1952) exposed the root systems of

nodulated Soybean plants to an atmosphere enriched with $^{15}N$. The subsequent distribution of the isotope in the hydrolysed acid extract of the nodules once again revealed that glutamic acid contained over twice as much $^{15}N$ as any other amino acid and over three times that of aspartic acid.

In 1952, however, Aprison & Burris, by restricting their isotopic analyses to the soluble nitrogen fraction of the nodule rather than the total nitrogen, obtained consistent evidence of fixation by excised legume nodules. The results indicated that fixation decreased rapidly with time, and ceased completely after six hours. The fixation after detachment is insufficient to increase the average $^{15}N$ content of the total nitrogen of the nodule by a significant amount. Using detached nodules Aprison, Magee & Burris (1954) further investigated the chemical pathway of fixation in Soybean nodules. The distribution of fixed $^{15}N$ was examined after exposure periods of 1 to 2 hours. As appreciable quantities of amide nitrogen were present, precautions were taken to avoid hydrolysis of the extract before fractionation. Despite this the free ammonia and the amide ammonia whether from asparagine or glutamine had much lower $^{15}N$ concentrations than glutamic acid, which contained the highest of any amino acid or ultra violet-absorbing compound examined. Aprison *et al.* attributed the low labelling of the free ammonia to its dilution by ammonia

produced catabolically by senescent tissue of the nodule. The results were therefore regarded as in accordance with the general picture of nitrogen fixation formed by examination of other organisms.

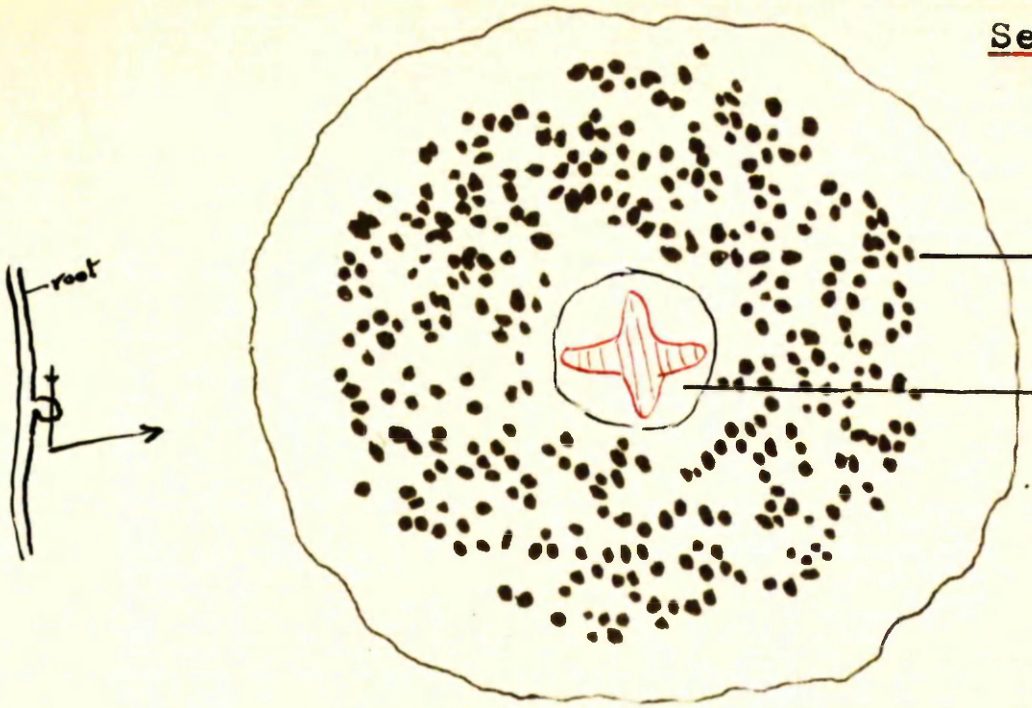
It would thus appear that the diverse systems examined by the Wisconsin workers, namely, aerobic and anaerobic bacteria, photosynthetic bacteria, blue-green algae and legume nodules possess the same end product of fixation, namely glutamic acid. This is considered by the Wisconsin workers to be in accordance with the proposed pathway for fixation given on page 62 , with the amino acid synthesis from ammonia being the main pathway in a quantitative sense. Although these workers have attempted to investigate further the suggested role of hydroxylamine in fixation, greater technical difficulties arise and so far no conclusive results have been obtained, while Virtanen (1956) has found it impossible to renew his earlier results concerning the presence of oximes in legumes.

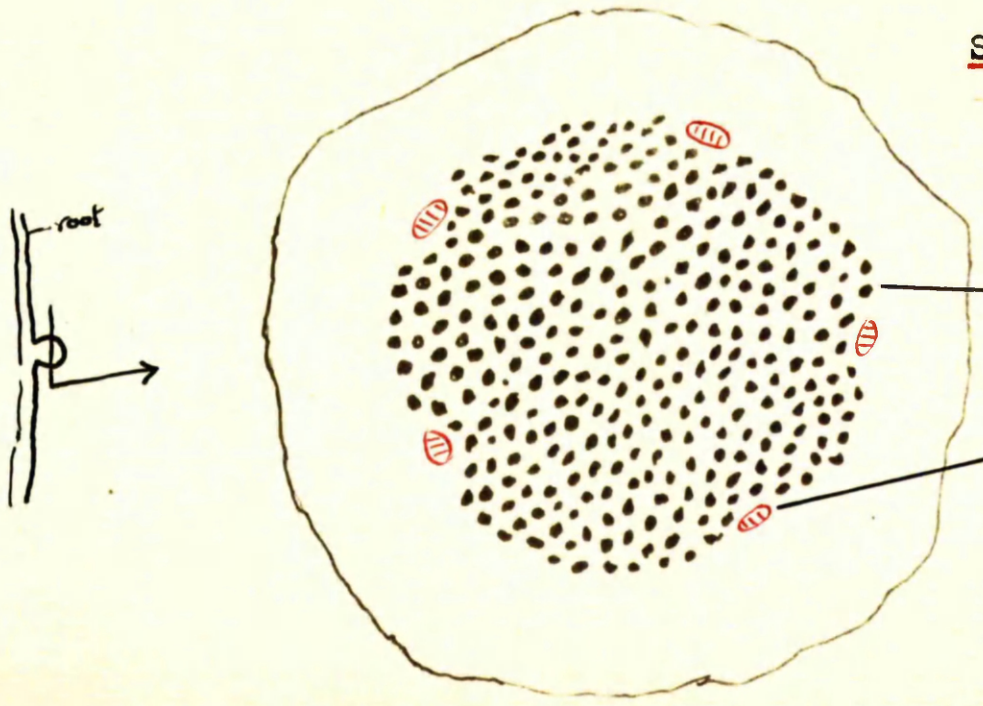
Most of the recognised nitrogen fixing systems have thus now been investigated. However, no attention had been paid to the non-legume root nodules. As mentioned in the General Introduction and from the results presented in Section I of this thesis, it is clear that these nodules possess the same physiological significance as do legume nodules. The non-legume nodules, however, differ greatly

from their legume counterparts in the nature of the endophyte and the organisation of the tissues (see Figure 2). It was thus of interest to investigate the mechanism of fixation in these nodules. It was considered that such an investigation gave the opportunity for a comparative biochemical study, which might well prove of value in elucidating the fixation process as a whole. By virtue of its greater capacity for fixation after detachment from the plant (Bond, 1957b) the non-legume nodule provides more favourable material than previously available for studying the symbiotic fixation process. Since the alder had been most extensively studied with respect to fixation in Glasgow, it was chosen for the first biochemical studies.

In preparation for isotopic experiments some preliminary knowledge of the nitrogenous constituents of the plant is required. The free amino acids of two European species of alder, A.glutinosa and A.incana, have been studied by paper chromatography by Miettinen & Virtanen (1952). Citrulline was shown to be the predominating free amino acid of the nodules, accounting for about 0.2% of the nodule dry weight in summer, rising to 0.9% in autumn and to almost 2.0% in winter. It also occurred in smaller quantities in the roots and leaves of non-nodulated plants grown on combined nitrogen, and hence it was not associated specifically with nitrogen fixation. This appears to be the first

Figure 2.


root
Section of
Non-Legume nodule.
large infected cells in cortex
vascular system - nodule represent modified lateral root.


root
Section of
Legume nodule.
central region containing large infected cells, surrounded by ring of vascular strands.

report of citrulline in higher plants since its discovery by Wada (1930) in the water melon. It became well known however, in the intervening years in animal metabolism as an intermediate in the Krebs "ornithine cycle". Other amino acids found in alder nodules by Miettinen & Virtanen were glutamic and aspartic acids, γ-amino butyric acid and traces of arginine and ornithine. No amides were found in the alder, a striking difference from the legume nodule in which asparagine and glutamine were abundant. Miettinen and Virtanen attributed to citrulline in the alder the role of nitrogen storage and transport usually attributed to the amides of other plants.

As the methods of Miettinen and Virtanen (loc. cit.) yielded only semi-quantitative data, a further quantitative survey of the individual amino acids present in the free state in the nodules of the alder was desirable. This was particularly so when subsequent $^{15}N$ investigations were planned, since ~~the~~ relatively large samples of nitrogen are required for assay in a mass spectrometer. The yield of individual amino acids to be expected from a given weight of nodules had thus to be determined.

The work to be described in this Section of the Thesis consists therefore of experiments with Alnus glutinosa and Myrica gale in which the nodules are exposed to excess $^{15}N$ and their nitrogenous fractions assayed for isotope content. Studies of the nature

of the nitrogenous substances present in the nodules of Hippophaë rhamnoides and Casuarina cunninghamiana have also been made but these nodules have not been exposed to $^{15}N$.

The work described in this part of the Thesis represents a joint investigation conducted by the author, Dr. G. Leaf and Dr. G. Bond. The latter suggested the investigation and provided the alder plants utilised, while he and the author jointly carried out the exposure of all the nodule material to excess $^{15}N$. The subsequent analytical and chromatographical operations were performed under the supervision of Dr. Leaf. In the case of the Alnus material the operations were carried out jointly with him, but the author personally performed the analytical operations on the material of the three other genera.

## METHODS.

### Plant Material Used.

Exploratory work on the extraction and fractionation of the free amino acids of *Alnus glutinosa* was carried out using field nodules collected from Milngavie and Stockiemuir, Dunbartonshire. For the main experiments involving exposure to excess free $^{15}N$ material was provided by first-year alder plants which had been reared from seed in the greenhouse. At the two-leaf stage the seedlings had been transplanted from the sand in which they germinated into nitrogen-free Crone's culture solution (pH 5-6) and inoculated in the usual manner (see General Methods, Section I). The nodule material was exposed to an atmosphere containing excess free $^{15}N$ in 1955 between 8th and 14th September, by which time the plants had attained a mean average height of 16 cm. Further exposures of nodules from similar material were made in August, 1956 and 1957. Typical plants used are shown in Plate 15.

*Myrica gale* nodules were likewise obtained from greenhouse-reared plants, in this case in their second season of growth. Typical plants are shown in Plate 10, Section I. Nodules were exposed to excess $^{15}N$ between 30th July and 9th August, 1956. Material for paper chromatographical analysis of *Hippophaë* and *Casuarina* was

Plate 15.

(x 1/8)

(x 4/7)

First year nodulated alder plants typical of those exposed to excess free $^{15}N$.

also obtained from plants reared in the greenhouse, in the latter case by Dr. G. Bond. Nodules of Alnus rubra were obtained from material sent by air-mail from America.

Exposure of Nodules to excess free $^{15}N$.

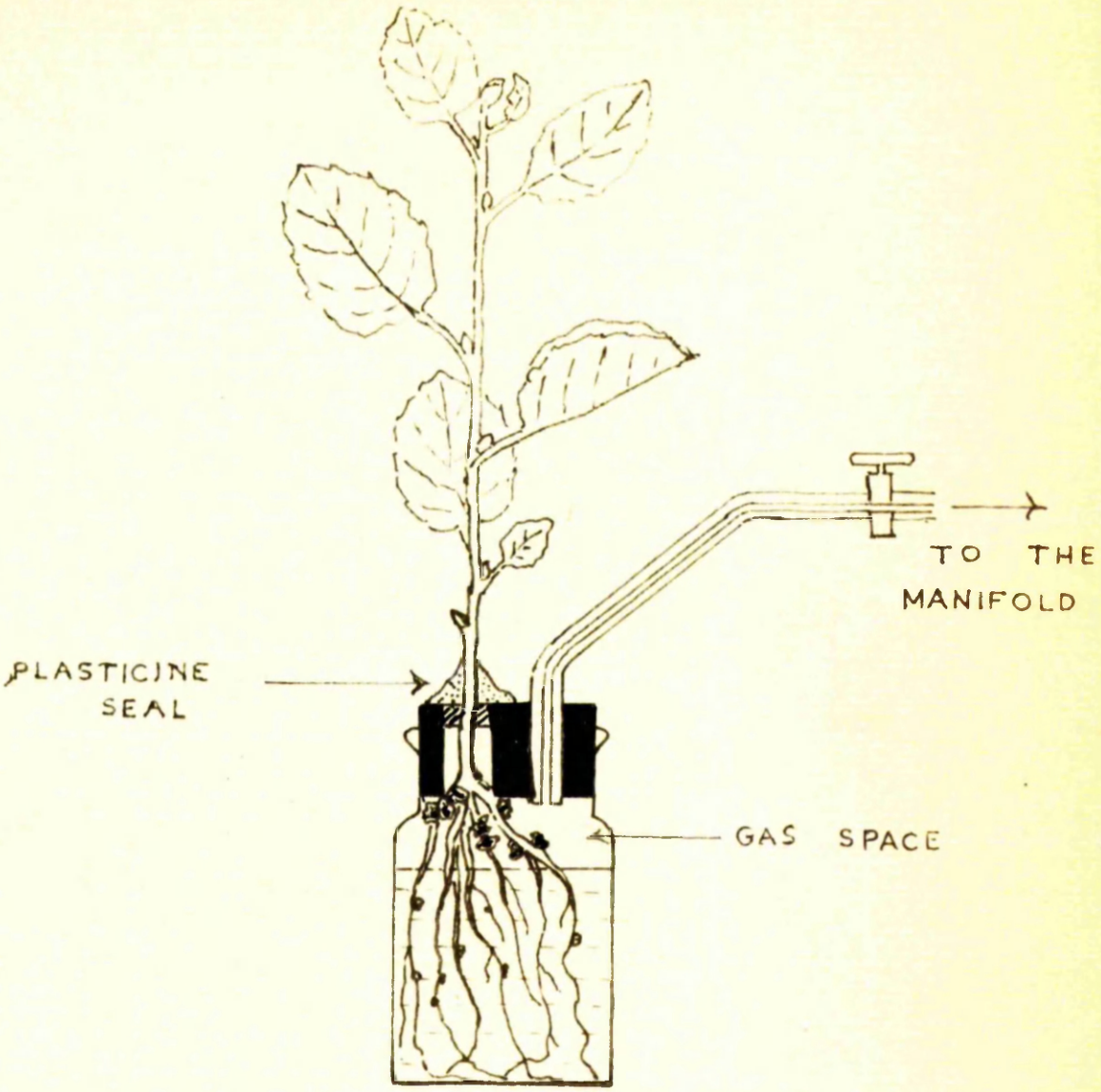
Although detached nodules are much simpler to work with than whole plants, it was desirable to ascertain whether detachment in any way altered the course of the fixation in the nodule. It must be remembered that when using detached nodules in short term experiments irregular metabolism due to injury may have an important effect, while in longer exposures there may be an unnatural accumulation of nitrogen compounds in the nodules since no translocation to the roots and shoots can occur. In the 1955 experiments with alder therefore, nodules were in some cases exposed to isotopic nitrogen before detachment from the plant and in other cases after detachment. In all subsequent isotopic work detached nodules were employed.

For such experiments gaseous nitrogen containing excess $^{15}N$ was prepared in a nitrometer by the action of sodium hypobromite on ammonium nitrate containing 36 atom per cent. $^{15}N$ in the ammonium radical. The nitrogen was transferred to a gas burette and thoroughly shaken with 5% sulphuric acid to remove any traces of labelled ammonia which might be present. Using this enriched nitrogen gas

mixtures of the desired composition were then prepared.

When attached nodules were to be exposed to excess $^{15}N$ the procedure described by Bond (1955) was followed. The plants intended for use were inserted at an early stage in growth into special 2 holed rubber stoppers designed to fit the bottles in which the root systems were to be exposed to the gas. Until this time the plants and stoppers were placed in large tubes of culture solution. When desired the stoppers and plants were fitted into wide necked bottles containing culture solution and a gas space of 30 ml., as shown in Figure 3. The base of the stem was sealed to the rubber stopper with plasticine. Ten bottles were then attached to a capillary manifold and the whole system evacuated to $^{3}/_{5}$ of an atmosphere five times, being refilled on each occasion with argon. This was considered the most effective procedure as under complete evacuation the plasticine seal does not always remain air-tight, while injury of delicate plant tissue may also result from complete evacuation. Finally the system was evacuated to half an atmosphere and the special gas mixture, 40% oxygen, 20% nitrogen (36 atom% $^{15}N$) and 40% argon, was admitted until the pressure was restored to normal. By this procedure the root systems were exposed to a gas mixture initially consisting of 20% oxygen, 10% enriched nitrogen and 70% argon. The bottles were left in open connection

**Figure 3.**

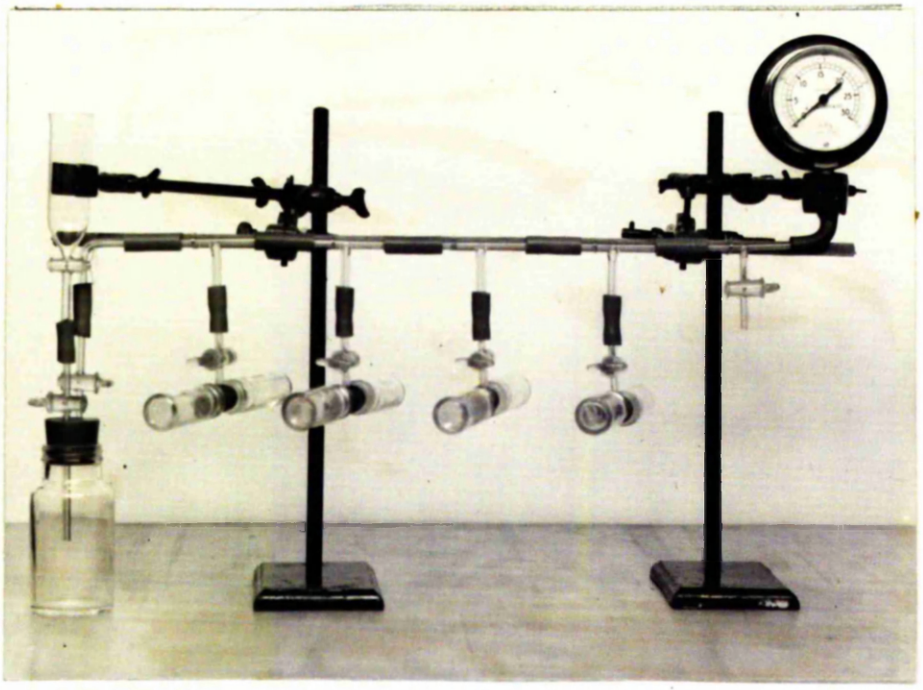



Arrangement used for exposing the root system of alder plants to an atmosphere containing excess free $^{15}N$. (X 1/2)

with the manifold for 3 hours, under the prevailing greenhouse conditions (average temperature 25°C). The plants were then removed from the bottles and the nodules picked off as quickly as possible and stored in solid carbon dioxide prior to extraction. Four such exposures were made in September (11-14th) 1955, the average weight of nodules obtained in each run being 5.8 gm.

When nodules were to be exposed to excess $^{15}N$ after detachment from the plant the nodules were placed in special tubes and those attached to the manifold as shown in Plate 16. The tubes employed were of 25 ml. capacity and 1 gm. nodules together with 0.5 ml. culture solution was placed in each. In early experiments 8 tubes were attached to the manifold for each exposure. This procedure involved removing nodules from an average of 37 plants, which took about 30 minutes. As the rate of fixation falls off after the nodule is detached from the plant, it was desirable to keep this interval to a minimum and in later experiments only 4 tubes were attached to manifold at one time, thus reducing the interval between detachment and exposure to 15 minutes at the most. After attachment to the manifold the whole apparatus was fully evacuated and the previously prepared gas mixture (20% oxygen, 10% nitrogen with 36 atom per cent. $^{15}N$, and 70% argon) admitted until atmospheric pressure was restored. The tubes were closed

# Plate 16



Apparatus used to expose "detached" nodules to an atmosphere containing excess free $^{15}N$.

(X 1/8)

Photograph by courtesy of Dr. G. Bond.

and removed to a 25°C incubator. After the desired exposure to the $^{15}N$, the tubes were opened and the nodules removed and stored in solid carbon dioxide prior to extraction.

Nodules used in experiments in 1955 were exposed to excess isotope for a period of 1½ hrs. The results of these exposures, together with further evidence on fixation by detached alder nodules obtained by Bond (1957b), led to the period of exposure in subsequent experiments being shortened to ½ hour. The shorter the exposure time the easier it should be to distinguish initial products of fixation. Myrica nodules were exposed to $^{15}N$ for periods of 2 to ¼ hour.

Extraction of Nodules.

Unlike the radioactive isotopes, the assay of stable isotopes in a mass spectrometer requires a relatively large amount of material. In the case of $^{15}N$, a minimum of 0.4 mgm. nitrogen is required. Since amino acids are normally present in living cells in the free state in only small proportions, an experiment using $^{15}N$ and designed to investigate the free amino acids specifically must be conducted on a very large scale. In their early experiments the Wisconsin workers obtained the necessary quantities of nitrogen by hydrolysis of their 4N HCl extracts. It is, however, possible that an amino acid which is present only in minute amount in the free state could possess a high

$^{15}N$ content. It is equally possible that large quantities of this amino acid could occur bound in peptide or protein of the cells. Isotopic assay of the amino acids after hydrolysis would record a low $^{15}N$ content for this amino acid, in no way reflecting its original high enrichment in the free state. On the other hand, amino acids present in relatively large quantities in the free state (e.g., glutamic acid) may not be diluted to an equal extent on hydrolysis and thus an ambiguous pattern is obtained for the distribution of the fixed $^{15}N$ in the cells. For this reason it was desired to investigate specifically the free amino acids of the alder nodule.

In the Wisconsin experiments with excised legume nodules extraction was by 0.067M phosphate buffer pH 6.5 and protein was precipitated with basic lead acetate. This was rejected in the present work due to the tendency of the precipitate to adsorb amino acids and peptides.

Miettinen & Virtanen (1952) extracted the free amino acids of the alder nodules with 70% alcohol. Such an extract was found in the present work to contain substances which on drying produced a dark gum and interfered with subsequent separation. The amino acids were separated by Miettinen & Virtanen from this resinous material by absorption on the ion exchange resin, Dowex 2. However, as a quantitative separation of the amino acids was desired, there

were two objections to the above procedure. In the first instance Dowex 2 would not retain the basic amino acids and ammonia quantitatively and secondly it was found that part of the citrulline was in some manner changed on absorption onto Dowex 2. The exact nature of this change has not so far been elucidated, but ornithine was produced together with a compound which was eluted ahead of citrulline from Dowex 50 and yielded ornithine and citrulline on hydrolysis.

Since aqueous solvents did not appear to extract gum producing materials, preliminary trials were therefore conducted using four such extractants, namely, 0.1N HCl, 3N HCl, 0.1M phosphate buffer pH 6.8, and water. It was finally found that 0.1N HCl gave the highest yield of nitrogen extracted (approximately 20% of total nodule nitrogen), and on lyophilisation yielded a white friable residue. The addition of ground glass during the extraction was not found to improve the yield of nitrogen extracted. The nodules were therefore homogenised in roughly 10 gm. lots in an ice-cooled Nelco blender with 0.1N HCl (9.0 ml. HCl to 1 gm. nodules) for 5 minutes. After centrifugation at 0°C, the residue was re-extracted with half the volume of 0.1N HCl initially used. The resulting supernatants were then combined, freeze dried, and finally taken up in 5 ml. citrate buffer pH 3.1. In this form the extracts

were stored at -6°C until required for fractionation.

Preliminary experiments showed that 10 gm. of Alnus glutinosa nodules was the maximum quantity, the extract of which could be conveniently fractionated on a 0.9 x 150 cm. column of Zeokarb 225 (as described below). Therefore, of the nodules exposed in September, 1955, two 10 gm. lots of detached nodules were extracted separately (Extracts 1 & 2). This resulted in some bulking of material exposed on different days. The same procedure was followed with the attached nodules (Extracts 3 & 4). In 1956 ten one gm. batches of detached nodules were exposed on the same afternoon and extracted together immediately afterwards (Extract 5). A similar procedure was also employed with the nodules picked in 1957 (Extract 6).

Nodules of Myrica gale were extracted also in 10 gm. lots as described for the alder nodules in 1956.

Paper Chromatographic Analysis of the Nodule Extracts.

Preliminary quantitative investigations of the amino acid composition of nodule extracts of Alnus glutinosa, Alnus rubra, Myrica gale, Hippophaë rhamnoides, and Casuarina were carried out by means of two dimensional paper chromatography. Samples of the extracts were examined both before and after hydrolysis at 115°C for 24 hours. The solvent systems employed were butanol - acetic acid - water in the

ratio 4:1:5 by volume in the first dimension followed by water-saturated phenol (in the presence of ammonia vapour and KCN) in the second dimension. Whatman No.1 filter paper was used throughout. The amino acids were detected by spraying with 0.2% ninhydrin in 60% ethanol to which 1% acetic acid and a trace of collidine had been added. The colour was developed in an oven at 90°C for five minutes. The presence of citrulline was confirmed by spraying with the p-dimethylaminobenzaldehyde reagent of Fowden (1951).

Fractionation of Components of Alnus glutinosa Extracts.

The nitrogenous components of the alder extracts were separated by ion exchange chromatography on a column of a sulphonated polystyrene resin by the procedure of Moore & Stein (1954). Zeokarb 225, the British equivalent of the Dowex 50 used by the above authors, was employed, the resin having a nominal degree of cross linking of 5%. As preliminary fraction of the extract on Dowex 2 had resulted in a low yield of citrulline the extract was applied to the column directly with no previous manipulation except concentration to a suitable volume. The pH was then adjusted to 2.0 and the charge applied to a 0.9 x 150 cm. column of the Zeokarb 225. Elution was by citrate buffer of continuously increasing pH and ionic strength. The effluent from the column was collected in 1.5 to 2.0 ml. fractions on an auto-

matic fraction collector by the drop counting method.

Portions (0.1 to 0.2 ml.) of alternate fractions were analysed for amino nitrogen by the photometric ninhydrin method of Moore & Stein (1948). The modified ninhydrin reagent suggested by Cocking & Yemm (1954) was used because of its greater stability. A plot of colour intensity against effluent volume was then made in which each peak represents one or more ninhydrin positive substances. These were then tentatively identified by their position of elution from the column as compared with the pattern obtained by Moore & Stein (1954) for a prepared mixture of 50 components. A preliminary analysis of a protein hydrolysate had established that 18 amino acids were eluted in the same order as found by Moore & Stein. However, as the methods of extraction employed extract peptide material as well as amino acids, the identity and purity of each peak was confirmed by paper chromatography.

The presence of salt interferes with paper chromatographic separations. As elution from the column was by citrate buffer, the salt had to be removed before the samples could be applied to the paper. An electrodialytic method using ion exchange membranes described by Wood (1956), was tried, but was found unsatisfactory as the removal of the citrate ions proved extremely slow compared with the sodium. Thus by the time the salt had been completely removed a large

proportion of the amino acids had also been lost. The acidic and neutral amino acid containing fractions were therefore freed from salt by passing them through a column of Dowex 2 (0.9 x 1.2 cm.) in the hydroxyl form. The column was then washed with water until the effluent was neutral, when the amino acids were eluted with normal acetic acid (Drèze, Bigwood & Moore, 1954). Citrulline had previously been found to be recovered in low yields from Dowex 2 but normally there was such a high concentration of citrulline present in the extracts that paper chromatograms could be run with no previous removal of salt from the appropriate fractions. Even where desalting was necessary some citrulline was recovered and therefore for qualitative purposes this technique was satisfactory. The above authors also described a method for freeing basic amino acids from salt by adsorption on a column of Zeokarb 225 in the hydrogen form. This method proved unsatisfactory. It was found that by using the Zeokarb in the ammonium form better results could be obtained. After the charge had been applied to the column (0.9 x 1.0 cm.) it was washed with 0.01N acetic acid and the basic amino acids eluted with 0.05M ammonia.

Salt free samples from each peak on the effluent curves were examined by two dimensional paper chromatography (as described previously) both before and after hydrolysis at 115°C for 24 hours with 6N HCl. It was found that the

amino acids were eluted from the Zeokarb 225 in the same sequence in each experiment, this sequence being similar to that of Moore & Stein (1954). In contrast to this, however, the ammonia peak varied in position. In a test run with a mixture of known composition ammonia occupied the position recorded by Moore & Stein, being eluted just ahead of ornithine. As no such peak was obtained on fractionation of the alder extracts, it was at first thought that these extracts contained insufficient ammonia for detection. As ammonia cannot be detected by paper chromatography, it was not immediately realised that it was being eluted together with another amino acid. In one fractionation the ammonia overlapped the ornithine peak while in the majority of others it was found with the $\gamma$-amino butyric acid. After this was realised, the ammonia was separated from these fractions by making them alkaline and aerating it into 2% boric acid. The free ammonia in Extract 6, however, was removed before it was placed on the column for fractionation by making the extract slightly alkaline with calcium hydroxide and aspirating the ammonia into boric acid.

Due to the high concentration of citrulline in the alder extracts there was a certain failure of resolution in that the citrulline tended to overlap both the glycine and alanine peaks. Since both these amino acids were present

only as traces, no further separation was attempted.

Moore & Stein (1954) reported that under their fractionation conditions on a long column of Dowex 50 glutamine was almost totally destroyed. Alder nodules were thus examined independently for the presence of glutamine and traces of asparagine. It was also thought that the extraction with 0.1N hydrochloric acid as employed above might decompose glutamine, hence a sample of nodules was specially extracted with water and the protein precipitated with tungstic acid. The free ammonia was removed by making a portion of this extract alkaline and aerating the ammonia into 0.02N sulphuric acid. It was then estimated with Nessler's reagent. Any glutamine present in the extract could be detected by removing the amide nitrogen of the molecule as ammonia. The method employed for this purpose is described on page 88 for Myrica extracts. Any ammonia so liberated could then be aerated into sulphuric acid and estimated as above. By similar means the presence of asparagine in the extract was also investigated. By these methods, however, no trace of either glutamine or asparagine could be found. Further investigations were then attempted by removing the free dicarboxylic acids (aspartic and glutamic) from an extract of the nodules on a column of Amberlite I.R.4B (see page 86). The extract was then hydrolysed and subsequently re-examined for the presence of

dicarboxylic acids, but none were found.

In the 1955 experiments each fraction from the column was also examined for the absorption of ultra-violet light of wavelength 260 mμ. This was carried out using a Unicam spectrophotometer (S.P. 500).

Degradation of the Citrulline Molecule.

The citrulline isolated from the alder extracts was found to contain a considerable excess of $^{15}N$ and as there are three nitrogen atoms in the molecule, it was of interest to find out whether this excess $^{15}N$ was evenly or otherwise distributed among the nitrogen atoms. It was planned to investigate this point by splitting the citrulline to ornithine and ammonia.

The removal of the carbamyl grouping from citrulline was first attempted by hydrolysis with normal sodium hydroxide. However, results were unsatisfactory and the high concentration of sodium interfered with the final Kjeldahl digestion. A modification of the method of Hirs & Rittenberg (1950) was consequently used. As the citrulline sample was initially in citrate buffer, the citrate was removed by precipitation with barium. The solution was then rendered 1.2N with respect to barium hydroxide and refluxed for 20 hours, the ammonia thus liberated being continuously aerated in a stream of ammonia-free nitrogen into 2% boric

acid. It was found necessary to increase the concentration of baryta from 0.05N to 1.2N to obtain complete hydrolysis, possibly because the concentration of citrulline was very much lower than in the experiments of Hirs & Rittenberg. Before Kjeldahl digestion of the ornithine residue, the barium was removed by precipitation with sulphuric acid.

Fractionation of Myrica gale nodule extracts.

In a preliminary experiment an extract of Myrica nodules was analysed as described above for Alnus extracts, but it was found that the quantity of asparagine present was such that the resolution of the column was reduced and the asparagine peak overlapped those of aspartic acid, serine and glutamine. Resolution could have been improved by the use of a larger column, but the method suffers from another disadvantage. Unlike alder, the Myrica extracts were shown by preliminary paper chromatography to contain some glutamine. Moore & Stein (1954) found that glutamine was to a large extent destroyed (25% yield) under their conditions of separation. Hence for Myrica extracts an alternative mode of fractionation was sought.

The acidic amino acids, aspartic and glutamic, were first separated from the neutral and basic ones by adsorption on a column of Amberlite I.R. 4B (80 x 0.9 cm.) using a modification of the procedure of Hirs, Moore & Stein (1952).

The column was prepared using a calcium acetate buffer rather than an ammonium buffer. It is critical for $^{15}N$ assay that the samples be uncontaminated by ordinary ammonia. The use of an ammonium buffer would not only dilute the free ammonia fraction but proved difficult to remove completely from the amino acid fractions. Calcium ions are readily removed from the effluent as the oxalate. The calcium buffer employed was 0.03M with respect to calcium and had a pH of 4.5. When the charge was applied to the column the neutral and basic amino acids passed straight through; aspartic and glutamic acids were retained and were subsequently separated from one another by gradient elution. The gradient was obtained by running normal acetic acid into a reservoir of 100 ml. capacity initially charged with the calcium acetate buffer pH 4.5 and stirred by a magnetic stirrer.

The calcium present in the fractions containing the neutral and basic amino acids was removed as the oxalate and the resulting solution concentrated to small bulk. The pH of this solution was adjusted to 5 and it was then applied to a short column (15 x 0.9 cm.) of Zeokarb 225. At this pH only tryptophan, ammonia and the basic amino acids were retained. These were separated using the buffer system described by Moore & Stein (1951).

The early fractions from this column containing glutamine, asparagine and the neutral amino acids were bulked

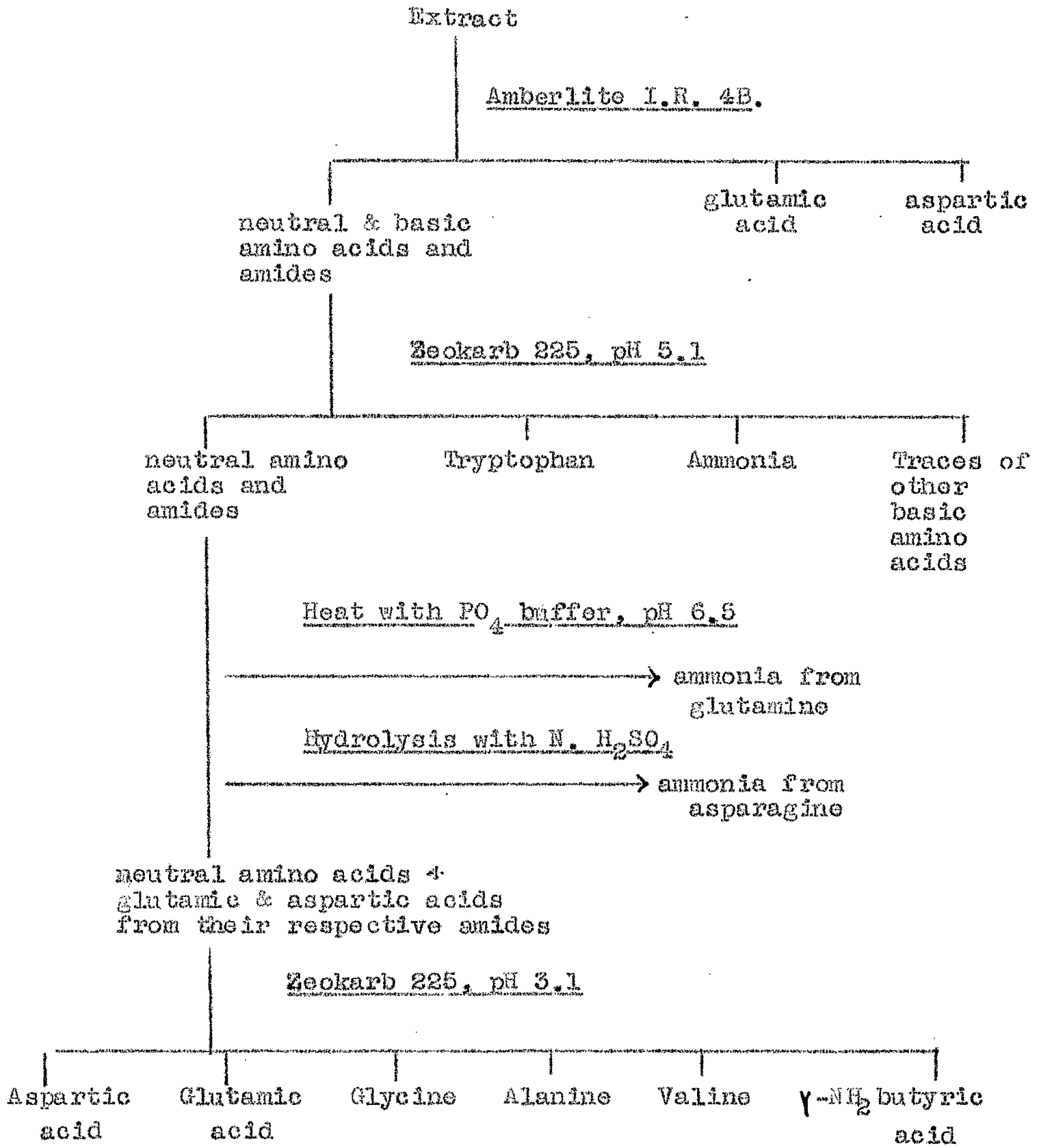
together. Phosphate-borate buffer was then added to give a final concentration of 0·03M $PO_4'''$ and pH of 6.5 and this mixture heated for two hours at 100°C. This treatment removes the amide nitrogen from glutamine (Pucher, Vickery & Leavenworth, 1935), as ammonia. The solution was then made alkaline with sodium hydroxide and the ammonia aerated into 2% boric acid. Difficulties were, however, encountered later when trying to free this solution from sodium salts. In subsequent experiments, therefore, the method was amended by the use of calcium hydroxide, the calcium being readily removable by precipitation as the oxalate. After aeration of the ammonia the solution was neutralised, made normal with respect to sulphuric acid and refluxed for three hours at 100°C. This procedure hydrolyses asparagine to aspartic acid and the pyrrolidone carboxylic acid formed from the glutamine to glutamic acid. The ammonia liberated from asparagine was aerated into boric acid.

The neutral amino acids together with aspartic and glutamic acids obtained from the hydrolysis of asparagine and glutamine respectively were finally separated on a column of Zeokarb 225. The technique already described for the Alnus extracts was employed here. Figure 4 summarises the fractionation procedure for Myrica extracts.

The above method of fractionation successfully overcomes the difficulty which arose previously due to the presence of glutamine and the large quantity of asparagine

Figure 4.

Fractionation Procedure for Myrica gale extracts.



in the extracts. However, it did possess one disadvantage. In preliminary paper chromatography of Myrica extracts a ninhydrin positive spot was detected in the arginine position. When the extract was fractionated first on Zeokarb 225 arginine was eluted in the expected position in the effluent curve together with tryptophan. On using the revised fractionation procedure, however, arginine could not be detected in its expected position in the effluent from the short Zeokarb column. It was later discovered that in fact the arginine was being retarded on the Amberlite I.R.4B resin and was eventually being eluted together with the aspartic acid. This retardation of the arginine appears to have been caused by the formation of a complex salt between the calcium of the eluting buffer and the guanidino group of the arginine.

The fractions from the Amberlite column containing the aspartic acid and arginine of Extract 2 were concentrated to small bulk and applied to a short column (1 x 7 cm.) of Zeokarb 225, the pH having been adjusted to 5.0. Using the procedure of Moore & Stein (1951), see page 87, the aspartic acid and the arginine were eluted separately.

Preparation of samples for Isotopic Analysis.

Assay of $^{15}N$ in a mass spectrometer requires the nitrogen sample to be in the form of elementary nitrogen.

The compound to be examined is first converted to ammonia by the Kjeldahl procedure and then to nitrogen by the action of alkaline hypobromite.

The contents of the tubes corresponding to a given peak on the effluent curve were combined, evaporated to small bulk and subjected to Kjeldahl digestion. Using the mercury catalyst of Hiller, Plazin & Van Slyke (1948), a six hour digestion was found sufficient to remove substances which might interfere in isotopic analysis (Rittenberg, 1946). The resulting ammonia was distilled into 2% boric acid, titrated with 0.05N HCl, evaporated to small bulk and stored for analysis of $^{15}N$.

In the fractionation of the Alnus extracts, the basic amino acids, particularly arginine, having high retention values on the Zeokarb 225, emerge late from the column eluted by buffer of very high salt content. Such fractions are extremely difficult to digest by the above means. The salt was removed from the arginine containing fractions by absorbing the arginine on a column of Zeokarb 225 in the ammonium form. The column was then washed with 0.01N acetic acid to remove the citrate ions and the arginine finally liberated with 0.05M ammonia. The ammonia was removed before Kjeldahl digestion by repeated evaporations in a vacuum desiccator after the addition of a small amount of sodium hydroxide.

The free ammonia fraction from the column was made alkaline and the ammonia aerated into 2% formic acid.

For spectrometric assay a minimum sample of 0.4 mgm. nitrogen is required. As some of the components on separation did not yield as much as this, the required amount was made up with ordinary ammonium sulphate, and the results obtained corrected for the dilution of the $^{15}N$. The maximum deviation obtained in control samples due to experimental error was 0.004 per cent. $^{15}N$ over the normal value. Thus an atom percent. excess of 0.010 is significant.

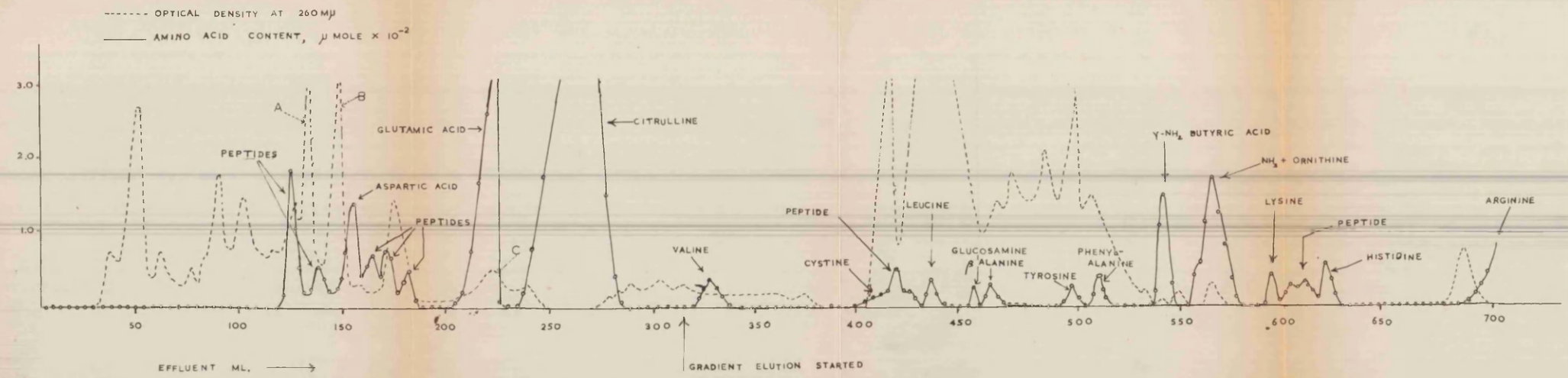
# R E S U L T S.

## Nitrogenous constituents of the Alder nodule extracts.

The analysis of the extract from one particular batch of Alnus glutinosa nodules (Extract 1) is shown in detail in Figure 5. These nodules had been detached prior to exposure to excess $^{15}N$ and in this case nodules exposed on one particular day had also been extracted together. The graph shows the distribution of compounds absorbing ultra-violet light at 260 mμ. and also of ninhydrin positive constituents.

It is clear that the alder extract contains many substances which absorb ultra-violet light. Some compounds are probably in the main purines or pyrimidines or their derivatives. The absorption spectra of these peaks, examined over the range 200 to 300 mμ., proved unrecognisable, except in three cases. These appeared to be homologous, peak A containing uridylic acid, B, cytidylic acid and C guanylic acid or derivatives of these substances. The remaining peaks in the effluent curve appeared to represent either mixtures of ultra-violet absorbing compounds or maybe more complex compounds whose absorption spectra are still unknown. The extinction values obtained for these compounds suggested, however, that there was not sufficient material present for isotopic assay, while the complex nature of the

FIGURE 5.




CHROMATOGRAM OF EXTRACT 1 — ALNUS GLUTINOSA NODULES. —

results also indicated that a great deal of work would be entailed in their separation and identification. Hence no further work was expended on these compounds.

On investigation of the ninhydrin positive peaks eluted from the column, it was found that in addition to free amino acids the extract also contained a number of peptides. Moore & Stein (1954) reported that, as well as amino acids, small peptides could pass through Dowex 50 resin and thus be separated on the same principle as the amino acids themselves. Those present in largest amount were mostly concentrated in the early fractions around and in a few cases almost completely obscuring the aspartic acid peak. As they were eluted from the column in this position these peptides presumably bore an overall negative charge due to the predominance of dicarboxylic amino acid residues. There was insufficient material for complete characterisation of these peptides, but paper chromatography showed that after acid hydrolysis all yielded aspartic and glutamic acids, alenine, serine and glycine. Others contained in addition threonine or a basic amino acid, either ornithine or lysine. Other simple peptides containing a preponderance of basic amino acid residues and hence bearing a more positive charge were found in smaller amounts in later fractions. Two of these peptides are of particular interest. One, which appeared as a spike in the cystine peak in Extract 1 (Figure 5), yielded

on hydrolysis glutamic acid, ornithine, citrulline, hydroxyproline, and an unknown spot near the valine position on a two dimensional chromatogram. Hitherto citrulline has not been encountered in combined form in the tissues of higher plants, although Smith & Young (1955) detected it in the protein fraction of the alga Chondrus crispus. The other peptide of interest occurred as a small peak immediately preceding leucine in Experiments 4 and 5. These fractions also contained ultra-violet absorbing material and it was only after hydrolysis that it was possible to separate the peptide material from that absorbing ultra-violet light, on a paper chromatogram. It thus appeared that the ultra-violet absorbing compound was linked to the peptide material. Such compounds are known to occur in bacteria although their function has not yet been elucidated (Park, 1952). From spectral analysis and chromatographic evidence the ultra-violet material was suspected to be a uridine derivative, while the peptide fraction after hydrolysis yielded glutamic acid, glycine, serine, alanine, valine and lysine. However, owing to the small quantity of material available, a fuller characterisation was not possible and further work is desirable

The free amino acid composition of the alder extracts is given in Table 11. The extracts from nodules exposed in September 1955 consisted mainly of citrulline, glutamic acid, γ-amino butyric acid and arginine in descending order of

Table 11.

Amino acid composition of extracts of alder nodules.

Results are expressed in terms of mgm. nitrogen per 100 gm. fresh weight of nodule material. A dash (-) indicates that there was insufficient material for confirmation and (+) indicates that the amino acid was shown to be present but the amount was not estimated.

| Extract No. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Exposure Type * | D | D | A | A | D |
| Duration of exposure to $^{15}N$ (minutes) | 90 | 90 | 180 | 180 | 30 |
| Aspartic Acid | 0.84 | + | 0.82 | 0.49 | + |
| Glutamic Acid | 4.65 | 1.54 | 1.71 | 1.32 | 1.90 |
| Citrulline | 53.0 | 5.60 | 17.20 | 9.05 | 6.64 |
| Valine | 0.27 | - | 0.21 | 0.05 | - |
| Cystine | 0.70 | - | - | 0.06 | - |
| Isoleucine | - | - | 0.09 | 0.13 | - |
| Leucine | 0.10 | - | 0.08 | 0.17 | - |
| Tyrosine | 0.27 | 0.47 | - | 0.18 | - |
| Phenyl Alanine | 0.18 | | - | - | - |
| $\gamma$-$NH_2$ Butyric Acid | 1.15 | 0.31 | 4.82 | 1.34 | 1.19 |
| Ammonia | 2.39 | 2.17 | | 3.04 | 0.80 |
| Ornithine | | 0.67 | 0.70 | 0.06 | 0.20 |
| Lysine | 0.70 | 0.31 | 0.53 | - | 0.24 |
| Histidine | 1.70 | 0.63 | - | - | 0.25 |
| Arginine | + | + | 3.52 | 3.52 | 1.18 |

* D indicates that the nodules were detached prior to exposure to $^{15}N$ while A indicates that the nodules were still attached to the plants when exposed to $^{15}N$.

The aspartic acid fractions were slightly contaminated with peptides.

magnitude. Smaller amounts of various other amino acids and of ammonia were also present. In accordance with the results of Miettinen & Virtanen (1952) no glutamine or asparagine was detected in the alder nodule extracts. However, a notable variation between extracts from different batches of nodules was obtained in respect of the absolute amounts of the various amino acids and hence of the total amino acid content. This variation is particularly obvious in the case of citrulline where results range from 6 to 53 mgm. citrulline nitrogen per 100 gm. fresh nodules in samples collected within a few days in September. Miettinen & Virtanen (1952) found that as winter approached and the alder plants began to shed their leaves, the concentration of citrulline rose rapidly in the nodules, to fall again in the spring, when the plant became active once more. Actual values obtained by these workers expressed in the same terms as above were 12, 18 and 120 mgm. citrulline nitrogen for nodules collected in May, June and January respectively. It seems that even early in September the alders in Glasgow were preparing for the winter dormancy. This might not affect all the plants at the same time hence giving rise to the variations between extracts. Certainly, since the alders were all from apparently uniform plants and all extractions were effected in exactly the same way, the variation in amino

acid content must be due either to physiological differences in the plants themselves or to differences in weather conditions which could give rise to variation in type of metabolism from day to day. The extract prepared from nodules exposed in August 1956, one month earlier than in the previous year, showed a low citrulline content as would be expected under summer conditions. In other ways this extract was similar to those of the previous year. To elucidate these fluctuations in the citrulline content of the nodules properly, however, an entirely different type of experiment would be necessary.

The qualitative examination of Alnus rubra nodules indicated that citrulline was again the predominant free amino acid. The distribution of other amino acids was also found to be similar to that of Alnus glutinosa. The results are recorded in Table 12.

Distribution of fixed $^{15}N$ in Alnus glutinosa nodules.

It was originally intended to pool corresponding fractions from different extracts to provide sufficient nitrogen for $^{15}N$ assay of as many constituents as possible. However, because of the variation in the composition of different extracts, it was thought best to have separate assays. This reduced the number of fractions available for assay.

The distribution of $^{15}N$ among the compounds separated

Table 12.

Comparison of the Free Amino Acid Composition of the nodules of some non-Leguminous Plants. The nodules were picked in late August - early September.

| | Alnus glutinosa | Alnus rubra | Myrica gale | Hippophaë rhamnoides | Casuarina |
|---|---|---|---|---|---|
| Aspartic Acid | 0.49 | + | 2.38 | + | + |
| Glutamic Acid | 1.32 | ++ | 6.16 | ++ | ++ |
| Asparagine | - | - | 16.80 | +++ | ++ |
| Glutamine | - | - | 2.80 | ++ | +++ |
| Citrulline | 9.05 | +++ | - | - | - |
| Serine | - | - | - | + | - |
| Glycine | - | + | 0.12 | - | - |
| Threonine | - | + | - | - | + |
| Alanine | - | + | 1.12 | ++ | ++ |
| Valine | 0.05 | + | 0.25 | + | + |
| $\gamma NH_2$ Butyric Acid | 1.34 | + | 2.16 | + | + |
| Histidine | - | + | 0.25 | ++ | + |
| Lysine | - | - | 0.12 | - | - |
| Ornithine | 0.06 | - | - | - | - |
| Arginine | 3.52 | + | + | + | - |
| Leucine | 0.17 | + | 0.42 | + | - |
| Tryptophan | - | - | + | - | - |

Where a quantitative examination has been made the figures represent mgm. nitrogen per 100 gm. fresh wt. nodules. Where only qualitative paper chromatographic investigations have been carried out, the concentrations of the amino acids have been indicated by +, ++ and +++ according to the faint, moderate or high colour intensity of the ninhydrin spots on the chromatograms. The colour intensity is a function of the concentration.

* C. cunninghamiana.

is shown in Table 13. Although fixation falls off after detachment of the nodule from the plant (Bond, 1957b), and despite the exposure period for the detached nodules being much shorter than that for the attached ones, it is the detached nodules which contain the greatest enrichment of $^{15}N$. The nitrogen fixed in nodules which are attached to the plant is quickly translocated to the shoot (Bond, 1956). When the nodules are detached, however, no translocation is possible and hence the nitrogen fixed accumulates in the compounds of the cells. Detachment also cuts off the nodules source of carbohydrates. However, over one and a half hours, the longest exposure period employed, this does not appear to have produced any marked effect. The distribution of $^{15}N$ shows a similar pattern whether the nodules were attached to or detached from the plant during exposure.

Of the fractions analysed the highest concentrations of $^{15}N$ were to be found in the free amino acids, the ultra-violet absorbing compounds and the peptides both showing a lower enrichment. In each batch of nodules extracted glutamic acid showed the greatest overall enrichment with $^{15}N$. It always exceeded that of the free ammonia. Other amino acids showing appreciable $^{15}N$ enrichment were citrulline aspartic acid and $\gamma$-amino butyric acid. It should be noted that although fractions containing amino acids sometimes also contained substances absorbing ultra-violet light, this

Table 13.

Distribution of excess free $^{15}N$ in the constituents of nodule extracts of Alnus glutinosa.

Results are given as atom per cent. excess over the normal value (0.375 atom per cent.).

| Extract No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Date of Exposure | 1955 | 1955 | 1955 | 1955 | 1956 | 1957 |
| Type of Exposure* | D | D | A | A | D | D |
| Period of Exposure (mins) | 90 | 90 | 180 | 180 | 30 | 30 |
| Peptides | 0.31<br>0.59<br>1.06 | | 0.09<br>0.16 | 0.08<br>0.06 | | |
| Aspartic Acid | 0.910 | - | 0.208 | - | - | - |
| Glutamic Acid | 1.200 | 0.956 | 0.450 | 0.427 | 0.250 | 0.156 |
| Citrulline | 0.590 | 0.870 | 0.241 | 0.342 | 0.179 | - |
| $\gamma$-$NH_2$-Butyric Acid | 0.660 | 0.348 | 0.176 | - | - | - |
| Arginine | - | - | 0.014 | 0.012 | 0.012 | - |
| Ammonia | - | - | - | - | 0.187 | 0.040 |
| U-V Absorbing Compounds | 0.230<br>0.591 | | 0.090<br>0.061 | | | |

* A - nodules attached to plant during exposure to $^{15}N$

D - nodules detached from plant prior to exposure to $^{15}N$.

contamination was insufficient to affect the isotopic analysis. For example, the substance overlapping the glutamic acid peak (Figure 5) was assumed to be guanylic acid on the basis of spectroscopic evidence. If this is so the ratio of guanylic acid nitrogen to glutamic acid nitrogen is only 1:400.

Citrulline was always found to have a considerable $^{15}N$, in one case (Extract 2), even approaching that of glutamic acid. As noted already, in the summer months, when the nodules are most active in fixation, the citrulline content of the nodules is low. It is interesting that in Extract 2 the concentration of citrulline is the lowest recorded in Table 11. As already mentioned, the citrulline peak tends to overlap the small glycine and alanine peaks; a check was therefore made to ascertain if this in any way affected the $^{15}N$ value recorded for citrulline. The tubes containing the citrulline from the column were arranged into groups before Kjeldahl digestion and analysed separately for $^{15}N$. No significant differences were obtained, showing that the quantities of glycine and alanine present were insufficient to affect validity of the $^{15}N$ assay for citrulline.

The citrulline molecule contained considerable excess $^{15}N$ and as it also contains three nitrogen atoms per molecule, it was of interest to examine the distribution of the $^{15}N$ throughout the molecule. A portion of the citrulline samples was thus degraded to ornithine and ammonia as

Table 14.

Distribution of $^{15}N$ in citrulline from Alnus glutinosa nodules exposed to $^{15}N$.

Results are given as atom per cent. excess over the normal value (0.375 atom per cent.).

| Extract No. | 4 | 5 |
|---|---|---|
| Exposure Type * | A | D |
| Duration of exposure to $^{15}N$ (mins.) | 180 | 30 |
| Citrulline total, found | 0.342 | 0.179 |
| " " , calc. | 0.324 | 0.181 |
| Carbamyl nitrogen | 0.578 | 0.390 |
| Ornithine nitrogen | 0.197 | 0.076 |

* A - nodules attached to plant during exposure to excess free $^{15}N$.

D - nodules detached from plant prior to exposure to $^{15}N$.

described in "Methods". The mass spectrometric results presented in Table 14 show that the ammonia nitrogen derived from the carbamyl grouping of citrulline was much more highly labelled than the ornithine nitrogens. A comparison of Tables 13 and 14 reveals the interesting fact that the carbamyl nitrogen of citrulline contains the highest concentration of $^{15}N$, exceeding even that of glutamic acid.

From the data available for Extract 4 it emerges that 60-70 per cent of the excess $^{15}N$ present in the extract is accounted for by the fractions shown in Table 13 subject to an addition calculated from other Extracts for $\gamma$-amino butyric acid and ammonia. It is presumed that there was a similar recovery in the other extracts.

Nitrogenous constituents of Myrica gale and other non-legume nodule extracts.

Myrica gale nodules were examined with a view to subsequent isotopic investigation and results are thus quantitative in nature, and in this sense can be compared with those of Alnus glutinosa. As the isotopic data obtained from the alder experiments indicated that it is in the free amino acids that the primary products of fixation are to be found, only ninhydrin positive substances from Myrica extracts were investigated.

Very little peptide material was detected in the extracts. This may have been due to the different mode of fractionation employed for Myrica or there may indeed have been much less in the extract. The preliminary fractionation on a Zeokarb 225 column as for Alnus, however, also indicated the absence of peptide material, while the free amino acids and amides extracted accounted for 63% of the total nitrogen extracted. In the alder nodules free amino acids accounted for only 20% of the total extracted nitrogen. The results for a typical Myrica nodule extract are given in Table 12, and Table 15 records the composition of the nodules which had been exposed to excess free $^{15}N$ for varying periods.

In each case the extract nitrogen contained 15 to 20 per cent of the total nodule nitrogen. Asparagine alone accounted for 27 to 30% of the total nitrogen of the extract. Tryptophan, glutamic and aspartic acids and ammonia were also present in considerable quantity together with smaller amounts of glutamine, arginine, $\gamma$-amino butyric acid, alanine, glycine, leucine, and valine. No citrulline was detected in Myrica nodules.

No great variation in the composition of the extracts, as was encountered with the alder material, was evident with Myrica. The pattern of free amino acids was very similar in all extracts examined. The Myrica nodules were harvested at the beginning of August, 1956, and thus earlier in the year

Table 15.

Amino Acid Composition of Extracts of *Myrica gale* nodules previously exposed to excess $^{15}N$.

The results are expressed in mgm. nitrogen per 100 gm. fresh weight of nodules.

| Extract No. | 1 * | 2 | 3 |
|---|---|---|---|
| Exposure to $^{15}N$ (minutes) | 120 | 60 | 45 |
| Aspartic Acid | 25.9† | 5.5 | 12.6† |
| Glutamic Acid | 6.2 | 6.4 | 5.6 |
| Asparagine | 18.9 | 27.5 | 18.7 |
| Glutamine | 2.9 | 2.8 | 2.6 |
| Glycine | + | 0.6 | + |
| Alanine | 0.5 | 1.5 | 1.3 |
| Valine | + | 0.4 | 0.4 |
| $\gamma$ -$NH_2$ butyric acid | + | 2.3 | 2.5 |
| Tryptophan | 2.6 | 3.0 | 6.6 |
| Arginine | ++ | 3.9 | ++ |
| Ammonia | 2.5 | 5.0 | 3.5 |

* Neutral amino acids not retrieved.

† Aspartic acid fractions contaminated with arginine. (+) indicates that the amino acid was shown to be present in traces, but the amount was not estimated.

than those of Alnus. At this time of the year the plants would be very active in fixation and there would be less likelihood of variation between nodules extracted within a few days of each other.

The yields of glutamic acid and ammonia obtained from the hydrolysis of glutamine agreed closely with the theoretical expectation, indicating that the treatment applied was not liberating any ammonia by the hydrolysis of other compounds or of glutamine in peptide material. A similar situation was encountered with asparagine.

In Extract 1 (Table 15) a large proportion of the neutral amino acids was lost owing to the addition of large quantities of sodium hydroxide at various stages in the fractionation procedure. As mentioned previously, the removal of this sodium on Dowex 2 prior to the final stage of fractionation led to a considerable loss of the neutral amino acids. In Extracts 2 and 3 calcium hydroxide was substituted for the sodium and the neutral amino acids recovered.

The results of the qualitative paper chromatographical examination of extracts of Hippophaë rhamnoides and Casuarina cunninghamiana are detailed in Table 12. In Hippophaë as in Myrica asparagine predominated while in Casuarina glutamine was dominant. The distribution of other amino acids in the nodules of both these plants was found very similar to that of Myrica apart from the fact that no tryptophan was detected.

Distribution of Fixed $^{15}N$ in Myrica nodules.

The distribution of $^{15}N$ among the compounds separated from the Myrica extracts is given in Table 16. Extract 2 was found to contain 82 per cent of the total $^{15}N$ fixed by the nodules, while Extract 3, prepared from nodules given a shorter exposure to the isotope, contained 89 per cent of the total fixed $^{15}N$.

In each batch of nodules extracted, the amide group of glutamine was found to contain the highest concentration of $^{15}N$. Asparagine in its amide group, glutamic acid and the free ammonia showed somewhat lower but substantial enrichments, while tryptophan and alanine also contained appreciable $^{15}N$. The free ammonia was never found to contain a greater enrichment than did the glutamine, but in Extract 2 it did exceed that of the glutamic acid. The glycine and arginine extracted showed very slight enrichment.

It has been mentioned previously that in Extract 1 the neutral amino acids were not retrieved, hence no $^{15}N$ data are available for $\gamma$ -amino butyric acid. In Extract 2 the $\gamma$-amino butyric acid fraction was unfortunately lost and in Extract 3 at the time of writing it has not been analysed.

Due to the method of fractionation employed for Myrica extracts, aspartic acid and arginine were found to be eluted together. This point has already been discussed on page

Table 16.

Distribution of excess free $^{15}N$ in the constituents of nodule extracts of Myrica gale.

Results are given as atom per cent. excess over the normal figure (0.375 atom per cent.).

| Extract No. | 1 | 2 | 3 |
|---|---|---|---|
| Period of exposure to $^{15}N$ (minutes) | 120 | 60 | 45 |
| Total nodule nitrogen | - | 0.069 | 0.070 |
| Extract nitrogen | - | 0.334 | 0.372 |
| Aspartic acid | 0.186 * | 0.427 | 0.049 * |
| Glutamic acid | 1.087 | 0.602 | 0.595 |
| Asparagine (amide N) | 1.127 | 0.506 | 0.560 |
| " (amino N) | 0.328 | 0.149 | 0.196 |
| Glutamine (amide N) | 3.140 | 1.450 | 1.684 |
| " (amino N) | 0.631 | 0.460 | 0.261 |
| Glycine | - | 0.054 | - |
| Alanine | 0.270 | 0.810 | 0.155 |
| Tryptophan | 0.595 | 0.359 | 0.388 |
| Arginine | 0.018 | 0.002 | - |
| Ammonia | 0.876 | 0.736 | 0.540 |
| Peptide | - | 0.036 | - |

* Aspartic acid peak contaminated with arginine.

of the "Methods". It was only in the case of Extract 2, however, that the two amino acids were satisfactorily separated and $^{15}N$ data acquired for each individually. A $^{15}N$ analysis for arginine is also available for Extract 1, although the only available value for aspartic acid was obtained before it was realised that there was arginine present. However, from Table 16 it is clear that arginine contains only a very low concentration of $^{15}N$ in excess of its normal value. This therefore explains the low values obtained for the "aspartic acid" fractions from Extracts 1 and 3.

The isotopic data are most complete for Extract 2 and it can be calculated that the compounds shown in Table 16 account for 67 per cent. of the total $^{15}N$ of the extract. Similar values can be obtained for the other extracts, and it would indeed seem that 65 to 70 per cent. of the $^{15}N$ fixed in compounds of the extract has been recovered in the free amino acids and ammonia.

# D I S C U S S I O N.

## The Nitrogenous Constituents of the Nodules.

A comparison of the free amino acid composition of the nodules of the non-leguminous plants examined (Table 12) reveals that apart from Alnus species the nodules contain either asparagine or glutamine as their predominant constituent, and in general their amino acid composition is similar to that found in the nodules of leguminous plants examined by Aprison, Magee & Burris (1954) and by Sen & Burma (1953). The nodules of the alder alone are devoid of asparagine and glutamine and contain in their stead citrulline.

The examination of the nitrogenous constituents of Alnus glutinosa nodules showed that in agreement with Miettinen & Virtanen (1952) citrulline is quantitatively the most important free amino acid present. Citrulline was also shown by these authors to predominate in the nodules of Alnus incana, while a similar finding is now reported in Table 12 for Alnus rubra. It would thus seem probable that the presence of citrulline is characteristic for Alnus as a genus.

The nodules of Alnus glutinosa were also found to contain appreciable amounts of glutamic acid, aspartic acid, and γ-amino butyric acid together with arginine and ornithine. The finding of free ornithine in plant tissues is unusual.

In animal metabolism, however, ornithine is well known and is closely related in metabolism to citrulline and arginine. Arginine is an important constituent of proteins in both plants and animals.

Since the finding of citrulline in the alder by Miettinen & Virtanen (1952) various other reports of its occurrence in the plant kingdom have been reported. Reuter & Wolffgang (1955) reported it as exuded in large quantities in the bleeding sap from roots and stems of species of both genera of the Betulaceae, Alnus and Betula, and also in the closely related genera Corylus and Carpinus. Later Bollard (1957) found citrulline to be a constituent of the xylem sap of 29 unrelated woody genera in 5 of which it was a major constituent and appeared to replace the asparagine and glutamine of other plants. Instances of the occurrence of citrulline are also forthcoming for the lower plants. Fowden (1951) detected it among the free amino acids of two species of Chlorella, Mansford & Raper (1954) found it to comprise 6.3 per cent. of the soluble nitrogen of Funaria hygrometrica, and Linko, Holm-Hansen, Bassham & Calvin (1957) have also reported its presence in certain blue-green algae.

This widespread occurrence of citrulline in the plant kingdom together with the fact that it has not been found in all non-legume nodules emphasises Miettinen & Virtanen's observation# that citrulline was not specifically related to

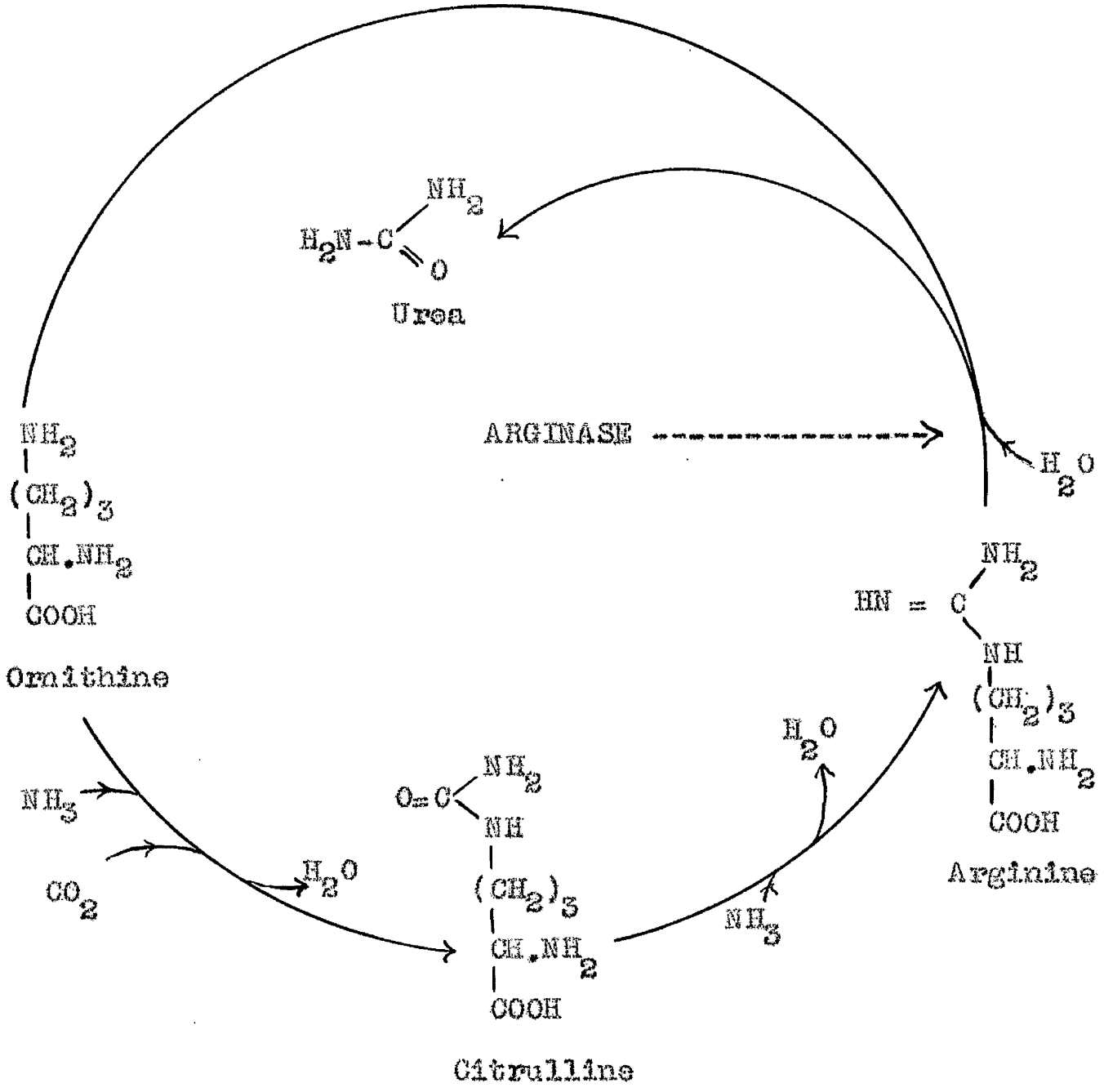
nitrogen fixation.

The metabolic function of citrulline is well known in animal tissues. Krebs & Henseleit (1932) found that ornithine, citrulline and arginine were intermediates in the production of urea in the mammalian liver. To explain these results Krebs formulated the "ornithine cycle" as shown in Figure 6. This cycle is now considered to be a special adaptation in mammals of the more widespread mechanism for arginine synthesis (Ratner, 1956).

The only direct evidence for the existence of a urea cycle in the plant kingdom is that given by Srb & Horowitz (1944) who demonstrated its presence by genetical techniques in the fungus Neurospora crassa. However, recent work of Coleman & Hegarty (1957), in which barley and white clover were fed with ornithine-$2^{14}C$, showed that enzymes capable of converting the ornithine to citrulline and arginine were present. An enzyme which could convert arginine to ornithine also appeared to be operative. Such findings indicate that many of the enzymes of the Krebs ornithine cycle are present in barley and white clover and the authors suggested that they may have a place in the nitrogen metabolism of these and other plants.

Miettinen & Virtanen (1953a) found no evidence for the participation of citrulline in such a cycle in the alder. The alder was shown to be devoid of arginine activity and

Figure 6.




Krebs' "Ornithine Cycle".

only negligible amounts of urea were detected. It was thus suggested that in the alder, citrulline replaced the amides of other plants and acted as a store of and as a means of transport for the nitrogen within the plant. Reuter (1957) substantiated this and extended the idea to other plants. The existence of citrulline as a major constituent of the xylem sap of such plants, although not definite proof, does support this theory. In a further investigation of the rôle of citrulline in the alder, Miettinen & Virtanen (1953b) reported slight but positive evidence of the transfer of citrulline nitrogen to $\alpha$-ketoglutaric acid in the leaves but not in any other part of the plant. The fact that such a reaction did not take place in the nodules was regarded as conforming to the suggestion that in citrulline the nitrogen fixed in the nodules was transported for use in other parts of the plant.

Linko *et al* (1957) likewise found no evidence for the operation of the urea cycle in the blue-green algae. All efforts to detect free arginine or urea in such algae were negative. These authors did, however, favour a cyclic process of some description for the formation of citrulline and suggested that it might function as a means of transfer of nitrogen from ammonia to the amino acids or possibly as a storage pool for the carbamyl group for nucleotide synthesis.

The isotopic data presented in Table 13 of this thesis show that arginine is very slightly enriched with $^{15}N$ compared

with citrulline. Thus if arginine is formed from citrulline in the alder nodules, as seems highly probable, its rate of formation must be extremely slow compared with the rate of formation of citrulline. This would therefore suggest that the citrulline in the alder nodules has some other rôle in addition to that of arginine synthesis, possibly that of translocation.

It has been shown that 25-30 per cent. of the soluble nitrogen of Myrica nodules extracted with 0.1N HCl was in the form of asparagine. Table 12 indicates that asparagine was also predominant in Hippophaë nodules and that in Casuarina glutamine took its place. Traditionally asparagine and glutamine were regarded as functioning in the higher plants as resevoirs for excess ammonia and as a means of transport for nitrogen from one part of the plant to the other. The distribution of other amino acids was essentially similar to that obtained in Alnus extracts, apart from no citrulline or ornithine being found. Myrica nodules did, however, differ from the others examined in that they contained tryptophan. Sen & Burma (1953) also reported tryptophan from legume nodule extracts. In general metabolism this amino acid is known as a precursor of the vitamin, nicotinic acid, several pigments, and an animal hormone. In plant metabolism in particular, however, tryptophan is of interest because of its relationship with the auxin indole acetic acid.

## The Pathway of Nitrogen Fixation in Alnus glutinosa.

As is shown in the Introduction to this Section, the isotopic studies of the Wisconsin group have provided two lines of evidence on the pathway of nitrogen fixation in general. Certain nitrogen fixing organisms, for example Clostridium, excrete a large proportion of the fixed nitrogen into the medium. When such cells were exposed to excess free $^{15}N$, this excretion was found to consist largely of ammonia containing an extremely high $^{15}N$ concentration. It was therefore concluded that this ammonia was an intermediate in fixation rather than a product of decomposition.

The study of the distribution of fixed $^{15}N$ within the cells of most of the known nitrogen fixers and in legume nodules invariably showed that glutamic acid contained the greatest concentration of $^{15}N$ of any compound isolated. This together with the finding that the isotope distribution was essentially the same whether the $^{15}N$ was supplied as molecular nitrogen or as ammonia was regarded as implying that ammonia was an intermediate in fixation. However, only in legume nodules was an unhydrolysed cell extract examined and the above work can be criticised on the grounds that random dilution of the free amino acids could occur. Another difficulty was that the labelling of the free ammonia was never found to exceed that of the glutamic acid.

In the above Madison experiments exposures of at least

one to two hours had been employed. Since the present work at Glasgow was begun, Allison & Burris (1957) in Wisconsin have tackled the problem of demonstrating the nature of primary products of fixation by using the approach employed by Calvin to elucidate the pathway of photosynthesis. It was argued that after an infinitely short exposure to $^{15}N$ 100 per cent. of the total $^{15}N$ fixed should be in the earliest products of fixation and with increasing exposure time the percentage of the total $^{15}N$ in these products should decrease. After demonstrating that in Azotobacter easily detectable levels of $^{15}N$ were present in a number of compounds in the cells and in the medium after one minute's fixation, the above authors showed by a kinetic analysis of the isotope accumulated from short steady state exposure of one to five minutes, that only the amide nitrogen of the cells and the ammonia of the medium fulfilled the conditions for the primary products of fixation. To the Wisconsin workers this constituted final proof of the intermediate rôle of ammonia in nitrogen fixation. It must be remembered, however, that this only applies so far to Azotobacter.

When considering the $^{15}N$ data obtained in Glasgow for the Alnus glutinosa material, it must be stated that the ideal proof that ammonia constitutes an intermediate in the fixation by these nodules would be the isolation of free ammonia containing the greatest enrichment of heavy nitrogen. In none

of the experiments was this found. The low labelling of the ammonia fraction was at first thought to be due to dilution during the fractionation process. In an effort to overcome this the free ammonia in Extract 6 was, as noted earlier, removed before the fractionation of the amino acids was begun. In this case the glutamic acid once more exhibited a greater enrichment than did the ammonia. Indeed in Extract 6 the proportion of $^{15}N$ in ammonia to that in glutamic acid was even lower than had been found in the other fractionation. However, it must be remembered that the nodule is a complex nitrogen-fixing system. It consists of endophyte cells in or on which the nitrogen is fixed together with host cells which are presumably unconcerned in the fixation but into which a great bulk of the products of fixation are passed and further metabolised for transport to other parts of the plant. Hence the ammonia and other nitrogen fractions extracted from the nodules by homogenisation probably consist of a complex mixture derived from different sources. Compounds containing newly-fixed nitrogen are obtained from the endophyte and neighbouring cells, whereas many host cells will supply much older unlabelled nitrogen. As there are many ways in which ammonia may be produced catabolically by the latter cells, it is not perhaps surprising that highly labelled ammonia has not been isolated from the nodule homogenates.

Since the free ammonia itself does not show the greatest $^{15}N$ enrichment, information concerning the rôle of ammonia in the fixation in alder nodules can only be gained from experiments of the present type by considering the nature of the constituents into which the fixed $^{15}N$ passes. In contrast to the many ways in which ammonia can be formed catabolically in living cells, there are few known in which free ammonia can be assimilated. The principal ones known are as follows:-

1. $\alpha$-keto glutaric acid + ammonia ⟶ glutamic acid

2. glutamic acid or aspartic acid + ammonia ⟶ glutamine or asparagine

3. carbon dioxide + $PO_4$ + ammonia ⟶ carbamyl phosphate ↙ citrulline ↘ pyrimidines

The alder nodule appears to possess no asparagine or glutamine and although the nucleotide constituents have by no means been rigorously investigated, preliminary experiments indicated that they do not possess as great enrichment as do the free amino acids. The general overall distribution of the fixed $^{15}N$ in the alder nodule shown in Table 13 indicates that glutamic acid contained the highest concentration of $^{15}N$. Other compounds showing marked enrichment were aspartic acid, $\gamma$-amino butyric acid, free ammonia and

citrulline. Superficially this isotope distribution agrees closely with the results obtained by the Wisconsin group for other nitrogen fixing systems. It would suggest that the fixation process in legumes and non-legume nodules at least is fundamentally similar. It could be argued that such results themselves point to ammonia as an intermediate in the fixation. However, the distribution of the $^{15}N$ in citrulline provides further and more convincing evidence in favour of this, for as noted already, a comparison of Tables 13 and 14 shows that the carbamyl nitrogen of citrulline possessed the highest labelling detected in the extract, surpassing even that of the glutamic acid.

In animal tissues and in bacteria the biosynthesis of citrulline as part of the Krebs ornithine cycle is envisaged by Hall, Metzenberg & Cohen (1956) as being as follows:-

1. $CO_2 + NH_3 + ATP \xrightarrow[Mg^{++} + \text{acetyl glutamic acid}]{\text{Enzyme I}}$ carbamyl phosphate

2. carbamyl phosphate + ornithine $\xrightarrow{\text{Enzyme II}}$ citrulline + ADP

The acetyl glutamic acid has been shown to be catalytically active in the synthesis of carbamyl phosphate, the ~~an~~ immediate precursor of the carbamyl group of citrulline. Thus the carbamyl grouping is derived directly from the ammonia and the carbon dioxide, not from the glutamate.

It should be noted that the synthesis involves a fixation of $CO_2$.

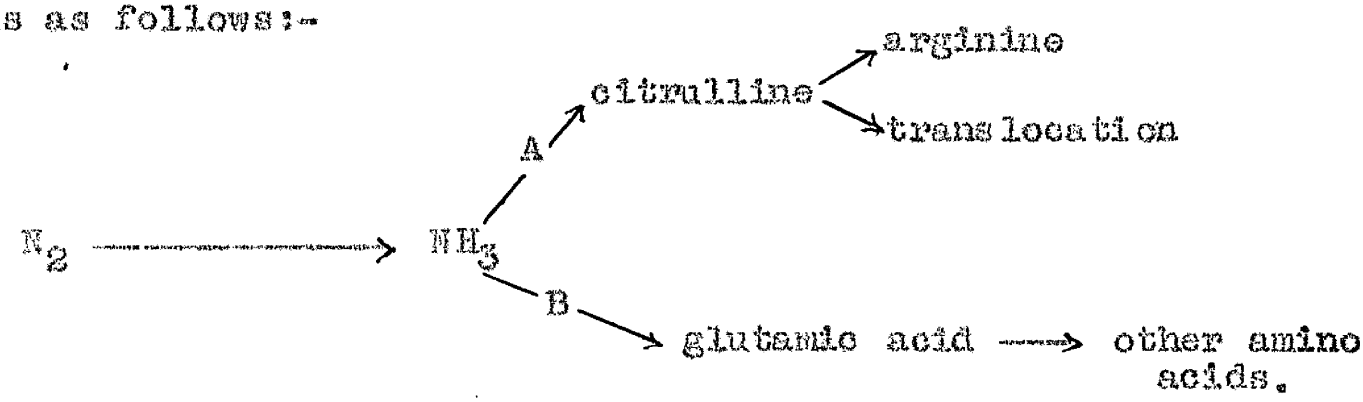
Linko, Holm-Hansen, Bassham & Calvin (1957) found that when $^{14}CO_2$ was supplied to Nostoc the $^{14}C$ was rapidly incorporated into the carbamyl grouping only of the citrulline. This result would appear to support a similar mechanism for the synthesis of citrulline in one branch at least of the plant kingdom, namely, the blue-green algae.

The distribution of $^{15}N$ fixed in the citrulline of the alder nodule is consistent with this mode of formation in the higher plants also. It moreover indicates that there must be a supply of highly labelled free ammonia in the nodule. Hence by virtue of its difference from other systems examined the alder provides new evidence that in nitrogen fixation the formation of ammonia precedes the formation of free amino acids.

The presence of such a mechanism for the synthesis of citrulline in the alder could support the suggestion of Linko et al (1957), already mentioned, that citrulline may function as an intermediate between ammonia and the amino acids. The lower labelling of the glutamic acid could then be accounted for by visualising the pathway of fixation being as follows:-

$N_2$ ⟶ $NH_3$ ⟶ citrulline ⟶ glutamic acid ⟶ other amino acids.

Miettinen & Virtanen (1953b), however, could find no evidence of the transfer of citrulline nitrogen to $\alpha$-ketoglutaric acid in the alder nodules. Such a scheme would, indeed, entail the synthesis of glutamic acid by a reaction hitherto unknown in metabolism and, while there is no reason why this should not happen, the data can be just as readily explained on the basis of known reactions. It is, therefore, suggested that a more likely pathway of assimilation in the alder is as follows:-




The higher labelling of the citrulline carbamyl nitrogen compared with that of glutamic acid may be explained if reaction A is more rapid than reaction B. The fact that the formation of citrulline from ornithine is reversible and both reactions may be operating to some extent at the same time may also explain the high enrichment of citrulline, as in this way the citrulline molecule forms an effective trap for the $^{15}N$. On the other hand the formation and breakdown of glutamic acid in the cells is quite distinct and possibly even occurs in different parts of the cells.

The much lower labelling of the ornithine-nitrogen of the citrulline molecule presumably indicates that the formation of that substance from ornithine is a much more rapid reaction than those leading to the formation of ornithine and that the precursors of ornithine were largely preformed before the experiment began. Other free amino acids with substantial $^{15}N$ enrichments are aspartic acid and γ-aminobutyric acid, both of which are in close metabolic relation with glutamic acid.

In considering the above interpretation of the data obtained for alder nodules, it has to be borne in mind that not all the excess $^{15}N$ in combination in the extracts was accounted for. It has already been noted that data available in respect of one particular Extract (No.4) indicate that the fractions isolated and assayed accounted for 60 to 70 per cent. of the total excess $^{15}N$ present. This is an aspect which will be considered again after the Myrica data have been discussed.

## Pathway of Nitrogen Fixation in Myrica gale.

Although only 15 per cent. of the total nodule nitrogen was extracted, it was found that of the $^{15}N$ fixed by the nodules of Myrica gale 80 to 90 per cent. was to be found in the extract. Thus in Extract 2 after exposure to $^{15}N$ for one hour 82 per cent. of the $^{15}N$ fixed by the nodules

appeared in the extract, while in Extract 3 after an exposure of 45 minutes 89 per cent. of the isotope fixed was in the extract. This confirms the expectation that it is in the soluble nitrogenous constituents that the primary products of fixation are most likely to be found. It can be calculated that the compounds isolated and analysed for $^{15}N$ (Table 16) account for 60 to 70 per cent. of the total $^{15}N$ of the extract. The major part of this $^{15}N$ accounted for is to be found in seven substantially labelled compounds, namely, glutamine, asparagine, glutamic acid, aspartic acid, free ammonia, alanine and tryptophan. These compounds must therefore be of importance if not as direct products of fixation then as early products of assimilation.

As noted earlier, Table 16 shows that the greatest excess of $^{15}N$ occurs in the amide group of glutamine. Asparagine in its amide group, glutamic acid and the free ammonia show somewhat lower but substantial enrichments, while alanine and tryptophan together with the amino groups of the amides also contain appreciable $^{15}N$. The glycine and arginine extracted show very slight enrichment.

The only known mode of synthesis of glutamine *in vivo*, elucidated by Elliott (1951) in homogenates of *Lupinus* seedlings and confirmed by Webster (1953) using extracts of bean seedlings, is as follows:-

$$\text{Glutamic acid} + NH_3 + ATP \xrightarrow{Mg^{++}} \text{Glutamine} + ADP$$

This reaction is very similar to that involved in the synthesis of citrulline and would again point to ammonia as an intermediate of fixation. Free ammonia with a greater enrichment of $^{15}N$ than the glutamine amide nitrogen has not, however, been isolated from the Myrica nodules, although in one case (Extract 2) the enrichment of the ammonia did exceed that of the glutamic acid. The distribution of the fixed $^{15}N$ in the extracts of Myrica nodules showing the greatest enrichment to occur in the amide group of glutamine would therefore appear to indicate a very close parallel between the fixation process in Myrica and in Alnus and to support the hypothesis that citrulline in the alder replaces the amides of other plants.

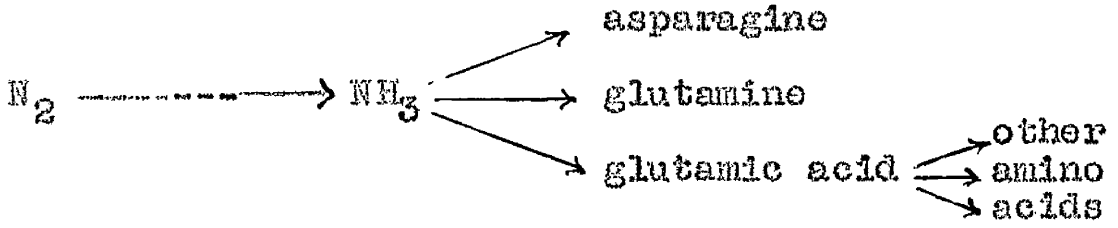
Asparagine also was found to carry a substantial concentration of $^{15}N$ in its amide nitrogen. Although not so much is known about the synthesis of asparagine as is the case for glutamine, it is thought to be similar to that of the latter substance. It is very difficult to compare the isotopic results for the two amides. The pool of asparagine in the nodules is very much greater than that of glutamine while nothing is known as to the relative rates of formation of these amides.

Other amino acids containing substantial excess $^{15}N$ include glutamic acid, aspartic acid, alanine and tryptophan.

Aspartic acid and alanine are normally considered as being closely related metabolically to glutamic acid and would hence be expected to contain much $^{15}N$. The rôle of tryptophan in nodular metabolism is of incidental interest owing to its connection with the auxin, indole acetic acid. It is possible that it is by means of auxin production that the endophyte is able to stimulate nodule production. It would be of interest to ascertain the distribution of $^{15}N$ between the nitrogen atoms of the molecule.

In each experiment the $^{15}N$ concentration in the amino groups of glutamine and asparagine is definitely less than that of the free glutamic and aspartic acids. This could be explained if the synthesis of the amides took place at a different location in the cell from that of the glutamic and aspartic acids.

The isotopic results would therefore suggest that in the Myrica nodule the fixation process could be represented as follows:-




The amino acid content of legume nodules is very similar to that of Myrica. The distribution of $^{15}N$ fixed under similar conditions in detached soya bean nodules after

exposure to excess $^{15}N$ for one hour as studied by Aprison, Magee & Burris (1954) was, however, rather different ~~to~~ from that found above. In the soya bean glutamic acid contained the greatest enrichment of $^{15}N$, the amide nitrogen whether from asparagine or glutamine being only poorly labelled. It is conceivable that in the two types of nodules the processes of fixation or assimilation might differ in detail. It has been noted already that structurally legume and non-legume nodules are quite different and that their endophytes are of different types. However, it must also be remembered that when legume nodules are detached from the plant the fixation falls off very much more rapidly than is the case with non-legume nodules. Thus the differences in the results obtained of the two types of nodule could be explained by the fixation in the legumes having fallen off after one hour while Alnus and Myrica were still actively fixing. More information on this point could be obtained by exposing the soya bean nodules for a much shorter time to $^{15}N$.

In conclusion it can be said that in both Alnus and Myrica nodules the bulk of the fixed $^{15}N$ appears in organic compounds which are likely to arise by reactions involving ammonia, pointing to the formation of that compound as an intermediate product of fixation. The ammonia appears to be the means by which the fixed nitrogen is incorporated into organic compounds, and it would thus be better to regard the ammonia as the end product of the fixation process and the citrulline and glutamic acid of Alnus AND the amides and glutamic acid in Myrica as the primary assimilation products.

As stressed already, free ammonia showing a labelling greater than that of any organic nitrogen has not been isolated from these nodules. This difficulty was also encountered in experiments at Madison and it has been suggested in some quarters that hydroxylamine rather than ammonia might just as easily be the end product of fixation. However, as no reactions have yet been discovered by means of which the nitrogen of hydroxylamine can be directly incorporated into glutamic acid, the amides or citrulline, this suggestion seems quite untenable in view of our results.

As mentioned already, it is necessary to bear in mind, however, that in both genera rather less than three-quarters of the fixed $^{15}N$ of the extracts was recovered. At present limitations of technique and incomplete knowledge of the full range of nitrogenous constituents occurring in plants make it

scarcely practicable to locate all the $^{15}N$, and there is thus the possibility that some so far undetected substance was present in the extracts in a highly-labelled form. Knowledge of such a substance might necessitate some re-interpretation of the data. Bach & Burris (1957) recently reported that a re-examination of soya bean nodules after exposure to excess free $^{15}N$ has revealed that dihydro-pyridazinone-5-carboxylic acid contained a high enrichment of $^{15}N$ frequently exceeding that of glutamic acid. They were led to look for this compound as a result of experiments in which labelled hydrazine had been fed to Azotobacter. The full implications of this report, which is of a preliminary nature, remain to be assessed.

Interesting and significant as the results of isotopic experiments with legume and non-legume nodules and with free-living nitrogen fixing organisms are, it is possible that the next major advances in the elucidation of the chemical mechanism of fixation will be achieved when methods of preparing cell-free extracts retaining the power of fixation have been perfected. The simpler system thus provided would greatly facilitate investigation, particularly perhaps of the nature of the intermediates, if any, preceding the ammonia which, on present evidence, appears to be the form into which the fixed nitrogen is brought prior to entry into organic combination. Many unsuccessful attempts reviewed by Burris

(1956) have been made to prepare such extracts, mostly from Azotobacter, but recently Mason, Takahashi, Hoch & Burris (1957) reported that essentially cell-free extracts prepared from that organism by a method of sonic oscillation showed a definite capacity for nitrogen fixation. Further studies along these lines may yield additional information concerning the mechanism of biological fixation of nitrogen.

## SUMMARY TO SECTION II.

1. The nitrogenous constituents, particularly the free amino acids, in 0.1N HCl extracts of the root nodules of *Alnus glutinosa*, *Alnus rubra*, *Myrica gale*, *Hippophae rhamnoides*, and *Casuarina cunninghamiana* have been investigated qualitatively by means of two-dimensional paper chromatography, both before and after hydrolysis. The examination of the extracts of the nodules of *Alnus glutinosa* and *Myrica gale* has been extended to a more quantitative basis by chromatography on ion exchange resins.

2. It was found that citrulline was the predominant free amino acid of the nodules of the *Alnus* species, while either asparagine or glutamine predominated in those of the other genera.

3. Nodules of *Alnus glutinosa* and *Myrica gale* were exposed for periods of 30 minutes to 3½ hours to an atmosphere containing excess free $^{15}N$, and the distribution of the fixed isotope in the nodule extracts then studied.

4. In *Alnus* the greatest concentration of $^{15}N$ was found in the carbamyl group of citrulline, whereas in *Myrica* it occurred in the amide group of glutamine.

5. Although the free ammonia of the extracts was never found to exhibit a greater labelling than that shown by the organic groups mentioned in Heading 4, the fact that the bulk of the fixed $^{15}N$ appeared in organic compounds which are likely to arise by reactions directly involving ammonia points to the formation of that substance as an early product of fixation, and as the form in which the fixed nitrogen enters into organic combination.

6. In respect of this importance of ammonia the path of fixation in the non-legume root nodules appears to be similar to that in legume nodules and free-living nitrogen fixers examined previously by other workers.

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