synthesis of cell-wall mucopeptide. Whether or not other substances, such as teichoic acid (Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958), which are associated with isolated cell walls in some organisms, are also synthesized under these conditions has not been tested. The method of mucopeptide preparation selected here is likely to remove them from the cell wall, and also the values for the radioactive content of the 'mixed trichloroacetic acid precipitates' agree with those for the isolated mucopeptide. Again, the treatment with hot 5% trichloroacetic acid in the preparation of mixed precipitates would certainly be expected to remove any teichoic acid.

SUMMARY

1. Staphylococci incubated in the presence of ^{14}C -labelled glycine, glutamate, alanine or lysine, either singly or together, incorporate these almost exclusively into the cell-wall mucopeptide.

2. This incorporation can be entirely accounted for by the increase in mass of cell-wall mucopeptide. This process is resistant to chloramphenicol.

3. The amino acids can be replaced by glucose and ammonium chloride with the strain of *Staphylococcus aureus* used.

4. The synthesis of cell-wall mucopeptide is inhibited by penicillin and bacitracin, neither of which inhibits protein synthesis.

5. Some evidence is presented which suggests that the small amount of mucopeptide synthesized in the presence of penicillin has an abnormal composition.

6. The relation of these observations to the mechanism of penicillin action and to a hypothesis concerned with protein synthesis is discussed.

We are indebted to Ruth Coyle and Ian Mathison for technical assistance given during the course of this work.

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Observations on the Composition and Metabolism of the Nitrogen-Fixing Root Nodules of *Myrica*

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(Received 4 December 1958)

In an earlier study of the fate of labelled nitrogen fixed by the root nodules of *Alnus* (Leaf, Gardner & Bond, 1958) the greatest abundances of ^{15}N were found in the carbamyl group of citrulline and the amino group of glutamic acid. This was taken to indicate that an early product of fixation was possibly ammonia, and that though the pattern of

nitrogen metabolism in *Alnus* differs in other respects from that of the leguminous plants and other nitrogen-fixing organisms, their systems for nitrogen fixation had this much in common. This paper describes similar work undertaken with the root nodules of *Myrica*, which have also been shown to fix nitrogen (Bond, 1951, 1958).

METHODS

Source of nodule material. Nodulated plants of *Myrica gale* L. (bog myrtle) were raised in the greenhouse in water culture in Crone's solution (Bond, 1951) (pH 5-6), free of combined nitrogen except for a very small amount provided during the period of nodule development. The latter was induced by inoculating the roots of the seedlings with a suspension of crushed *Myrica* nodules collected from the field. In the early stages of plant growth the Crone's solution was supplied at one-quarter of the normal concentration, and was later increased to one-half of the latter; growth was much superior to that obtained on previous occasions when the full strength solution was employed.

Exposure to excess of ^{15}N . Exposure of nodules to excess of ^{15}N was effected in the summer of the second year of growth of the plants, at a stage when the latter were some 40 cm. in height. The nodules were rapidly detached from the plants, dried roughly with filter paper, and samples of approximately 1.3 g. placed, together with 1 ml. of culture solution, in Universal Containers of capacity 25 ml. These were immediately charged to atmospheric pressure with a gas mixture containing 10% of nitrogen (with 36 atoms % ^{15}N), 20% of oxygen and 70% of argon. Exposures were made on three different days in the period 1-9 August, a total of about 10 g. of nodules being taken on each occasion. The exposures were begun at the same hour (3 p.m.) on each day, and on different days varied in duration from 2 hr. to 45 min.; during exposure the nodules were held at a temperature of approximately 25°, and were subsequently bulked and plunged into solid carbon dioxide.

Extraction. The nodules were extracted with 9 vol. of 0.1N-HCl in a blender cooled in ice. The suspension was centrifuged at 1000 *g* for 20 min. at 0° and the extract lyophilized.

Fractionation. An extract of *Myrica* nodules was examined by two-dimensional paper chromatography, with butanol-acetic acid-water (4:1:5, by vol.) followed by saturated aqueous phenol in the presence of ammonia, and a preliminary quantitative determination of its composition was made by the method of Moore & Stein (1954), which was used for alder extracts (Leaf *et al.* 1958). The further use of this method was precluded by the finding that the extract contained glutamine, which is destroyed to a serious extent on such columns (Moore & Stein, 1954), while asparagine was present in such high concentration that it overlapped both aspartic acid and glutamine. A different method of fractionation was therefore developed to avoid these difficulties. The procedure is summarized in Fig. 1. In a multistage fractionation of this sort, care must be taken to avoid the accumulation of salts which are difficult to remove from the amino acid fractions after they are separated. In a similar process Hirs, Moore & Stein (1952) used buffers prepared from ammonium acetate, which was subsequently removed by vacuum sublimation. In a study involving ^{15}N it was thought desirable to avoid ammonium salts, and buffers based on calcium acetate were therefore used. Calcium is readily removed by precipitation with oxalic acid, and acetic acid by evaporation. This proved satisfactory except that calcium interfered with the separation of aspartic acid and arginine, presumably by the formation of an acidic complex with the arginine. This difficulty was easily overcome when it was recognized, but before this two samples containing a

mixture of aspartic acid and arginine had been degraded to ammonia.

The first step in the fractionation procedure consisted in separating aspartic acid and glutamic acid from the other constituents of the extract, and from each other, by chromatography on a column of Amberlite IR-4B. The column (1 cm. $\times$ 80 cm.) was poured in calcium acetate buffer (Ca^{2+} 0.03 M, pH 4.5) and washed with the same buffer until the resin was in equilibrium with it. The extract (5 ml., pH 4.5) was then added and the column developed by gradient elution, that is with a solvent of constantly changing composition produced by running *N*-acetic acid into a reservoir (capacity 100 ml.) initially filled with the calcium acetate buffer with which the column had been prepared. The neutral and basic amino acids other than arginine passed straight through this column and their further separation is described below. This first peak was followed by glutamic acid and then by a third peak which was found to contain aspartic acid and arginine. These two amino acids were readily separated by chromatography on a short column of Zeo-Karb 225 at pH 5. The appropriate fractions were concentrated to 5 ml., and after adjusting the pH of the solution to 5 it was run on to the column of Zeo-Karb 225 (1 cm. $\times$ 7 cm.) previously equilibrated with disodium citrate (0.2 M, pH 5). The aspartic acid was washed off the column with a small volume of the same buffer and the arginine was then eluted with 2 M-disodium citrate.

The early fractions from the Amberlite column, containing the neutral and basic amino acids other than arginine, were mixed and freed from calcium by the addition of one

Fig. 1. Fractionation of soluble nitrogen of *Myrica* nodules.

equivalent of oxalic acid and centrifuging of the insoluble calcium oxalate. The supernatant was lyophilized and the residue taken up in 5 ml. of water. After adjustment of its pH to 5, this solution was placed on a short column of Zeo-Karb 225 (15 cm. $\times$ 1 cm.) prepared as described by Moore & Stein (1951). The column was developed with the sodium-containing buffers recommended by these authors and yielded tryptophan, free ammonia and basic amino acids well separated from one another.

The neutral amino acids, including asparagine and glutamine, were not retarded by this last column and passed through in a single band. The fractions containing them were transferred to a Van Slyke aeration tube in order to remove the amide groups from glutamine and asparagine. This was achieved by the method of Pucher, Vickery & Leavenworth (1935). Sodium hydroxide and phosphate buffer were added to give pH 6.5 and a phosphate concentration of 0.03 M, and the solution heated in a boiling-water bath for 2 hr. to decompose glutamine. The solution was then cooled, and adjusted to pH 9 with calcium hydroxide and the ammonia swept by aeration into boric acid (2%). The residual solution was acidified with sufficient sulphuric acid to make it N with respect to acid and the tube heated in a water bath for 3 hr. After cooling the solution was adjusted to pH 9 by the addition of calcium hydroxide and the ammonia from asparagine aerated into boric acid.

The mixture of amino acids now remaining in the reaction tube was separated by chromatography on a long column of Zeo-Karb 225 (150 cm. $\times$ 1 cm., Moore & Stein, 1954). Before addition to the column the solution was treated with oxalic acid until no further precipitation occurred, the precipitate of insoluble calcium salts was centrifuged off and the supernatant solution concentrated to 5 ml. and adjusted to pH 2.5.

The details of chromatographic technique used during these fractionations were similar to those described earlier (Leaf *et al.* 1958), as were also the methods of preparation for isotopic assay.

RESULTS

As indicated above, three experiments were carried out, each involving the exposure of nodules to excess of ^{15}N and their subsequent analysis.

The nodules contained some 0.7% of total nitrogen (fresh weight basis) of which 15–20% was soluble in dilute acid. The distribution of this soluble nitrogen among amino acids and ammonia is shown in Table 1. In the analysis of the extract from Expt. 1 a number of amino acids were lost during the final stage of the fractionation though they were known to be present as a result of paper chromatography of the original extract. In this experiment also, arginine was only partially separated from aspartic acid. Some arginine was obtained free from aspartic acid but not vice versa. Visual examination of the paper chromatograms of the original extract suggested that the major part of the nitrogen of the mixed fraction was contributed by the arginine. In Expt. 3 the mixed arginine-aspartic acid fraction was inadvertently degraded before separation of the two amino acids.

Here also the greater part of the nitrogen was from arginine.

In Expt. 2, where the recovery of amino acids and ammonia was most complete, these constituents accounted for 63% of the extracted nitrogen. The nature of the remainder is unknown. There was close agreement between the ninhydrin determinations and the determination of total nitrogen by the Kjeldahl method, suggesting that undefined nitrogenous substances were present in the isolated fractions in amount insufficient to interfere with the isotopic analyses.

A sample of the residue, left after extraction of the soluble nitrogen, was hydrolysed with hydrochloric acid (15 hr. at 115°) and the hydrolysate examined qualitatively by paper chromatography. In addition to the seventeen amino acids commonly found in protein hydrolysates hydroxyproline was also found. Comparisons of paper chromatograms of the hydrolysate with controls prepared from a gelatin hydrolysate indicated that hydroxyproline accounted for some 2–4% of the insoluble protein of the nodules.

All the substances listed in Table 1 which were present in sufficient amount were assayed for ^{15}N and the results obtained are presented in Table 2. A high proportion of the ^{15}N taken up by the nodules was present in the extract. For Expt. 2 this proportion was 82% and for Expt. 3, where the exposure was shorter, 89%. Of the ^{15}N in the extract approximately 60% was recovered in the compounds analysed in Expts. 2 and 3. In all experiments the highest abundance of ^{15}N was found in the amide group of glutamine which

Table 1. *Amino acid composition of extracts of Myrica gale nodules*

Results are expressed as mg. of N/100 g. (fresh weight) of nodules. + indicates that the amino acid was shown to be present but the amount was not determined. Aspartic acid fractions in Expts. 1 and 3 were heavily contaminated with arginine. The experiments were carried out on 1, 2 and 9 August respectively.

	Expt. 1	Expt. 2	Expt. 3
Time of exposure to ^{15}N (min.)	120	60	45
Aspartic acid	25.9	3.5	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
γ -Aminobutyric acid	+	2.3	2.5
Tryptophan	2.3	3.0	6.6
Lysine	0.2	0.4	0.2
Arginine	+	3.9	+
Histidine	0.3	0.4	+
Ammonia	2.5	5.0	3.5

Table 2. *Distribution of ^{15}N in the constituents of the nodule extracts*

Results are given as atom % excess over the normal figure (0.375 atom %). The aspartic acid fractions in Expts. 1 and 3 were heavily contaminated with arginine.

	Expt. 1	Expt. 2	Expt. 3
Time of exposure to excess of ^{15}N (min.) ...	120	60	45
Total nitrogen of nodule	—	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
Asparagine (amino N)	0.328	0.149	0.196
Glutamine (amide N)	3.140	1.450	1.684
Glutamine (amino N)	0.630	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

showed a degree of enrichment quite striking for nodule tissue. The abundance of ^{15}N in the asparagine amide nitrogen was lower and approximately equal to that of the amino nitrogen of free glutamic acid. The enrichment of the amino nitrogen of the amides was in all cases less than that of the amide nitrogen and less than that of the corresponding free amino acid. The enrichment of the tryptophan nitrogen is remarkably high but it has not been possible as yet to determine how the ^{15}N is distributed between the two nitrogen atoms of the molecule.

DISCUSSION

Among the products of hydrolysis of the protein fraction of *Myrica* nodules was, as noted above, hydroxyproline. In animal tissues this latter amino acid occurs in certain structural proteins such as collagen. The observations of Steward and his collaborators suggest that, though the mechanism of formation of this amino acid is similar in plant and animal tissues (Steward & Pollard, 1958), its occurrence in the former is more restricted (Steward, Thompson & Pollard, 1958). Their findings of its abundant occurrence in the proteins of crown gall of *Kalanchoë* sp. and tobacco-leaf tumours is of interest here since it is not unreasonable to assume a relationship between such structures and a root nodule.

Of the amino acids found in the protein fraction only nine are represented among the free amino acids of the nodules (Table 1) and the concentrations of these are spread over a wide range. This finding of an unbalanced amino acid composition conforms to what is now a familiar pattern for extracts of plant tissues (e.g. see Steward & Pollard, 1956, 1957; Oland & Yemm, 1956; Bathurst, 1953,

1954a; Miettinen & Virtanen, 1952; Leaf *et al.* 1958). The reasons for the accumulation of certain amino acids in relatively high concentration are not known, but presumably reflect in some way metabolic idiosyncrasies of the cells concerned. This has suggested that nitrogen-fixing tissues such as root nodules might show distinctive features of amino acid composition. The available data do not substantiate this. Legume nodules were found to have a general similarity in this respect (Hunt, 1951; Sen & Burma, 1953; Bathurst, 1954b), but the work of Virtanen & Miettinen (1953) revealed dramatic differences between the nodules of pea and those of alder. The results of the present investigation show that though *Myrica*, like *Alnus*, is not a legume the pattern of amino acids in its nodules is more akin to those of the legume nodules than to that of *Alnus* nodules. The latter contain high concentrations of citrulline but no asparagine or glutamine (Miettinen & Virtanen, 1952; Leaf *et al.* 1958); *Myrica* nodules contain no citrulline but, like legume nodules, contain generous amounts of the amides. The nodules of *Myrica* differ from those of the pea, however, in that they do not contain detectable amounts of homoserine. It appears therefore that the amino acid composition of the nodule sap is determined by the nature of the host plant. This is what may be expected if the nitrogen fixed is presented to the plant in a form normally assimilated by plants without nodules.

It is generally assumed that the accumulations of amino acids and amides act as a reserve supply of nitrogen for the synthesis of protein and other essential substances. There is, however, little evidence to show how readily available are these reserves to the plant. In some instances such substances may accumulate because they are metabolically inert and, once formed, however slowly, remain unchanged. In this case their nitrogen would not be readily available to the plant.

The present findings with respect to the distribution of ^{15}N have some bearing on this point, for they show that with the exception of arginine all the substances present in appreciable amount incorporated this isotope quite rapidly. It is perhaps significant that arginine behaves exceptionally here, for the same was found in the work on *Alnus* (Leaf *et al.* 1958). Yemm & Folkes (1958) suggest that in certain plants, such as the pea and apple, arginine may serve a function similar to that generally ascribed to the amides, asparagine and glutamine. This appears to be unlikely in either *Alnus* or *Myrica*. It would therefore be interesting to determine the rate of turnover of the nitrogen of arginine in plants where it is a more conspicuous constituent of the soluble nitrogen.

The main object of the present investigation was to secure evidence on the mechanism of nitrogen

Fig. 2. Possible routes of assimilation of fixed nitrogen in *Myrica nodules*.

fixation in *Myrica nodules*. In examining the results in Table 2 from this point of view, it is notable that the nitrogen atoms showing the highest abundance of ^{15}N are, with the exception of those of tryptophan, all in chemical groups which might be expected on the basis of comparative biochemistry to be derived fairly directly from free ammonia (Fig. 2). Admittedly there is some uncertainty as to the origin of asparagine in plants. Webster & Varner (1955) studied the formation of this amide in lupin seedlings and concluded that it was formed by a reaction between ammonia and aspartic acid, catalysed by an enzyme similar to that involved in the synthesis of glutamine (Elliott, 1953). As pointed out by Steward & Pollard (1956), the concentrations of substrates required by this enzyme were, however, very much higher than those for the glutamine-synthesizing enzyme, so high indeed as to throw doubt on its physiological significance.

In *Myrica nodules* it appears that asparagine may be formed at least as rapidly as is glutamine. Though the abundance of ^{15}N is always greater in the amide group of glutamine than in that of asparagine, when account is taken of the relative amounts of the two amides present it becomes evident that the amount of ^{15}N incorporated into the amide group of asparagine is some $3\frac{1}{2}$ times that incorporated into the amide group of glutamine. This cannot be taken as a direct measure of the relative rates of synthesis, but it suggests that asparagine may be synthesized at least as rapidly as is glutamine. The data do not give any indication of the mechanism of asparagine synthesis, but would be compatible with either a more active mechanism of the type described by Webster & Varner (1955) or one involving transamidation from glutamine, such as was found in animal liver by Mardashev & Lestrovaya (1951). Whichever reaction sequence is involved the asparagine receives its nitrogen fairly directly from ammonia. Asparagine and glutamine have long been considered to act as repositories of nitrogen in excess of that required for immediate anabolic requirements. In *Myrica nodules* the amide groups of these compounds appear to act as traps into which the nitrogen, newly fixed and apparently in the form of ammonia, drains as it is formed.

The evidence of the distribution of ^{15}N in the amino acids thus strongly supports the view that ammonia is an obligatory intermediate between fixation and assimilation of nitrogen. Apparently in discord with this view is the observation that ammonia itself does not show a higher abundance of ^{15}N than any other fraction. This is a common finding in work of this type (see Leaf *et al.* 1958, for references), and is usually explained by the assumption that there are two pools of ammonia in the tissue, one receiving newly fixed nitrogen and the other inert in this respect. Inherent in this is a second assumption that the same effect does not obtain, at any rate to the same extent, with the amino acids and amides. This explanation receives support from the work of Allison & Burris (1957) with *Azotobacter* and such a situation is even more likely in a nodule in view of the greater structural complexity.

SUMMARY

1. Nodules of *Myrica gale* were exposed to excess of ^{15}N , after detachment from the plant, for periods of 45–120 min. The soluble nitrogen compounds were then extracted from the nodules and separated by chromatographic procedures.
2. The amino acid composition of the extract was thus determined and it was found that asparagine was the predominant amino acid. Glutamine and various other amino acids were present in smaller amounts.
3. The highest enrichment with ^{15}N was found in the amide N of glutamine and the next highest in the amide N of asparagine and amino N of glutamic acid.
4. The data are considered to support the view that the fixed nitrogen passes through the form of ammonia before entry into organic combination.

The authors are indebted to Dr R. H. Burris, of the University of Wisconsin, for his kindness in carrying out the ^{15}N assays reported in this paper. One of the authors (I.C.G.) held a Carnegie Research Scholarship during the period of the investigation.

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Oxidative Enzyme Systems in Ossifying Cartilage

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(Received 11 December 1958)

Previous studies into the metabolism of cartilage have been directed mainly towards the anaerobic phase of carbohydrate metabolism and little is known about the presence of aerobic enzyme systems in this tissue. Early work indicated that cartilage was almost anaerobic in nature, and Bywaters (1937) and Lutwak-Mann (1940) showed that in articular cartilage oxygen consumption was virtually absent. The latter author was also unable to demonstrate histochemically the presence of the cytochrome system, a finding which was subsequently confirmed by Follis & Berthrong (1948). Cartilage before ossification shows little vascularization and Cartier (1951a) stated that the resultant low oxygen concentration accounted for the relatively high concentration of lactic acid in cartilage. Dixon & Perkins (1952) demonstrated the presence of the condensing enzyme and aconitase in cartilage, but found that isocitric dehydrogenase activity was very low. This, it was claimed, explained the accumulation of citrate in hard tissues.

Hiatt & Shorr (1953) were, however, able to provide histochemical evidence for the existence of dehydrogenase systems in rachitic rat cartilage by using 2:3:5-triphenyltetrazolium chloride. Follis & Melanotte (1956) confirmed this finding, and in addition showed that the reduction of the dye was accelerated by certain intermediates of the Krebs cycle.

Various attempts to demonstrate manometrically

the presence of dehydrogenase systems have been made and Boyd & Neuman (1954) demonstrated the presence of succinic dehydrogenase in embryonic chicken cartilage. Laskin & Engel (1956) confirmed this finding, but their results showed that glucose, pyruvate, citrate, α -oxoglutarate and fumarate did not affect the respiration of young metaphyseal bone slices. These results would indicate the absence of the Krebs cycle as an entity. The presence of succinic dehydrogenase has also been shown by Castellani & Zambotti (1954), who used a colorimetric procedure.

Recent work (Whitehead & Weidmann, 1959) on the effect of parathyroid extract on metabolism of adenosine triphosphate indicated the possible importance of oxidative phosphorylation in cartilage, but owing to the uncertainty in the literature as to the presence of an oxidative metabolism it was difficult to ascertain the significance of this suggestion. To clarify this situation, in the following work oxidative systems in ossifying cartilage were studied. The distribution of dehydrogenase and cytochrome oxidase systems was examined histochemically, and the nature of the various enzymes involved deduced from manometric studies. Oxidative phosphorylation in cartilage was also investigated by examining the effect *in vivo* of the injection of 2:4-dinitrophenol on the incorporation of ^{32}P into adenosine triphosphate.