LIII. INVESTIGATIONS ON THE ROOT NODULE BACTERIA OF LEGUMINOUS PLANTS

XXII. THE EXCRETION PRODUCTS OF ROOT NODULES. THE MECHANISM OF N-FIXATION

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The excretion of N-compounds from leguminous root nodules has been the subject of many years' research in this laboratory. Apart from its great significance in nature and agriculture the excretion has also rendered possible a closer examination of the chemical processes of biological N-fixation which have been completely obscure. As established in several publications[1] from this laboratory the excretion in certain cases assumes such proportions that over 80 % of the total fixed N is excreted from the nodules to the medium by young legumes. This fact and the special composition of the excreted products show that the excreted N-compounds are products of N-fixation and not secondary products of protein decomposition. A thorough chemical investigation of the excretion products was therefore necessary for the elucidation of the mechanism of N-fixation.

The results of our studies hitherto on the chemical composition of excretion products have been described in brief preliminary communications and summarizing lectures. A detailed report of the methods of analysis and of the course of the research has, however, not been presented. In this paper we give such a report.

The biological aspects of the excretion of N-compounds will not be discussed in detail here; we refer in this respect to our earlier contributions.[2] From the general course of the work, however, the following may be mentioned. Peas were grown in a sterile culture system developed by us, using generally N-free quartz sand, and later also cellulose, as a medium. The composition of the nutrient solution was as follows: $Ca_3(PO_4)_2$ 0·25 g., $CaSO_4$, $2H_2O$ 0·25 g., $MgSO_4$, $7H_2O$ 0·39 g., KCl 0·25 g., $FeCl_3$ (5 % solution) 3 drops in 1000 ml. of distilled water. Peas were inoculated with an effective strain of legume bacteria isolated from the root nodules of pea (strain HX in this laboratory). The growth of the peas depended, in the absence of nitrogenous fertilizers, entirely on the N-fixation in the root nodules caused by the bacteria. In the sterile system the N-compounds excreted from the root nodules to the medium could decompose only through the action of the legume bacteria, and these decompose the excreted N-compounds only very slowly in a definite manner (cf. below). The

[1] The summary of the results obtained in this laboratory up to the end of 1937 in the field of N-fixation as well as the especially great bearing of legumes on agriculture and human nutrition have been described in A. I. Virtanen's lectures delivered in the University of London and now published in book form under the title, *Cattle Fodder and Human Nutrition with special reference to Biological Nitrogen Fixation*, Cambridge, 1938. References are also included in the book.

[2] Especially we refer to our articles in the *J. agric. Sci.* **27**, 332 and 584 (1937).

excretion products could therefore be isolated from the medium at the end of the experiment period unchanged, or changed in a definite manner.

In ordinary pot cultures, even when N-free medium is used for inoculated peas (or other legumes), the excretion products cannot be isolated as such, because various and numerous micro-organisms present in the medium decompose the excretion products thoroughly, forming ammonia and nitrate. The use of a sterile culture system is therefore a necessary condition for the chemical elucidation of excretion products.

The plant experiments have been carried out with Dr Synnöve v. Hausen.

Experimental

Extraction and characterization of the excretion products

The experiment in 1932 [Virtanen *et al.* 1933] showed that when inoculated peas were grown in quartz sand in sterile culture system, without N-nutrition, and the N-compounds excreted into the medium were isolated by removing the roots carefully from the same, and extracting the sand with boiling water, the extract obtained contained the following N-fractions (Table I).

Table I

	N, mg.	N, % of total N
Total N in the extract	61·50	100·00
Amino-N	47·60	77·40
Ammonia-N	0·00	0·00
Amide-N	2·03	3·30
Volatile bases-N	1·68	2·73
Melanin-N	1·26	2·05

This preliminary experiment already showed that the major part of the nitrogen was amino-N. Since hydrolysis with HCl did not increase the amount of amino-N, the solution contained only free amino-acids and no peptides. Among the individual amino-acids an attempt was made to determine aspartic acid but with negative result. As was noticed later, this failure was due to the fact that the determination was made in the solution after ammonia determination according to Van Slyke, when aspartic acid is precipitated with alcohol and removed from the solution. The presence of volatile bases and melanin-N in the extract led to the assumption that the extraction with boiling water had probably caused decomposition in the primary excretion products.

We therefore made experiments with another excretion method. In a continuously acting apparatus sand was placed in a tinned metal dish, capacity 60 l. A glass tube near the bottom of the dish was led into a boiling flask, in which the water, acidified with H_2SO_4 to *p*H 2–3, was kept boiling. Steam was passed through a condenser, from which the water dropped on to the sand in the metal dish. In the flask the extracted compounds remained in the boiling solution for several days as the extraction in this apparatus was slow. Even this method therefore offered great possibilities for the decomposition of N-compounds. This extraction method was employed in the following experiment.

In the spring of 1935 peas were grown in our greenhouse in 25 Woulff's bottles (3 l.) each containing 4 kg. quartz sand. Two inoculated peas were grown in each bottle. The cultures were harvested in the middle of the flowering period, and the sand was washed from the bottles using as little water as possible. The root particles were carefully removed from the sand. The sand from all bottles (100 kg.) was extracted together for about 100 hr. in the manner

described. The extract was concentrated *in vacuo* to 500 ml. and filtered. The small precipitate contained 10 mg. N, and the clear filtrate, extract II, 577·5 mg. N. N was determined in both cases by the Kjeldahl method.

The filtrate contained the following amounts of amino-N [Van Slyke]:

After 5 min. reaction time:

In 10 ml. (1) 9·55 mg.
In 10 ml. (2) 9·49 mg. } In 500 ml. 476·0 mg. amino-N.

After 30 min. reaction time:

In 10 ml. (1) 10·05 mg.
In 10 ml. (2) 10·10 mg. } In 500 ml. 504 mg. amino-N.

The increase of amino-N during 30 min. reaction time indicates that the filtrate apparently contained also some other N besides α-amino-N.

Volatile bases were determined in 25 ml. of the solution according to Van Slyke's distillation method. The distillate used 1·1 ml. of 0·1 *N* H_2SO_4 corresponding to 1·54 mg. NH_3-N.

Ammonia-N was determined by the same distillation method also in 25 ml. Ammonia was determined in the distillate by Nessler's reagent. 0·3 mg. NH_3-N was found. Accordingly:

500 ml. contained 6·0 mg. NH_3-N,
500 ml. contained 24·8 mg. N of other volatile bases.

Amide-N was determined in the solution after volatile bases had been distilled according to Van Slyke. Conc. HCl was added to the solution up to 20%. The mixture was heated on the water bath for 14 hr. after which the major part of the HCl was evaporated *in vacuo*. The residue was nearly neutralized with 20% NaOH, made alkaline with MgO and NH_3 was distilled over. The distillate used 0·8 ml. of 0·1 *N* H_2SO_4 corresponding to 1·12 mg. NH_3-N. 500 ml. thus contained 11·2 mg. "amide-N".

Table II illustrates the quantities of different N-fractions in extract II.

Table II

	N, mg.	N, % total N
Total N	577·5	100·0
Amino-N	504·0	87·3
NH_3-N	6·0	1·0
Volatile bases-N	24·8	4·3
"Amido-N"	11·2	2·0

In this extract the amount of amino-N was considerably higher than in the extract of the preliminary experiment. The extraction method seemed thus to have a marked effect on the composition of the N-compounds. Since in extract II also the N-compounds remained for a long time in boiling solution at *p*H 2–3, decompositions were still possible.

In extract II the following qualitative tests and determinations were made:

1. Ninhydrin reaction was positive.
2. Folin's amino-acid reaction with 1:2-naphthoquinone-4-sodium sulphonate was positive.
3. Klein's arginine reaction was negative.
4. Winkler's tryptophan reaction was negative.
5. The determination of organic sulphur was negative; thus sulphur-containing amino-acids were not present.
6. Pauly's diazo reaction for histidine and tyrosine was negative.

7. Millon's reaction was negative.

8. The enzyme aspartase (dry preparation of propionic acid bacteria) formed ammonia abundantly in the extract. According to our experiments the said dry preparation does not split off ammonia in the presence of toluene from any amino-acids except from *l*-aspartic acid. Therefore it was evident that *a large quantity of aspartic acid was present* in the extract. Quantitative determination was not made.

9. 100 ml. of the extract (= 115·5 mg. N) neutralized to *p*H 7 were extracted for 100 hr. with *n*-butyl alcohol in Dakin's vacuum extraction apparatus. Only 0·5 mg. N was removed by the butyl alcohol. Thus it was evident that mono-amino-monocarboxylic acids and proline were not present in the extract.

10. A test was made to find out whether a part of the N in the extract was precipitable with phosphotungstic acid. 50 ml. of the extract (= 57·8 mg. N) were neutralized with NaOH, whereupon 15 g. of phosphotungstic acid were added and the solution brought to the boiling point. The solution was kept for 48 hr. at 37° before the precipitate was separated. Another sample of 50 ml. was taken and 10 ml. of conc. HCl and 15 g. of phosphotungstic acid were added. The solution was kept in the ice-box for 48 hr. N determinations in the precipitates of the two experiments gave the following results:

(1) 24·0 mg. N or 42 % of total N,
(2) 28·6 mg. N or 49·7 % of total N.

Since a considerable part of the N was precipitated with phosphotungstic acid, it was evident, in view of the above observations, that the extract contained, in addition to aspartic acid, either diamino-acids or other non-α-amino-acids.

Extract II already contained nearly 90% of the total N in the form of amino-N. The presence of melanin-N and volatile bases indicated that some decomposition had probably taken place during the extraction in boiling solution. Therefore we employed in the following extraction a new method, in which all heating was prevented. This procedure, which we have since used regularly, is illustrated in Fig. 1.

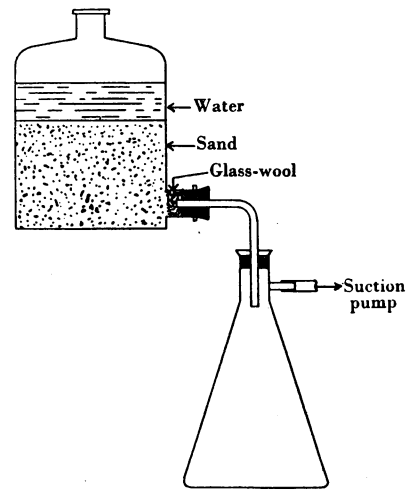


Fig. 1.

The sand from 10 Woulff's bottles, each containing 4 kg. quartz sand and 2 inoculated peas grown in summer 1935 without N-nutrition, was washed from the bottles with a small amount of water and the roots were removed simultaneously. The sand was extracted in portions of 8 kg. with cold water (about 15–20°) in the apparatus shown in Fig. 1. 3 l. of water acidified with H_2SO_4 to about *p*H 4 and containing some toluene were mixed well with the sand, whereupon the water was sucked into a filter flask. The extraction was repeated 10 times with the same water. Then a new washing was carried out in the same manner with another 3 l. of water. Each 8 kg. portion of sand was thus finally washed with 6 l. of acid water containing toluene, and the total amount of 40 kg. of sand with 30 l. of water. In this manner 80 % of the excreted N was recovered from the sand (cf. below).

The solution was evaporated to 500 ml. *in vacuo* on the water bath at 40°. The small precipitate formed during the evaporation was filtered off, and the filtrate was poured into a 500 ml. measuring glass and made up to this volume with water (extract III). 500 ml. of the extract contained (Table III):

Table III

	N, mg.	N, % of total N
Total N	259·0	100·0
Amino-N (30 min. shaking time)	256·0	98·8
NH_3-N	Traces	—
Volatile bases-N	Traces	—

The extraction at lower temperature showed that the N excreted from the root nodules was almost entirely amino-N and that the other N-compounds found in the previous extracts were evidently ascribable to the decomposition processes occurring during the extraction.

In this extract the same qualitative determinations were first made as in that described above. The results were identical.

Isolation and characterization of individual excretion products

Isolation of aspartic acid. 5 ml. of 10 % milk of lime and 250 ml. of 96 % alcohol were added, with good stirring, to 20 ml. of extract (= 10·36 mg. N), and kept for 1 hr. (Foreman's precipitate). The mixture was filtered and the precipitate washed carefully with 90 % alcohol, whereupon N-determination was made. The precipitate contained 5·25 mg. N or 50·7 % of the total N. The extract thus contained a monoamino-dicarboxylic acid. Attempts to identify glutamic acid as hydrochloride and as Zn salt, gave negative results. On the other hand we succeeded in isolating aspartic acid as the Cu-salt in the following manner. Amino-acids were precipitated from 50 ml. of extract (= 25·9 mg. N) according to Neuberg. The extract was made alkaline with 10 % Na_2CO_3. Small amounts of 25 % Hg acetate and 10 % Na_2CO_3 were added alternately so that the solution was kept constantly alkaline; 6 vol. of alcohol were added, the solution was filtered and the precipitate suspended in water and decomposed with H_2S. The filtrate was evaporated *in vacuo* to a small volume (about 25 ml.) and boiled with freshly precipitated $Cu(OH)_2$. It was filtered hot and kept in the ice-box. After a while the Cu-salt began to precipitate. Filtration was carried out after some hours, the precipitate was carefully washed with cold water and dried in a desiccator over H_2SO_4. The Cu-salt weighed 150 mg. Cu was determined according to Abderhalden & Schnitzler [1927] and N according to micro-Kjeldahl. (Found: Cu, 22·97, 23·30; N, 5·10, 5·03 %. $C_4H_5O_4NCu$, 4·5 H_2O requires Cu, 23·06; N, 5·08 %.)

The salt obtained was thus the *Cu salt of aspartic acid.* To confirm the fact, aspartic acid was also determined in the Cu salt by means of the enzyme aspartase.

190 mg. Cu salt were dissolved in 25 ml. of 0·1 *N* H_2SO_4 and Cu was precipitated with H_2S. H_2S was evaporated from the filtrate. 10 ml. of *M*/15 phosphate buffer (*p*H 7), 10 ml. of a suspension of *B. fluorescens liquefaciens* (from 375 mg. of dry bacterial preparation) and 10 ml. of toluene were added. The volume of the solution was made up to 150 ml.

The parallel solution consisted of 100 mg. of *l*-aspartic acid, 10 ml. of phosphate buffer, 10 ml. of bacterial suspension and 10 ml. of toluene, made up to 150 ml.

The control solution for the determination of the NH_3-content of the bacterial suspension was otherwise the same as the parallel solution, but did not contain aspartic acid.

All three experimental solutions were kept for 5 days at 37°, after which NH_3 was determined. According to our experience liberation of ammonia from aspartic acid by aspartase reaches practically its maximum during this time, and the reaction *l*-aspartic acid $\rightleftarrows$ fumaric acid + NH^3 attains its equilibrium.

The following amounts of ammonia were found in the different solutions:

	Bacterial suspension without aspartic acid	Bacterial suspension + 100 mg. *l*-aspartic acid (= 10·53 mg. N)	Bacterial suspension + filtrate corresponding to 190 mg. Cu salt (= 9·69 mg. N)
NH_3-N, mg.	4·76	10·50	9·80
NH_3-N, formed from the substrate	—	5·74	5·04
NH_3-N, % of the N in the substrate	—	54·50	52·00

The data show convincingly that the isolated Cu salt was Cu *l*-aspartate. It is known that aspartase does not split ammonia from *d*-aspartic acid.

Several methods were used for the quantitative determination of aspartic acid in the extract. The aspartase method gave the following result.

40 ml. of the extract (= 20·01 mg. N) + 10 ml. of a suspension of *B. fluorescens liquefaciens* (prepared from 375 mg. of dried bacteria) + 10 ml. of *M*/15 phosphate buffer (*p*H 7) + 10 ml. of toluene were made up with water to 100 ml. During 5 days at 37°, 5·6 mg. NH_3-N were formed (control, with bacterial suspension alone, subtracted). Since, under the same experimental conditions with the same bacterial material, *l*-aspartic acid forms 54 % NH_3-N of the total N, the amount formed in the extract, 5·6 mg. NH_3-N, corresponds to 10·4 mg. *l*-aspartic acid. Consequently, *52 % of the total N in the extract (= 20·01 mg. N) was* l-*aspartic acid-N*.

When amino-dicarboxylic acids were precipitated from the extract according to Foreman as the Ca salts, 50·7 % dicarboxylic acid-N was found. Since the aspartase method gave 52 % aspartic acid-N of the total N, it was evident that *the whole Foreman-fraction was* l-*aspartic acid*.

Aspartic acid was determined in still another way, viz. by oxidizing it first to malic acid and further to acetaldehyde. According to Fürth *et al.* [1932], the extract was treated with nitrous acid, followed by permanganate oxidation (the ordinary lactic acid determination). Acetaldehyde is only formed in this manner from α-alanine and aspartic acid. The acetaldehyde formed corresponded to 48·5 % of the total N in the extract. In the absence of α-alanine this N belongs to *l*-aspartic acid.

2 ml. of extract + 75 ml. of water + 0·5 ml. of conc. HCl were heated on the water bath. 15 ml. of 2·5 % $NaNO_2$ were added drop by drop from the burette during 20 min., after which 15 ml. of 7·5 % urea were added in the same manner to remove the excess of nitrite. The solution was made up to 250 ml. and the oxidation with permanganate was accomplished in 50 ml.

1·80 ml. of 0·008 *N* iodine solution were used, corresponding to 0·317 mg. acetaldehyde. From the whole extract (500 ml. = 259 mg. N) 396·5 mg. acetaldehyde were formed, corresponding to 126 mg. N or 48·5 % of the total N.

The different methods, Foreman's precipitation, liberation of ammonia by aspartase and the oxidation of aspartic acid after treatment with nitrous acid, thus gave the following amounts of aspartic acid.

Foreman's precipitation ...	50·7 %	aspartic acid-N of the total N
Aspartase method	52·0	,, ,,
Oxidation to malic acid and further to acetaldehyde ...	48·5	,, ,,

The results of the different methods are in good agreement. Thus approximately 50 % of the N in the whole extract is aspartic acid-N. As was shown by the aspartase method, the aspartic acid in question is the *l*-form. Glutamic acid was not found in the extract.

Characterization of the amino-acid precipitable with phosphotungstic acid

Nearly all of the rest of the N in the extract was precipitated with phosphotungstic acid. 50 ml. of the extract (=25·9 mg. N) were precipitated in acid solution with 15 g. of phosphotungstic acid. The solution was heated to boiling and then kept in the ice-box for 48 hr. 12·0 mg. N were precipitated or 47·1 % of the total N. Aspartic acid-N together with the N precipitated with phosphotungstic acid thus formed 97–99 % of the total N. On the basis of the qualitative experiments described above it was evident that the N precipitated with phosphotungstic acid belonged either to a diamino-acid or to a non-α-amino acid. In order to elucidate this point some additional determinations were made.

Formaldehyde titration. 8 ml. of the extract, freed from phosphoric acid and CO_2, used 2 ml. of 0·1 *N* NaOH corresponding to 2·8 mg. amino-N. The whole amount of amino-N was 4·10 mg., thus only 68·3 % of the amino-N had been fixed through formalin. In view of this fact a part of the amino-N must be in a position other than α with regard to carboxyl.

Carbon determination in the extract was made according to Osburn & Werkman [1932]. 25 ml. of extract (=12·95 mg. N) gave 156·0 mg. CO_2, whence the atomic C : N ratio was 3·8 : 1. In aspartic acid this ratio is 4 to 1, and consequently this ratio in the remaining amino-acid or acids in the extract must be smaller than 3·8 : 1, and in fact considerably smaller because the extract contains also some N-free organic compounds (cf. below).

Absorption spectrum. Amino-acids were precipitated according to Neuberg. The mercury precipitate was decomposed with H_2S, and the solution was evaporated *in vacuo* to a small volume. The final solution contained 10 mg. N in 100 ml. The absorption spectrum in the ultraviolet was photographed. No absorption maxima were noted, showing the absence of tryptophan, tyrosine and phenylalanine. A strong end absorption occurred rising abruptly at 20 mμ but this is characteristic of all aliphatic amino-acids.

Preparation of a picrate from the amino-acid fraction precipitable with phosphotungstic acid did not succeed. A dilute alcoholic solution of picric acid formed a precipitate, but it contained mostly potassium picrate, and no pure and characteristic amino-acid picrate could be isolated.

Taking into account all the facts hitherto observed, there was reason to assume that the amino-acid precipitable with phosphotungstic acid was a diamino-acid, probably lysine, provided that the amino-acid in question was a normal amino-acid present in proteins. Attention was therefore paid to the determination of lysine. This amino-acid is difficult to characterize and is therefore often determined indirectly. In order to develop a specific method for the determination of lysine we considered the possibility of splitting the carboxyl group from the lysine with the formation of cadaverine, which is easy to determine. As we have previously reported [1937], an almost quantitative production of CO_2 from lysine was accomplished by a strain of *B. coli* and the corresponding amount of cadaverine was obtained. Applying the same method to our extract we could not obtain cadaverine. From one of our extracts (extract IV), which contained 32·3 mg. N in 30 ml., we precipitated aspartic acid as the Ba salt according to Foreman. The precipitate contained 15·54 mg. N or 48·3 % of total N. Alcohol was evaporated from the filtrate and Ba was removed

as sulphate. The obtained filtrate was evaporated to 25 ml., 2 g. of moist *B. coli* (aseptically isolated) and 25 ml. of glycerol nutrient solution were added. No formation of cadaverine occurred within 3 weeks at 37°. From the parallel experiment, to which lysine was added, 90% of the theoretical amount of cadaverine was isolated. This proved that the N-fraction precipitating with phosphotungstic acid could not contain lysine. Putrescine, which is formed from ornithine by the same bacteria, was also excluded.

In the absence of all the known α-amino-acids, which might possibly have passed into the N-fraction precipitable with phosphotungstic acid, the unknown amino-acid must be one which is not found among the products of protein hydrolysis. Our attention was now directed to β-alanine. It appeared that the *legume bacteria split off quantitatively one carboxyl group from* l-*aspartic acid with the production of* β-*alanine* [Virtanen & Laine, 1937]. The presence of β-alanine among the excretion products of root nodules was thus quite natural. The following methods were used for the identification of β-alanine in our extracts.

The formation of ethyl acrylate. All the extracts investigated, and their N-fractions precipitable with phosphotungstic acid, gave, when treated with HCl and alcohol according to Abderhalden & Fodor [1913], a strong smell of ethyl acrylate. β-Alanine can be qualitatively determined in this manner.

The butyric acid compound of β-*alanine.* Przylecki & Kasprzyk [1937] have introduced a method for the characterization of basic amino-acids by means of their butyric acid compounds. 100 ml. of the extract (*p*H about 4) from sterile sand cultures of inoculated peas, containing 69·0 mg. N, were extracted in a percolator with ether for 3 days to remove the ether-soluble substances (cf. below). The aqueous solution, containing all the amino-N, was evaporated *in vacuo* almost to dryness, whereupon anhydrous Na_2CO_3 and anhydrous Na_2SO_4 were added. The dry mass was extracted in a Soxhlet apparatus with abs. methyl alcohol for 48 hr. 28·1 mg. N or 40·7% of the total N in the extract were obtained in the methyl alcohol, which was evaporated to dryness *in vacuo*. The residue was extracted with abs. butyric acid for 4 hr. and then filtered. The butyric acid solution contained 20 mg. N. Ether was added to the solution, causing the formation of a fluffy precipitate containing N. On washing with ether the precipitate became syrupy. It was dissolved again in butyric acid and precipitated once more with ether. The precipitate was washed with ether and dried in a desiccator. Its M.P. was about 180°. The butyric acid compound prepared from β-alanine melted at 204°. The mixture of both melted at 190°. On microscopic examination the butyric acid compound prepared from the extract was found to contain crystals typical of the butyric acid ester of β-alanine. A part of the preparation was, however, amorphous and the low M.P. was evidently due to this. Various salts in the extract originating from the nutrient solution of peas, were partly dissolved in methyl alcohol and butyric acid, and obviously prevented complete purification of the butyric acid compound.

The butyric acid compound gave, when treated with HCl and alcohol, a distinct smell of ethyl acrylate.

Oxidation of the extract with ninhydrin. α-Alanine is quantitatively oxidized with ninhydrin to acetaldehyde; aspartic acid forms at the most 10% of the theoretical amount; other normal amino-acids do not form acetaldehyde, whereas β-alanine forms 10–34% of the theoretical amount in different experiments [Virtanen & Laine 1938].

From an extract of quartz sand cultures of peas (extract V) aspartic acid was removed according to Foreman. The filtrate which contained 12·1 mg. N gave, on oxidation with ninhydrin, 6·46 mg. acetaldehyde or 17% of the theoretical

amount (1 N atom corresponds to 1 mol. acetaldehyde). This result therefore supports the conclusion that β-alanine forms at least the major part of the N-fraction precipitable with phosphotungstic acid.

Whether the whole fraction consists of β-alanine does not directly appear from our experiments. The absence of all known amino-acids implies, however, that β-alanine is the only amino-acid in the fraction. This fact is confirmed also by the ratio of aspartic acid to the fraction precipitable with phosphotungstic acid in the sand during different stages of growth of the pea (cf. below).

Nitrite- and oxime-N in the extract

As has been mentioned above, the extraction of quartz sand cultures of inoculated peas with acid water at room temperature produces extracts in which nearly all (90–99 %) the N is amino-N as determined according to Van Slyke using 30 min. reaction time. In the autumn of 1935 we detected the presence of NO_2-N in the extract by means of α-naphthylamine and sulphanilic acid according to Blom [1926]—later we used the more sensitive reagent atoxycocaine instead of sulphanilic acid according to Jendrassik & Falcsik-Szabó [1933]. Before extraction also some nitrite was generally found in the sand cultures. In every case, however, the NO_2-content of the concentrated extract was many times higher than before extraction of the sand. During extraction and especially during concentration of the extract some N-compounds must thus have formed NO_2-N. This is evident from the following experiment.

Sterile system. 2 Woulff's bottles; 4 kg. quartz sand; 2 inoculated peas in each bottle. N-free nutrient solution. After 40 days' growth the plants were harvested and roots carefully removed from sand.

4 peas		
Dry wt., g.	N, mg.	N, excreted in sand, mg.
2·040	60·6	90·9

In this experiment much more N was excreted into the sand than had been taken up by peas. Solution sample taken from the sand gave a very slight nitrite reaction.

The sand was washed five times, in the apparatus described above, with a total of 16 l. tap water. After every washing NO_2-N was determined in the washings, which were then evaporated *in vacuo* to 100 ml. The air passed through the solution during evaporation was washed with alkali to remove possible N-oxides. The washing waters contained the following amounts of NO_2-N:

1.	4 l. washing water	0·16 mg.	NO_2-N
2.	3 ,,	0·15	,,
3.	3 ,,	0·05	,,
4.	3 ,,	0·01	,,
5.	3 ,,	0·00	,,
	Total	0·37 mg.	NO_2-N

100 ml. of concentrated extract contained 1·72 mg. NO_2-N; thus its amount had increased during concentration from 0·37 to 1·72 mg. Also during the washing of the sand a considerable increase of NO_2-N had already occurred, but the amount cannot be expressed quantitatively, since the determination of nitrite in sand was not quantitative.

Examining the course of formation of NO_2-N we noticed that Endres [1935] had recently found oxime-N in *Azotobacter* cultures. Employing this method (hydrolysis of the oxime at 100° for 6 hr. in 3*N* H_2SO_4, followed by oxidation of the hydroxylamine formed with iodine to nitrite at room temperature) we found oxime-N in our extract also. In sand cultures it usually amounted to 1–2 % of

the total excreted N. NO_2-N was apparently formed from oxime-N, since in the concentrated extract oxime-N was decreased and NO_2-N increased. Later, when the chemical nature of the oxime was known, it was possible to prove experimentally the formation of NO_2-N in sterile cultures of peas from the oxime in question (cf. below).

Since the oxime is partly decomposed during slow extraction and particularly during long vacuum evaporations of large volumes of liquid, we started to use finely divided cellulose as a medium for the peas in order to isolate the oxime. 500 g. finely divided cellulose were placed, together with a sufficient amount of N-free nutrient solution, in a 1·5 l. suction flask. Two peas were grown in each flask. The sterile system was the same as with sand. All the excreted N-compounds were removed from the cellulose by 2–3 washings with a little water. In our experiments the nutrient solution was first pressed from the cellulose, after which the latter was washed three times with 1·5 l. of distilled water, altogether 4·5 l. The washings were added to the nutrient solution and evaporated as rapidly as possible *in vacuo* to 100 ml. Even then decomposition of oxime occurred to some extent as indicated by the following experiment.

Two parallel experiments with inoculated peas. Period of growth, 1 Mar.–23 April, 1936. Cellulose used as a medium.

	Peas		Medium			
No. of exp.	Dry wt., g.	N, mg.*	Excreted N, mg.	Oxime-N mg.	NO_2-N, mg.	Oxime-N + NO_2-N % of excreted N
1	1·370	36·5	7·3	0·06	0·10	2·2
2	1·291	34·4	6·7	0·12	Traces	1·8

* N in seeds is subtracted.

The total amount of oxime-N and nitrite-N in numerous cellulose cultures investigated was regularly 1–2 % of the excreted N. Only in one experiment did this amount rise to 10 %. In cultures of ripening peas, when N-fixation in the root nodules ceases, oxime-N is no longer found. Amino-N amounted, in cellulose cultures, to 95–98 % of the excreted N.

The isolation and characterization of the oxime was accomplished as follows.

Isolation and characterization of oxime

Isolation as Cu salt. From several cellulose cultures of peas the N excreted from the root nodules was extracted with water in the manner outlined above. 200 ml. of concentrated extract were made acid and extracted for 24 hr. in a percolator with ether, which removed 4 mg. oxime-N. The ether was evaporated of and the residue dissolved in a little water. A small amount of this solution was precipitated according to Foreman; all the oxime-N was thereby removed, whence the oxime was apparently also a dicarboxylic acid.

From the major part of the solution the Cu salt was prepared, by addition of 10 % $CuSO_4$ and alcohol. The precipitated Cu salt was dried at 50°. 20 mg. of green-coloured salt were obtained.

For comparison the Cu salt was prepared in the same way from synthetic oximinosuccinic acid. Both salts were similar in appearance.

Analyses: Cu was determined according to Abderhladen & Schnitzler [1927].

Cu salt prepared from the extract gave on analysis 31·6 % Cu.

Cu salt prepared from oximinosuccinic acid gave on analysis 31·1 % Cu.

The analytical data are in very good agreement. The Cu salt of the oximinosuccinic acid contains theoretically 30·5 % Cu. Low Kjeldahl N-values were

found for both the synthetic Cu salt and that of oximinosuccinic acid isolated from the extract owing to the fact that only a small part of the oxime-N is determined by this method. Both the Cu salts contained oxime-N as determined according to Endres [1935], but the values were in both cases too low owing to the fact that oxime-N is partly oxidized in the presence of Cu. In the synthetic Cu salt 66 % and in the isolated Cu salt 79·6 % of the theoretical amount of oxime-N were found.

Characterization of oxime through its reduction to aspartic acid. Ether solution containing 2 mg. oxime-N, prepared from pea cultures as described above, was evaporated and the residue dissolved in 10 ml. of methyl alcohol. A small amount of finely divided platinum was prepared, and saturated with hydrogen in the cold hydrogenation apparatus of Hückel. The methyl alcoholic solution of the oxime was added and hydrogenated. The hydrogen uptake was 60 ml. in 3 hr. The solution was filtered and evaporated to dryness, the residue dissolved in water and extracted thoroughly with ether in a percolator to remove N-free substances such as succinic acid formed from fumaric acid (cf. below). The aqueous solution was evaporated to a small volume and boiled with freshly precipitated $Cu(OH)_2$. It was filtered hot and kept in the ice-box. A green-coloured Cu salt separated which, after drying in a desiccator, weighed 10 mg. and gave on analysis 23·20 % Cu and 5·66 % N. Cu-aspartate requires 23·06 % Cu and 5·08 % N. Since aspartic acid can be formed on reduction only from oximinosuccinic acid, this must have been the oxime isolated with ether from the cellulose culture of peas.

N-free C-compounds in the extract

N-free dicarboxylic acids. After reduction of the oxime the N-free residue left in the ether was dissolved in water and precipitated with $AgNO_3$, yielding the *Ag salt of succinic acid.* The great excess of hydrogen taken up in the reduction had therefore been used in the reduction of fumaric acid to succinic acid.

Fumaric acid could be isolated with ether from an extract of sand culture of peas, which was kept until all the oxime was decomposed. The amount of fumaric acid was small, 25 mg. of the crude substance being obtained from an extract which contained 120 mg. N. It is possible that in addition to fumaric acid some malic and succinic acids were present in the extract, but these were not investigated more closely.

Amount of aspartic acid among the excretion products at different stages of growth

When sand was washed with water to isolate the excreted N-compounds, it was found that these were very difficult to remove. The thorough extraction, outlined above, with cold water slightly acidified with H_2SO_4 (*p*H 3–4) usually led to the recovery of about 80 % of the excreted N-compounds in the solution. The possibility therefore existed that the remaining 20 % of the N was to be found in compounds other than those in the extract. This fact deserved full attention when theoretical conclusions were drawn.

We therefore tried to transfer all the N-compounds from the medium to the aqueous solution. This was easily attained when finely divided cellulose was used as a medium. Already with two washings all excreted N was recovered from the cellulose in the aqueous solution. The excretion was, however, lower in cellulose cultures than in sand cultures, varying usually from 10 to 20 % of the total fixed N, so that in order to obtain the same quantity of N for investigation much more cellulose cultures would have been required than sand cultures.

Besides, since quartz sand was mostly used as a medium in our experiments, we tried to study as completely as possible the particular N-compounds excreted in such media.

In continuing the extraction experiments we could ascertain that the extraction was generally the more complete the earlier the peas were harvested. Using for extraction water which was acidified with HCl to *p*H 3–4 we succeeded in recovering from young pea cultures, harvested before flowering, practically all the excreted N in the aqueous solution, as shown by the following experiment (Table IV).

Table IV

3 l. Woulff's bottles; 4·8 kg. dry quartz sand and 2 l. N-free nutrient solution in each bottle. 2 Torsdag peas in each bottle, inoculated with strain HX. Period of growth 17 Sept.–22 Oct. 1937. Plants were still not in flower at the end of the experiment. Roots were removed and the sand from all bottles was mixed together. 8 kg. of sand were extracted at a time with cold water in the apparatus shown in Fig. 1. Water was acidified with HCl to *p*H 3–4. All aqueous extracts were evaporated together *in vacuo* to 200 ml.

						In the aqueous extract		
No. of exp.	Dry wt. of plants g.	N in plants mg.*	N excreted in sand, mg.*	Total fixed N, mg.	Extent of excretion	N, mg.	N, % of the excreted N in sand	Aspartic acid-N % of total excreted N
1	1·116	14·5	16·3	30·8	52·9			
2	0·940	14·1	5·0	15·0	33·3			
3	1·115	11·5	16·3	27·8	58·6			
4	0·936	10·4	10·1	20·5	49·3	64·6	99·8	74·7
5	1·115	14·9	5·0	20·0	25·0			
6	1·382	18·2	5·0	23·2	21·5			
7	0·699	13·8	7·0	10·8	64·8			

* Control subtracted.

In this experiment 74·7 % of excreted N was *l*-aspartic acid-N. This amount was appreciably higher than in previous experiments. The difference is due, as will be seen from the data below, to the fact that aspartic acid decreases while β-alanine increases at later stages of growth, a result which is to be expected. Since in the earlier experiments the plants had been in flower, the amount of aspartic acid was already smaller.

In the extract obtained from the experiments presented in Table IV the same qualitative amino-acid analysis was carried out as described earlier in connexion with extract II; the results were identical. With the exception of aspartic acid the extract thus contained no other amino-acids normally present in proteins. β-Alanine could be detected in the extract, since acetaldehyde was formed in the ninhydrin reaction after removal of aspartic acid. In addition, the extract contained some nitrite- and oxime-N:

	% of total N
N precipitated according to Foreman (*l*-aspartic acid-N)	74·7
N precipitated with phosphotungstic acid (β-alanine, calculated)	23
Oxime- and nitrite-N	2

On the basis of this result, the root nodules would not excrete other N-compounds than those found in our investigations, unless the phosphotungstic acid fraction contains some unknown amino-acid in addition to β-alanine. The large amount of aspartic acid in the extract is obviously due to the fact that the cultures were harvested at a very early stage, before flowering. The following series of experiments (Table V), in which the cultures were harvested at different

Table V

The experiment was arranged in the same manner as that in Table IV.

Period of growth	Stage of growth	'Dry wt. of plants, g.	N in plants, mg.*	N excreted in sand mg.*	Tota fixed N, mg.	Extent of excretion	In the aqueous extract: N, mg.	In the aqueous extract: N, % of the excreted N in sand	In the aqueous extract: Aspartic acid-N, % of the N in the extract
30. iii.–24. iv. 37	Before flowering	1·319	28·0	9·1	37·1	24·5	28·8	95·1	63·2
		1·648	44·3	13·0	56·3	23·1			
		1·449	33·3	8·2	41·5	19·7			
30. iii.–3. v. 37	At start of flowering	3·456	105·4	35·4	140·8	25·2	118·3	90·9	51·7
		2·947	79·9	51·8	131·7	39·3			
		4·011	118·6	43·7	162·3	27·0			
30. iii.–13. v. 37	In full bloom	3·438	112·1	16·3	128·4	12·8	56·1	87·2	47·0
		3·449	104·0	20·6	124·6	16·5			
		4·460	122·9	27·4	150·3	18·2			
30. iii.–28. v. 37	Pods developed	7·904	167·2	7·7	174·9	4·4	33·1	85·0	35·7
		8·453	159·9	20·2	180·1	11·2			
		8·382	204·4	11·0	215·8	5·1			

* N of the uninoculated controls (14·0 mg.) subtracted.

times, shows clearly that the percentage of aspartic acid-N in the excreted N decreases while β-alanine increases towards the end of the growth, as is to be expected.

This experiment illustrates clearly that the aspartic acid content is higher in younger cultures than in older ones. The experiment also shows that during later stages of growth the pea is able to absorb from the medium through its roots the N-compounds which have been excreted at the earlier stages. In this connexion aspartic acid is of major importance; according to our findings it is an excellent N-source for the legumes.

It is interesting that in older cultures of inoculated peas oxime-N is no longer found.

In 1935–36 we determined the excretion products regularly in pea cultures harvested at flowering stage and obtained extracts which contained about 50% aspartic acid-N. The above data show, however, that early in growth *l*-aspartic acid is the main excretion product, and that it is later partly replaced by β-alanine, so that the amount of *l*-aspartic acid decreases continuously with the age of the cultures. In associated cultures of inoculated peas and non-legumes e.g. barley (sterile system) the amount of aspartic acid is generally higher than in cultures of peas alone, since the non-legume cannot utilize aspartic acid appreciably but can use β-alanine.

Discussion

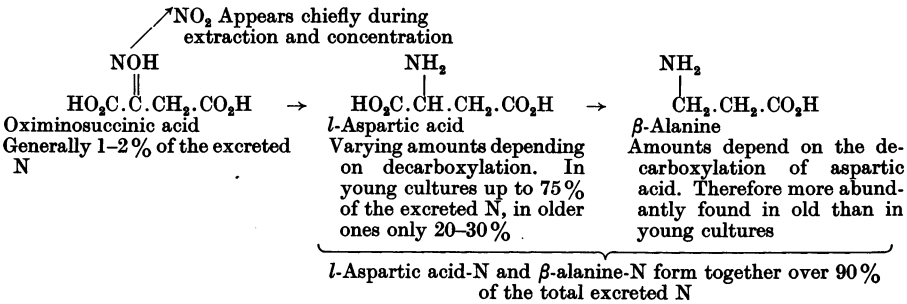
In the medium of inoculated peas grown in a sterile culture system N-compounds appear immediately after nodule formation. The N-compounds have been shown to be excreted from the root nodules and not from the roots. Their amount may increase so much that the major part of the total fixed N is diffused from the root nodules to the medium and the host plant can utilize only a small part of the fixed N. Such being the case, the chemical nature of the excreted N-compounds is of especial interest from the standpoint of the mechanism of N-fixation.

The excreted N is chiefly amino-N. In young pea cultures the major part of the excreted N is accounted for as *l*-aspartic acid. In addition to aspartic acid

also an amino-acid precipitable by phosphotungstic acid has been found among the excreted N-compounds and has been shown to be β-alanine, which is formed from *l*-aspartic acid by the legume bacteria. Other amino-acids have not been found in the medium of inoculated peas. In addition to amino-N the medium contains some $>C{:}NOH$-N and in most cases also some NO_2-N; their total amount together is generally 1–2 % of the total excreted N. The nitrite-N is formed from oxime-N, the oxime in question being oximinosuccinic acid.

The excreted N-compounds and their mutual relations appear from the following:

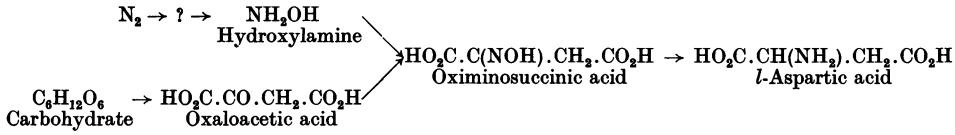
The excretion products of the root nodules



Taking into account the often exceptionally high rate of excretion as well as the chemical composition of the excreted N-compounds and their mutual relations, the excreted compounds must be regarded as the products of N-fixation. As β-alanine is a secondary decomposition product of aspartic acid, the actual percentage of aspartic acid-N in excreted N is over 90 %. On the other hand, as the ratio of excreted N to total fixed N may rise to 80 %, it can be said that approximately 75 % of the total fixed N may be excreted in the form of *l*-aspartic acid. Thus it is impossible that the excretion products of the root nodules are products of decomposition of bacterial proteins, since the content of aspartic acid in proteins is far from such amounts, being generally below 10 %. According to our determination the N in root nodules contains about 10 % aspartic acid-N. In addition the legumes use aspartic acid excellently as their N-source and on the basis of the facts hitherto known it seems likely that the legumes receive their N-nutrition from the root nodules actually in the form of aspartic acid. Thus the percentage of aspartic acid-N in the total fixed N would rise close to 100 %. *The fixation of atmospheric N in the root nodules thus leads to* l-*aspartic acid, which consequently is a primary fundamental amino-acid.*

The mechanism of the formation of aspartic acid is explained by the isolation of oximinosuccinic acid. This oxime, which is easily reduced to aspartic acid, is obviously the precursor of aspartic acid. Oximinosuccinic acid is formed from hydroxylamine and oxaloacetic acid. Even in extremely dilute solutions oxaloacetic acid and hydroxylamine react instantaneously at slightly acid or neutral reaction so that hydroxylamine cannot be detected in a solution which contains an excess of oxaloacetic acid. No other carbonyl compounds investigated by us react so vigorously with hydroxylamine as does oxaloacetic acid. Oximinosuccinic acid must therefore be formed in the root nodules from hydroxylamine and oxaloacetic acid. Experimentally we have been able to show that oxaloacetic acid is found in leguminous plants; the host plant thus supplies the acceptor for hydroxylamine. The fact that hydroxylamine has not been found among the excretion products of root nodules is easily explicable, owing to its rapid

reaction with oxaloacetic acid. On the basis of the experimental facts the course of biological N-fixation occurring in the leguminous root nodules is as follows:



This course of the reaction is in agreement with all the facts hitherto known; the results cannot be interpreted otherwise on the basis of the present knowledge. How hydroxylamine is formed from atmospheric nitrogen has not yet been experimentally proved. It can be assumed hypothetically that a di-imide is formed as a primary reduction product ($N_2 \rightarrow HN{=}NH$) which then forms hydroxylamine by addition of two water molecules. The reduction of the nitrogen molecule, "N-fixation" in a stricter sense, requires obviously a specific enzyme, which is found in the N-fixing bacteria. The reaction of hydroxylamine with oxaloacetic acid is a non-enzymic process. On the other hand, the reduction of oximinosuccinic acid to *l*-aspartic acid is an enzymic reaction. It has not yet been discovered from which compounds in the plant the hydrogen required for the reduction is transferred to the substrate to be reduced, and which enzymes act in the reduction process.

On the basis of the above facts N-fixation requires the presence of oxaloacetic acid. The important role of this C_4-dicarboxylic acid in N-fixation offers an explanation of the symbiosis of legumes and intranodular bacteria, which has hitherto remained mysterious. It has been possible to show synthetically the decisive role of oxaloacetic acid in N-fixation and thus to throw more light on our idea of the reaction mechanism of N-fixation. Excised root nodules do not fix nitrogen in aqueous solution and even in solutions containing glucose N-fixation is either nil or very slight, but in a neutral solution of oxaloacetic acid a vigorous N-fixation occurs already in a few hours [Virtanen & Laine 1937]; simultaneously oxime-N and amino-N appear in the solution. In a series of 25 experiments distinct N-fixation has been accomplished in all the experiments with excised root nodules in presence of oxaloacetic acid. We are going to describe these experiments more closely in another communication.

Aspartic acid is, according to our results, a primary amino-acid formed in the N-fixation. Other amino-acids must therefore be formed from this fundamental amino-acid. In the pea plant the reaction:

$$HO_2C.CH_2.CH(NH_2).CO_2H + CH_3.CO.CO_2H \rightleftarrows HO_2C.CH_2.CO.CO_2H + CH_3.CH(NH_2).CO_2H$$

takes place rapidly, so that the formation of other amino-acids from aspartic acid is easily understood [Virtanen & Laine, 1938, 2]. In plants the reamination (*Umaminierung*) occurs at least partly in the same manner as, according to Braunstein & Kritzman [1937], it does in animal tissues. It is surprising that no glutamic acid is formed in N-fixation by root nodules, although glutamic acid occupies a special position in amino-acid synthesis. In v. Euler's laboratory [Adler *et al.* 1938] several important investigations have been carried out during the last year on the dehydrogenation of glutamic acid and on the reamination taking place thereby, which according to these findings is caused in micro-organisms and plants by a specific glutamic acid dehydrogenase.

The reaction mechanism of the N-fixation occurring in free-living *Azotobacter* is obviously similar to that occurring in the intranodular legume bacteria. The

excretion of N-compounds by *Azotobacter* is, however, so slight that it would be impossible to conclude anything from it, if the results attained with legume bacteria did not justify the conclusions by analogy. The great difference in the excretion by *Azotobacter* and by intranodular legume bacteria is obviously due to the fact that the former uses the products of N-fixation largely for the formation of its cell protein, while the latter excrete the major part of them. We shall give later a detailed report of our experiments with *Azotobacter*.

Summary

The N-compounds excreted from the root nodules of leguminous plants have been isolated and characterized.

Over 90 % of the excreted N is amino-N. In addition, 1–2 % oxime-N and some nitrite-N is found.

The major part of the amino-N is present as l-*aspartic acid*, if peas are harvested at a young stage, long before flowering. In ageing cultures the amount of aspartic acid decreases.

The other amino-acid found among the excretion products is *β-alanine* which is slowly formed from *l*-aspartic acid by the legume bacteria. Its formation explains the decrease of aspartic acid in ageing cultures.

The oxime appearing among the excretion products is *oximinosuccinic acid*; nitrite-N is formed from this oxime.

Some *fumaric acid* is detected as an N-free excretion product.

The often exceptionally high amount of excreted N (60–80 % of the total fixed N) and the special nature of the excreted N-compounds prove that these N-compounds are products of N-fixation. An idea has thus been obtained, on the basis of the excreted N-compounds, of the mechanism of biological N-fixation.

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