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" SOME ASPECTS OF SYMBIOSIS OF LEGUMINOUS PLANTS AND NODULE BACTERIA ".

-being a Thesis presented by

JANNIS THOMAS VANTSIS

for the Degree of Doctor of Philosophy of the University of Glasgow.

December 1948.

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The work was carried out under the direction and supervision of Dr. George Bond, to whom the writer wishes to express his most sincere thanks not only for the keen interest and continuous suggestions regardings his research but also for the trouble which he took in procuring the material and tools for the work at a time when everything was in acute shortage, and for the opening of his private library to the writer.

The writer apologises for the fact that the thesis is not written in very good English. He has received assistance in this connection from several friends, including Dr. Bond, but realises that there is still much to criticise, due to the thesis being written in what is, to the writer, a foreign language.

December 1948.

Department of Botany,
University of Glasgow.

GENERAL INTRODUCTION.

In spite of the remarkable progress that the science of Chemistry has made in the synthesis of organic substances, man is still absolutely dependent on the vegetable production of the soil for his own food and for the food of domestic animals. Any improvement in the production of the soil is a new victory against the disastrous results of starvation from the effects of which the world is still suffering severely to-day, even three years after the end of the 1939-1945 War

One of the greatest scientific achievements in the past century in this field was undoubtedly the discovery of the meaning and the use of fertilizers. Nitrogenous fertilizers are particularly important as most cultivated soils are short of nitrogen, and the increase of the crop due to its application is always considerable. This is the reason why the discovery of methods for the synthesis of ammonia was accepted with relief by the world as a means of cheaper and more abundant production of food. More than thirty years after the general use of synthetic nitrogen it is almost unbelievable that despite the evergrowing increase in the manufacture of synthetic nitrogen, production is extremely small, corresponding to an insignificant percentage of the total nitrogen returned yearly to the soil. The main supplies of this fundamental element are still provided by the symbiotic and non-symbiotic nitrogen-fixing micro-organisms. Considering the fact that the activities of the nitrogen

fixing micro-organisms are thus integrated with all terrestrial life it is easily understood why so much thought has been devoted to their study over the past sixty years.

A greater understanding of the life and the mechanism by which these micro-organisms fix the free nitrogen of the atmosphere, apart from the theoretical point of view, will eventually bring by its application to agriculture a notable increase in the production of the soil, thereby giving into the hands of man a formidable weapon to use in his struggle against the menaces of famine and starvation, which according to the warnings of eminent scientists are not only temporary but are bound to be more permanent and acute in the near future due to the continuous increase of the human population and to the simultaneous maltreatment and deterioration of the soil productivity.

Since the discovery by Hellriegel and Wilfarth (in1886) of the function of the nodules in legumes, and the subsequent isolation of the micro-organism by Beijerink (in 1888), extensive work has been done on the life and the needs of the micro-organism outside and inside the nodule as well as on the conditions under which nitrofen fixation occurs and a brief review of the most outstanding achievements in this field is considered as useful.

The root nodule bacteria have been placed in a special genus Rhizobium, which is generally held to occupy a systematic position close to Bacillus radiobacter, and further the bacteria have been separated in groups (species) each one of

which is able to infect and produce nodules on a limited number of host legumes- legumes of the same cross- inoculation group. (Fred, Baldwin, and McCoy 1932).

The life cycle of the organism outside the host plant and in the nodule has been studied and the way by which the organism enters the root hair and causes the formation of nodules is quite well known.

Different strains of the nodule organism have been classified as efficient or inefficient according to their ability to fix nitrogen when they are in nodulated legumes, and although at the beginning the inefficient strains were thought to be unable to fix nitrogen, recent studies (Chen and Thornton 1940) showed that the so-called inefficient strains are equally good fixers of nitrogen per unit of time and active bacterial mass, but due to the small size of the active bacterial mass in the nodule and to the short life at this stage and the rapid disintegration, the quantity of nitrogen actually fixed by them is almost negligible.

Since in the soil a legume crop usually obtains its nodules from a mixed population of legume bacteria varying in their effectiveness towards the production of nodules in the host plant, as well as in their efficiency to fix nitrogen, the relative effectiveness of the nodule bacteria has been studied and some strains have been found as dominant, when they compete with other ones. By isolation of strains combining dominant effectiveness and efficiency, the problem of providing the cultivated legumes with efficient nodules in soil

lacking such efficient micro-organisms greatly progressed by the inoculation of the seeds with these efficient and effective strains. (Thornton 1936).

As soon as the nodule bacteria enter the roots through the root-hairs, the cells of the root cortex become meristematic and by division produce the young nodule. Cell division extends beyond the cells that are actually infected and is perhaps due to the secretion of some diffusible stimulant by the bacteria.

Now the way by which the bacteria are distributed through the cells of the young nodules differs in various legumes but the result is that soon the cytoplasm of the infected cells in the centre of the nodule becomes closely packed with bacteria, usually in swollen branched form, the so called bacteroids, and this bacterial tissue is presumed to be the seat of the nitrogen fixation. (Thornton 1936).

Little progress has been made towards solving the problem of how the nitrogen is fixed in the nodules. The nodule organism is not able to fix nitrogen outside the host plant, and since the fixation of nitrogen in the nodules is closely connected with the active photosynthesis of the host, and evidently with the continuous supply of carbohydrates to the nodule tissue, attempts have been made to determine the provided and consumed energy for the process of fixation of the unit of nitrogen. Relative figures for the respiration of the unit of mass of nodule-tissue, and non-nodulated roots, (Bond 1941.), revealed that the respiration of the nodule

tissue is significantly higher than the corresponding respiration of the non-nodulated mass of roots, and gross figures for the required energy for the fixation of one mg. of nitrogen are reported as high as 19 mg. of carbohydrates in Soya plants growing in water cultures.

As regards now the primary and intermediate products through which the nitrogen fixation takes place, the occurrence of excretion of nitrogenous substances by nodulated legumes growing in sterile cultures in some environments, enabled the Helsinki workers to put forward the theory that the fixation takes place probably through hydroxylamine-oxime of oxalacetic acid -and finally through aspartic and glutamic acid. (Virtanen 1938, 1947).

Whatever the primary and intermediate products of the nitrogen fixation may be the question arises in which way are the fixed nitrogenous substances released to the host cytoplasm. Whereas earlier theories supported the hypothesis that the release took place as a result of a digestion of bacteria through the enzymes action of the host cytoplasm, the prevalent theory now is that the bacteria excrete the fixed nitrogen to the host cytoplasm as soon as they start fixing nitrogen, and that this excretion keeps pace with the fixation amounting to about 90 per cent of the fixed nitrogen. (Bond 1936, Jensen 1948.).

Apart from the nitrogenous substances that the bacteria release to the host cytoplasm, under special conditions (probably when the fixed nitrogen exceeds the quantities that

can be utilised by the synthesised carbohydrates of the host plant) a quantity of the fixed nitrogen is excreted from the young healthy nodules to the surrounding rooting medium and the phenomenon of such excretion has been studied extensively in an attempt to elucidate the proper conditions of the environment for the occurrence of it. (See Section II of Thesis).

The symbiosis of bacteria and legumes is a delicate equilibrium and with a slight change of the environmental conditions the equilibrium is disturbed and the bacteria soon become parasitic organisms. Two factors to which special importance has been given for the maintenance of this equilibrium are the total or soluble carbohydrates synthesised by the plant, (Allison 1935), and the ratio of the total or soluble carbohydrates to the total or soluble nitrogen of the plant, (Wilson 1940). Fixation according to the last theory takes place only when this ratio is within a narrow range and any deviation from this narrow range giving as a result relative excess or shortness of carbohydrates, soon upsets the equilibrium.

The discovery of the red pigment in the healthy nodules and the identification of it as haemoglobin,(Keilin and Wang 1945), is one of the latest achievements in this field, and the suggestion about the probable connection of the red pigment with the process of fixation is very interesting. (Virtanen et al 1947). Finally the finding that nodulated legumes require especially the presence of Molybdenum for the function

of nitrogen fixation (Anderson and Thomas 1946)must be mentioned.

Although as a result of extensive work many aspects of the complex problem have been elucidated, much still remains to be done, and in the present thesis a small contribution to some aspects of the problem has been attempted. The thesis may be divided into three sections presented separately under the following titles:

I. Effect of charcoal on growth of legumes in sand culture with observations on excretion of fixed nitrogen from nodules.

II. Further experiments of excretion of fixed nitrogen from nodules.

III. Nitrogen nutrition of non-nodulated clover plants.

Section I.

Effect of charcoal on growth of legumes in sand culture with observations on excretion of fixed nitrogen from nodules.

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I N T R O D U C T I O N.

The practice of cultivating mixed cultures of legumes and non-legumes is a very old system applied in primitive agriculture all over the world since ancient times and the opinion that the non-legume benefits from the adjacent legume is supported by farmers in most countries. There is an axiom in science that the experience of the race must never be disregarded unless experiments prove their bases to be unsound.

Lipman (1912)is probably the first investigator who tried to demonstrate with experiments that non-legumes benefitted strongly from association with a legume and among the other earlier investigations on this subject that of Stallings (1926)must be mentioned. But to Virtanen and his associates goes the credit for a thorough investigation of the problem and for obtaining the explanation of the beneficial on the non-legume in mixed cultures.

Virtanen and his co-workers in a series of papers (Virtanen 1938, 1947) proved definitely that in Helsinki healthy nodulated legumes excrete a considerable amount of the fixed nitrogen very early and at a time when no question of disintegration of nodules may arise, this amount being sometimes as large as 80% of the total fixed nitrogen.

Soon after this many workers all over the world tried to confirm Virtanen's results and although they followed his methods almost exactly and sometimes even used the same medium, the same varieties of seeds and the same strains of bacteria, most of them failed to detect any excretion at all,

or only succeeded in detecting a very small one, far below the excretion reported by the Helsinki investigators. Literature is full of papers reporting negative results and a number of theories have been devised trying to explain why under one environment the excretion is almost a common phenomenon, whereas under another, exactly the opposite is the rule.

The present writer working in Greece at the Institute of Plant Breeding, Thessaloniki, has had the opportunity of seeing experiments with mixed cultures in pots and fields carried out by Papadakis (1939,1940), and although these experiments were carried out in pots with soil instead of the sand usually used and their final results may be questioned, nevertheless early observations on the appearance of the plants suggested the acceptance of probable excretion. An experiment carried out by the writer in 1940-41 at the low-nitrogen fields of the Institute, showed that in these fields at least with some of the small-seeded legumes, especially lentils, wheat growing in mixture strongly benefited from the adjacent legumes. The appearance of wheat in all the plots with lentils was far better than that of the wheat growing alone and this superiority was already clear from almost the first weeks when no question of disintegration of nodules could have arisen.

The War stopped further experiments and in 1945 soon after the Peace, the writer, with the confidence that apart from Helsinki the phenomenon of excretion could also be detected in Greece, reached Glasgow as a scholar of the

British Council and started research under Dr Bond's supervision in the Botany Department of Glasgow University.

Since Bond (1938,1939,1941a)during several years work in the Glasgow environment failed to detect any appreciable excretion, further work on the problem in Glasgow was considered as useful only with the modification of the rooting medium and in general, of the environment. The paper reporting experiments by the Russians, Gukova and Butkevitch (1941) with a mixture of sand and charcoal was the starting point for the experiments described below.

Gukova and Butkevitch, using mixture of a sand with 6 per cent granulated charcoal (type not stated)in pot cultures (type of pot - glazed or unglazed not indicated),reported that inoculated Soya beans watered with Hellriegel's solution containing only one tenth of the normal quantity of nitrogen, produced twice as much total yield as Soya beans grown in sand without charcoal;the weight of nodules being nearly doubled, and the yield of beans being nearly tripled.

In non-inoculated soya beans supplied with the full Helriegel's solution, the presence of charcoal produced a considerable decrease in the yield, probably (the authors suggested) because the nutrient salts were introduced in amounts calculated with reference to the weight of the sand. Thus since the weight of the sand alone was 7.4 kg. per pot, whereas the weight of the sand plus charcoal was only 5.0 kg. the amounts of available nutrient salts were considerably less in the pots with charcoal. The advantage of having more

nutrient salts when there was no charcoal in the sand could not be turned to profitable account by the inoculated plants, because they were lacking nitrogen.

The effect of the charcoal on nodulated plants was attributed to the loosening of the substratum and to better aeration, and the fact that this change had no favourable effect on the cultures without inoculation, was interpreted as meaning that the effect of increased aeration was connected with the influence on the bacteria in the nodules, rather, than on the activity of the root system itself.

In the same paper the authors by changing the temperature of the rooting medium from 30° C. to 24° C. obtained over a 50% increase in the total yield of inoculated soya beans and since the non-nodulated soyabeans gave similar yields at both temperatures, the favourable influence of the alteration in the temperature was attributed mainly to producing better conditions for nitrogen fixation. Discussing these results the authors put forward the hypothesis that the discrepancy in results of nitrogen excretion obtained by the Helsinki investigators and the other investigators all over the world, might be explained by the assumption that the conditions of aeration and temperature in the Helsinki experiments were more congenial for nitrogen fixation; the excess of fixed nitrogen being excreted.

Although the use of charcoal mainly as a means for the improvement of aeration and drainage is more or less common in horticulture very few scientific investigations are

available. Tincker in unpublished experiments* with Lilies found that addition of charcoal to a clay medium had a remarkably favourable influence on the formation of new bulbs. The charcoal used amounted to 10 - 20 per cent of the clay and its favourable influence was attributed to a mechanical improvement of aeration and drainage.

Prianitchnikow and Domontovitch (1926) reported the results of an unpublished experiment conducted during 1916 by T.V.Janushkin on the influence of finely-ground charcoal (type and percentage not stated)on the yield of different plants grown in sand cultures supplied with various nutrient solutions. According to these results the yield of barley, oat and rye in pots with charcoal was increased by 100 to 300 per cent over the yield in pots without charcoal. The explanation given for these striking results was that the charcoal, through its adsorbing capacity for hydrogen and hydroxyl ions, prevented the injurious influence of a shift of reaction either towards acidity or alkalinity.

Papadakis (1940a, 1941) reported experiments with added charcoal with wheat and corn and the same author referred to experiments carried out by Zinzandre (1932)and Holynski (1928) according to which addition of activated charcoal either to water cultures or to the soil, increased the yield of the crops.

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* The writer wishes to express his gratitude for the supply and the permission for the use of the unpublished data.

There are in commerce two common types of charcoal, animal and wood, namely according to their origin. Activated charcoal is usually prepared from common wood charcoal by special treatment involving heating for long hours at high temperature (925° C.) with a limited access of air. The process of activation is accompanied by a decrease of the bulk density, increase of porosity and probably by some modification on the carbon molecules themselves. Adsorptive power is greatly increased. The efficiency of a particular birch wood charcoal as regards its adsorbing capacity for a poisonous gas was tested before and after the process of activation and it was found that with the activation the adsorbing capacity for the gas was increased 550 per cent (British Encyclopaedia).

A mixture of sand with activated charcoal appeared a very promising rooting medium combining two essential factors stressed by Virtanen and his associates (1938, 1940) as indispensable for the occurrence of excretion, namely,

1) high adsorbing capacity of the medium and

2) good aeration.

The combination of those two factors in a medium would otherwise have been very dificult since by using a finer and finer sand and even more by mixing it with clay or kaolin as was done by Ludwig and Allison (1940), with the increasing adsorption of the medium, the first factor was fulfilled but at the same time the aeration of the medium was worsened. In the other way by use of coarse sand the aeration of the medium was promoted but this was obtained at the expence of the

adsorbing capacity.

It was decided consequently, on the basis of the paper by Gukova and Butkevitch, (a) to attempt to confirm and investigate further the beneficial effect of charcoal on the growth of legumes and to determine how specific any such effect is for nodulated plants in particular, and (b) to investigate whether any such increased growth of nodulated plants is in fact attended by excretion.

M E T H O D S.

Open pot cultures were used with glazed or unglazed earthenware pots, the latter type being always new and unused; the capacity of the containers varied from two to eight litres. Sand of two types has been employed, namely a relatively coarse horticultural sand (Bedfordshire), and a finer industrial silver sand kindly supplied by General Refractories Ltd. from the quarry at Glenboig, Lanarkshire. Two deliveries of Bedfordshire sand differed somewhat in coarseness and colour and are distinguished as Yellow and White Bedfordshire sand.

The mechanical analysis of the sands was as follows:

Size of particles.	Yellow Bedfordshire coarse.	White Bedf. coarse.	Glenboig fine.
Larger than 2.4 mm	1.6	0.3	0.0
1.2 - 2.4 mm	6.5	3.8	0.0
0.6 - 1.2 mm	34.0	24.8	0.2
0.3 - 0.6 mm	53.8	47.7	29.3
0.15- 0.3 mm	3.3	21.8	62.0
Smaller than 0.15mm	0.8	1.6	8.5

The total nitrogen of the sand, although amounting to 11 - 17 mg N. per kg. dry sand, apart from one consignment employed in one experiment, was unavailable for the plants, as is shown by the fact that the growth of barley, oats, and non-inoculated legumes in the sand was extremely poor.

Four kinds of charcoal have been tested: ordinary animal and wood charcoal with particles 0.5-3.0 mm in diameter, as well as powdered animal charcoal and two kinds of activated charcoal, namely, a granular type and a powdered type (manufacturer's numbers 193 and 189 respectively). The activated

charcoals were supplied by Sutcliffe,Speakman & Co. Ltd., with the kind confidential information that the raw material was coal. Different consignments of the two types of activated charcoal are distinguished in the following account by the letters a,b,c,etc. The samples of charcoal employed contained from 0.4 - 1.0 % nitrogen but as the growth of barley showed and as analysis of the charcoal at the beginning and the close of the experiments confirmed, the nitrogen was in a form unavailable for the plants.

The dry sand and the appropriate kind and amount of charcoal (the amount of charcoal being expressed as a percentage of the dry wight of the sand), were thoroughly mixed prior to filling the pots.

The pots and the rooting medium were usually employed without previous autoclaving, but in certain experiments pots with the medium were autoclaved for 3 hours at 15 lb. pressure and additional precautions were taken to avoid the chance of infection of the legume with the nodule organism. Thus, as soon as the seeds were germinated, the surface of the sand was covered with 1-2 kg of sterilised gravel 0.2- 1 cm in diameter,and the watering of the medium, with sterile solution or water, was carried out through 2 or 3 glass cylinders, 1" by 3", inserted into the medium and projected through the gravel; the glass cylinders being closed with corks. (Fig. No1).

Hiltner's (Virtanen and V.Hausen,1935), or Rothamsted (Thornton 1929),nutrient solution free from nitrogen,

F i g u r e 1.

The photograph shows the applied arrangement -covering the surface with gravel and watering through glass cylinders- in order to avoid chance nodulation.

strengthened with the addition of 1 cc per litre of A-Z minor elements solution (Templeman 1941)with or without Molybdenum Oxide, was added, bringing the moisture of the sand to about 80% of its water holding capacity. the moisture was kept near to this point throughout the time of the growth by weighing the pots every two or three days and restoring the transpired and evaporated water, alternately, with added nutrient solution or distilled water. Since the pH of the Rothamsted solution with the autoclaving dropped to 5, in order to provide all the experiments with nutrient solution with pH approximately 6.5,the formula of the Rothamsted solution, when it was autoclaved, it was slightly altered,by substituting the 0.3 gm per litre of mono and dipotassium phosphate with 0.7 gm per litre of dipotassium phosphate, and reducing the content in potassium chloride from 0.7 gm per litre to 0.56 gm.

New Zealand Maple pea (Pisum arvence) has been used in all experiments as the leguminous plant, with barley (Spratt Archer variety) as detector plant for excretion.

Macroscopically undamaged seeds of definite weight were selected from a sample. The seeds were sterilised on the surface by first immersing them in absolute alcohol for 2½ minutes and afterwards in 0.1% solution of mercuric chloride for 6 minutes for the peas, or 2½ minutes for the barley, with continuous shaking. Six rinses with sterile water follow ed and the seeds were left soaking overnight in the water of the last rinse. Prior to sowing, the surface-sterile seeds of

peas were inoculated by immersion in a suspension in sterile water, of young subcultures of the effective strain NX Virtanen. The number of seeds sown was always in excess of the actually required plants, to allow for poor germination.

As soon as sufficient seedlings of the legume were germinated, the excess of seedlings or non-germinated seeds were carefully removed and the same day, surface-sterilised barley seeds were sown among the legumes and in the control pots; the number of barley plants being always double in the control pots. In experiments carried out in 1947 and 1948 this technique was slightly modified, the seeds of legume and detector plant being sown simultaneously. This results in additional difficulty in the process of thinning, but it was considered preferable in order to give the opportunity to the young legume and detector plants to begin their life under conditions more like those existing with mixed cultures in nature.

Immediately after thinning, the pots were transferred to an open situation in the University gardens, where they were protected on rainy days with frames of wood and glass substitute (fig.2,3). In this way the plants were grown under almost natural out-door conditions. The precaution was taken to change the place of the pots frequently, in order to equalise exposure to light and wind.

To avoid any release of nitrogen from decaying cotyledons the shrivelled cotyledons of the peas were carefully removed at the end of the fourth week.

Figure 2.

Figure 3.

The photographs 2 and 3 show the site of the experiments and the method of protection against rain. (covers removed in Fig. 2).

Full records were kept during all the growth period and the plants were usually harvested after 10-13 weeks, when the first pods were formed. The shoots of legume and barley were harvested separately and so were dried at 98° C. to practically constant weight.

The rooting medium was allowed to become air-dry and the roots of the two kinds of plants were carefully separated and the number of nodules counted. The fine particles of sand attached to the roots were removed with the help of a brush and water and the roots were subsequently dried as with shoots; the dried material was kept in air-tight jars with vaselined stoppers till the time of analysis.

The total nitrogen of the plant was determined with the Kjeldahl method as modified by Ranker (1925, 1926).

For the determination of the total nitrogen in dry samples of sand, practically freed from roots, the procedure described by Bond (1938) was followed.

D A T A .

EXPERIMENT I. Two kinds of charcoal, ordinary animal (granular type) and activated charcoal in a mixture of 3 parts granular sample No 195a, to one part powdered sample No 189a, were tested. Twelve earthenware glazed pots 8.5" in diameter by 11" in height with a tubulure in the side near the bottom, were used and in each pot at the bottom and around the tubulure four pounds of washed coarse gravel were added for the promotion of aeration. The pots were divided in 3 lots of 4 pots each, and whilst each pot of the first lot was filled with 24 pounds of Glenboig sand, each pot of the other two lots was filled with 24 pounds of mixture of the same sand plus 6% (the amount used by Gukova and Butkevitch) of animal or activated charcoal.

Three pots of each lot were sown with mixed cultures, 8 peas plus 8 barley, whilst the fourth pot was used as control with 16 barley alone.

Hiltner's solution free from nitrogen, with the addition of 1% calcium carbonate, as was recommended by Hausen(1936), was added to all pots, bringing their moisture to 16% of the dry weight of the medium, and the plants were watered during the first month with this solution or with distilled water; but since at the end of the first month the pH of the media was alkaline (7.4-7.8), further addition of calcium carbonate was omitted from the beginning of the second month till harvest time.

After 3 weeks growth, the peas in the lot with animal charcoal had a remarkably different appearance from the peas

in the other lots, their leaves being smaller and showing marked marginal reddening. Roots from these peas examined macroscopically appeared perfectly healthy. With the progress of time the differences in top-growth between the peas in the pots with animal charcoal and the peas in the other lots was gradually accentuated, the growth of the peas in animal charcoal being far behind the growth of the peas of the other lots, the size of their leaves being only one third of the size of the leaves of the other lots and the reddish marginal colour with spots on all the leaves being more and more intence. (fig 4).

As regards the peas in the two other lots, they were more or less similar in growth and appearance with a possibility that the peas in the pots with activated charcoal were slightly greener (fig. 4).

Compared with the peas, the reaction of the barley plant to the charcoal was entirely different; although all the barley in the control pots and in the mixed cultures showed the symptoms of nitrogen hunger, the barley in the pots with animal charcoal were definitely larger and greener plants (fig. 5, 6), whilst the growth of the barley in the pots with activated charcoal was the poorest of all (fig. 7).

It is considered necessary to mention a few words about the pH of the rooting media. The measurements for this experiment were carried out with colorimetric methods, not particularly accurate, but since the differences were persistent, the results can be considered trustworthy enough. When the

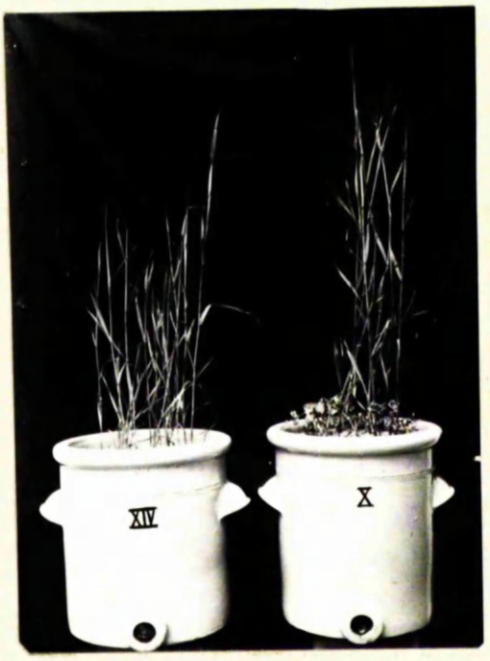
F i g u r e 4.

Cultures of peas and barley from Experiment I. Pots I and VIII in sand alone, pots II and VII with 6 per cent animal and activated charcoal respectively.

FIGURE 5.

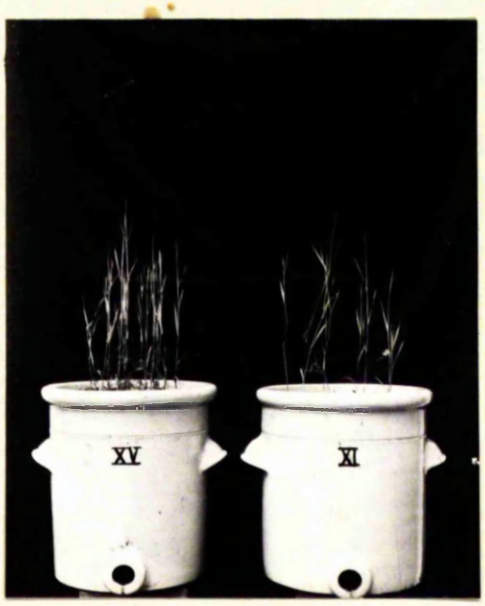
Growth of barley in control pot (barley alone) on the left, and in mixed cultures (peas plus barley, peas removed for purposes of photograph) on the right. Rooting medium sand alone. (From exprt. I.).

FIGURE 6.



Growth of barley in control pot (barley alone) on the left, and in mixed cultures(peas plus barley) on the right. Rooting medium sand plus 6% animal charcoal.

F i g u r e 7.



Growth of barley in control pot (barley alone) on the left, and in mixed cultures (peas plus barley) on the right. Rooting medium sand plus 6% activated charcoal. (From exprt. I.).

peas were 4 weeks old, the pH of the sand with animal charcoal was more alkaline(pH 7.8) than the pH of the sand in the other lots (pH 7.4). The pH remained in the above levels till harvest time.

Table Ia gives the harvest data for the peas. It can be seen that the growth in the pots with sand alone and sand plus activated charcoal was very good, the nitrogen fixed per plant being about 90-100 mg. or 700-800 mg per pot. There is a slight increase in the dry matter and the total nitrogen in the peas in the pots with sand plus activated charcoal, in comparison with the peas in sand alone, but since the increase is statistically insignificant, the experiment failed to show increase in the growth with the added activated charcoal. The dry matter of the peas in sand plus animal charcoal was only 1/6th and the nitrogen fixed only 1/15th of the corresponding figures for the peas in the other two media. The animal charcoal acted as a very strong depressing factor on the growth of peas.

The figures for the number of nodules are of some interest. The number of nodules in peas with activated charcoal was only 1/3rd of the number of nodules in peas in sand alone. They were, however, of greater average size, as is indicated by consideration of the dry weight data, and were concentrated near the tap roots.

Table Ib gives the results for the barley. Unfortunately, due to shortage of pots the control pots (barley alone) were without replicates, and the results can be

Table Ia.

Data of experiment I. Growth period 2nd May -6th August 1946. 8 peas inoculated with HX strain and 8 barley per glazed pot containing 24 lbs. rooting medium. Sand Glenboig fine. Data shown in table are per pot and for the peas only.

Rooting medium.	No of pot.	Dry wt. gm.	Total* fixed nitrogen, mg.	Dry wt. nodules, mg.	Number of nodules.
Sand alone	1	25.57	792	336	3210
" "	2	20.54	683	316	--
" "	3	22.59	687	296	2540
Mean for 3 pots.		22.83	721	316	2880
Sand plus 6% activated charcoal.	4	27.72	833	287	840
" " " "	5	23.95	781	233	810
" " " "	6	19.39	641	260	-
Mean for 3 pots.		23.67	752	260	830
Sand plus 6% animal charcoal.	7	4.15	61	134	1020
" " " "	8	3.77	53	170	2130
Mean for 2 pots.		3.96	57	152	1580

* The column total fixed nitrogen indicates the total nitrogen in the plants minus the nitrogen in the seeds.

Table Ib.

Data of experiment I, for the barley. Mixed cultures 8 peas and 8 barley per pot, 16 barley alone in the control pots. Data are per pot for mixed cultures, and the half of the yield for the control pots, corresponding to the growth of 8 barley

Rooting medium.	Culture.	No of pot.	Dry wt. gm.	Nitrogen %	Nitrogen minus N. of seeds mg.
Sand alone	Mixed cult.	1	2.41	1.04	18.7
" "	" "	2	2.08	0.97	15.8
" "	" "	3	2.31	1.06	17.0
		Mean for 3 pots.	2.25	1.03	16.5
" "	Control, barley alone.	13	2.15	1.07	16.6
Sand plus 6% activated charcoal.	Mixed cult.	4	1.03	0.97	3.6
" " " "	" "	5	0.98	0.98	3.2
" " " "	" "	6	0.89	0.92	0.9
		Mean for 3 pots.	0.97	0.95	2.6
" " " "	Control, barley alone.	14	1.16	0.81	3.0
Sand plus 6% animal charcoal.	Mixed cult.	7	4.21	1.32	49.2
" " " "	" "	8	4.09	1.45	51.6
" " " "	" "	9	3.51	1.42	43.4
		Mean for 3 pots.	3.91	1.40	48.1
" " " "	Control, barley alone.	15	1.32	1.68	24.1

considered as having some significance only because they can be connected with previous experiments carried out in a similar environment and conditions by Bond (1938,1939,1941a). As in those previous experiments,here also it is quite clear that there was not the slightest gain in dry matter or in nitrogen in barley grown in mixed cultures with peas in either of the two media sand or sand plus activated charcoal, in comparison with the dry matter and the total nitrogen of the barley in the corresponding control pots. Assuming that any excreted nitrogenous substance could be used by the barley,either directly or indirectly, after disintegration to a more available form through the existing micro-organisms in the rooting medium, the conclusion is drawn that in those two media there was no evidence of excretion at all(fig 5,7).

There was a considerable decrease in growth (both dry matter and total nitrogen)in barley grown in sand plus activated charcoal in comparison with the growth of barley in sand alone (fig.7),and this decrease can probably be explained with the hypothesis that the activated charcoal adsorbing part of the available nitrogen in the sand, left the medium poorer in nitrogen than in the sand alone.

The results of the pots with animal charcoal were entirely different. Both the barley in mixed cultures and in the control pot gave a higher dry matter and total nitrogen than barley grown in sand alone, suggesting that the animal charcoal itself provided the medium with a small quantity of available nitrogen (fig. 6). At the same time since the

barley in the mixed cultures exceeded the barley in the control pot by 24 mg nitrogen, there was the possibility that under the special conditions created by the addition of animal charcoal, the phenomenon of excretion probably occurred (fig. 6). A further experiment with the addition of animal charcoal is described later.

EXPERIMENT II. In this experiment, which overlapped with the first one, the effect of wood charcoal and again of activated charcoal was tested under somewhat different conditions as regards pots, sand and quantities of charcoal.

Twelve unglazed earthenware pots, 9" in diameter were divided into three lots, the pots of the first lot being filled with yellow Bedfordshire sand alone, the pots of the second with mixtures of sand with 2, 4, 6, and 8 per cent activated charcoal (mixture three parts to one granular charcoal No 195b and powdered charcoal No 189b), and the pots of the third lot with mixtures of sand with similar percentages of ordinary granular wood charcoal.

Owing to different specific weight of the charcoal and the sand, in this experiment, all the pots were filled with the same volume of medium, and the following table gives the exact weight of the rooting medium in each pot:

Rooting medium	Weight gm.	Rooting medium	Wgt. gm.
Sand alone	8,500		
Sand plus 2% activat.	8,400	Sand plus 2% wood charcoal	8,050
" " 4% "	8,300	" " 4% "	7,600
" " 6% "	8,200	" " 6% "	7,150
" " 8% "	8,050	" " 8% "	6,800

Rothamsted solution free from nitrogen with the add-

ition of 1 cc. per litre of A-Z minor elements solution plus molybdenum oxide was used, bringing the moisture all the pots to about 11% of the dry weight of the medium. This 11% moisture was near the water-holding capacity of the coarse sand but was only a smaller percentage of the water holding capacity of the medium with charcoal, and this difference introduced an undesirable lack of uniformity; this matter is considered in the discussion below. The moisture was restored to this point every two or three days by adding alternately the above solution or distilled water.

Nine of the twelve pots were used as mixed cultures with 6 peas and 6 barley in each pot, and the remaining 3, containing respectively sand alone, sand plus 4% activated charcoal and sand plus 4% wood charcoal, were used as control pots with twelve barley plants in each.

From the fourth week on, there was a conspicuous difference in the growth of the peas in the various media, and with the progress of time the growth in the pots with mixtures of charcoal became, every day, better than the growth of the peas in sand alone. As regards the barley, apart from the fact that the barley in the pots with the activated charcoal was the poorest of all, and the barley in the wood charcoal slightly better than the barley in the sand alone, there was no trace of any superiority of barley living in mixed cultures, in comparison with the corresponding barley in the control pots.

The pH of the fresh nutrient solution was about 6.5

and the table below gives the pH of the medium, six weeks after the beginning of the experiment:-

Rooting medium	pH	Rooting medium	pH
Sand alone	6.4	Sand plus 2% wood charcoal.	7.0
Sand plus 2% activated	6.8	" " 4% "	7.1
" " 4% "	6.8	" " 6% "	7.3
" " 6% "	6.9	" " 8% "	7.3
" " 8% "	7.1		

Thus there was a slight increase towards alkalinity in the pH of the medium with the increasing quantities of charcoal, the wood charcoal being more effective in causing this change than the activated.

Table IIa gives the harvest data for the peas. The favourable effect of charcoal on the growth and the fixation of nitrogen is well marked. The best growth and fixation occur in pots with small percentages of activated charcoal, namely 2 and 4 per cent, while the opposite holds for the mixtures with wood charcoal, the best growth being at 8% mixture. (fig.8). An increase in fixed nitrogen can be seen up to 84%. It is unfortunate that due to the shortage of pots and material, there was only one pot with sand alone and mixed cultures. In any case, these results can be considered trustworthy, since they were confirmed by further experiments with replicates. It must be pointed out that although the stimulating effect of charcoal on the growth is exerted to both tops and roots, the increase in dry matter is more pronounced in the tops than in the roots, and treatment with both charcoals had as effect an enlargement of the ratio top/root from 9.3 in peas grown in sand alone to 11.5 in peas grown in mixture with charcoal.

F i g u r e 8.

Experiment II. Mixed cultures peas plus barley. Left pot sand alone, middle pot sand plus 2% activated charcoal, right pot sand plus 8% wood charcoal.

Table IIa.

Data of experiment II, June 15 - September 28, 1946. Six peas inoculated with NX strain and six barley per unglazed pot containing 6.8-8.5 kg. medium. Sand Bedfordshire coarse yellow. Data shown in table are per pot & are for the peas only.

Rooting medium.	No of pot.	Dry wt. gm.	Total* fixed nitrogen, mg.	Dry wt. nodules, mg.	Number of nodules.
Sand alone	21	17.55	594	535	5700
Sand plus 2% act. charcoal.	22	32.66	1090	678	4640
" " 4% "	24	30.24	1007	542	3130
" " 6% "	26	27.84	933	455	1680
" " 8% "	28	21.72	713	300	810
Sand plus 2% wood charcoal.	23	24.07	727	612	7140
" " 4%	25	25.52	845	567	5050
" " 6%	27	18.92	616	382	3890
" " 8%	29	29.10	971	467	4850

* The column total fixed nitrogen indicates the total nitrogen in the plant minus the nitrogen in the seeds.

Although a strict comparison of the results of this experiment with the results of the first one is not possible, since there were many differences in the conditions under which the two experiments were carried out, including the employment of different samples of activated charcoal (though with the same code number), nevertheless it must be emphasised that in this experiment yellow coarse Bedfordshire sand was employed, whereas in the first one the much finer Glenboig sand was used, and, as results of subsequent experiments suggest the beneficial effect of charcoal on the growth of peas may probably be connected with the coarseness of the sand employed.

It will be observed that the effect of activated charcoal on the number and size of nodules is the same as in the first experiment. With increasing quantities in the medium, the number of nodules decreases considerably while their size increases, and in the 8% mixture the number of nodules amounts to 1/9th only of the number of nodules in the sand alone. The effect of wood charcoal on the nodulation is not quite clear, although large quantities of wood charcoal in the mixture tend to decrease the number of nodules.

Table IIb gives the harvest results for the barley. In spite of increased growth and nitrogen fixation by peas treated with charcoal, there is no sign of excretion, since barley grown in mixed cultures failed to exceed the corresponding barley in the control pots in dry matter or total nitrogen. (fig 10). The activated charcoal had a depressing effect,

F i g u r e 9.

Experiment II. Effect of charcoal on the growth of barley. Barley grown alone in control pots. Left sand alone, middle sand plus 4% activ. charcoal, right sand plus 4% wood charc.

F i g u r e 10.

Experiment II. Left barley alone with 4% activ. charc. , middle barley with peas in 2% , and right barley with peas in 8% activated charcoal.

Table IIb.

Data of experiment II, for the barley. 6 peas and 6 barley per pot, in mixed cultures, 12 barley alone in the control pots. The data are per pot except for the control pots for which the half of the yield of the pot is recorded.

Rooting medium.	Culture	No of pot.	Dry wt. gm.	Nitrogen minus N. of seeds.
Sand alone	Mixed cult.	21	1.15	11.3
" "	Control, barley alone.	30	1.96	17.0
Sand plus 2% act. charcoal	Mixed cult.	22	0.98	6.7
" " 4%	" "	24	0.86	4.7
" " 6%	" "	26	0.86	4.1
" " 8%	" "	28	0.83	5.1
" " 4%	Control, barley alone.	31	1.20	6.1
Sand plus 2% wood charcoal.	Mixed cult.	23	1.41	15.4
" " 4%	" "	25	1.97	25.5
" " 6%	" "	27	2.00	23.6
" " 8%	" "	29	1.94	19.6
" " 4%	Control, barley alone.	32	2.13	19.9

as it had in the first experiment, suggesting that with its strong adsorbing capacity it competed with the barley for the available nitrogen inthe sand, whereas the wood charcoal not having strong adsorbing capacity, left the barley to use all the available nitrogen in the sand, and furthermore, added a few mg. of available nitrogen to the medium (fig. 9, 11).

EXPERIMENT III. In 1947 a number of experiments were set up mainly to study further the effect of activated charcoal on peas and on excretion. The effect of activated charcoal on the growth of peas was clear in experiment II, which was carried out with yellow coarse Bedfordshire sand. In order to investigate whether the influence of charcoal on the growth of the peas was dependent or independent of the coarseness of the sand, two kinds of sand were now used, i.e. yellow coarse Bedfordshire and fine Glenboig.

In this experiment granular activated charcoal sample No 193c was used alone i.e. without mixture with powdered type, as was done in the previous experiments. Three percentages of of the activated charcoal were used: 1, 2, and 3%, since in experiment II the best results were given by percentages of this order.

Since Virtanen and Torniainen (1940) stressed the importance of the porosity of the pots for the aeration and the occurrence of excretion, 32 extremely porous unglazed earthenware pots were selected from a number of unused pots, using the test of the above investigators. This test consists in placing the pots in water up to the rim and measuring the

F i g u r e 11.

Experiment II. Left barley alone with 4% wood charcoal, middle barley with peas in 2% and right barley with peas in 8% wood charcoal.

time taken for the water to penetrate to the inner surface. The time for the penetration of water at 9^{0} C. was 20 to 40 minutes, a time comparable with that reported by Virtanen and Torniainen for their most porous pots. By using these porous pots, it was hoped that any existing small excretion could be promoted.

Six peas and six barley were sown the same day in each pot with mixed cultures, whereas twelve barley were sown in each of the control pots. Rothamsted solution, free from nitrogen, plus 1 cc. per litre A-Z minor elements solution plus molybdenum oxide was again used.

Contrary to expectations, almost from the beginning the growth of the peas in mixtures of coarse sand plus activated charcoal, especially in the pots with the large percentages of charcoal, was inferior to that in the pots with sand alone. As regards the peas in the pots with Glenboig sand, the growth was similar both with charcoal and without.

Measurements of the pH of the medium again showed that the effect of the presence of charcoal in the medium was a slight increase of the pH (a few decimals of one unit of the pH scale) towards the alkaline side.

Table III gives the analytical results. It is clear that the charcoal added in percentages of 2 and 3% had a depressing effect on the peas grown in the Bedfordshire sand, due possibly to some toxic substance present in the charcoal. Thus although the charcoal employed in this experiment was supplied under the same code number as the granular sample

Table III.

Data of experiment III, May 5 - August 14 1947. 6 peas inoculated with HX strain and 6 barley per unglazed pot containing 8 kg. of rooting medium, 12 barley alone in the control pots. The data are per pot except for the controls in which the half of the yield of the pot is recorded.

Rooting medium.	Culture.	No of replicates.	PEAS Mean dry wt. gm.	Mean* fixed nitrg. mg.	Mean No of nodul.	BARLEY Mean dry wt. gm.
Bedfd. sand alone.	Mixed cult.	3	13.0	378	1710	1.57
" " "	Barley alone	1				2.74
" " plus 1% act. charc.	Mixed cult.	2	12.1	342	170	1.04
" " " 2%	" "	2	5.9	108	25	0.75
" " " 3%	" "	2	8.1	198	85	0.81
" " " 2%	Barley alone	1				0.67
Glenboig sand aln.	Mixed cult.	3	14.3	400	3600	2.36
" " "	Barley alone	1				2.44
" " plus1% activ.charc.	Mixed cult.	2	15.3	384	300	1.20
" " " 2%	" "	2	11.1	240	60	0.83
" " " 3%	" "	2	14.3	354	430	0.85
" " " 2%	Barley aln.	1				0.80

* The column fixed nitrogen indicates the total nitrogen in the plant minus the nitrogen in the seeds.

used in 1946, its effect on peas was different from that in experiment II. The fact that the charcoal had no depressing effect on the peas grown in the Glenboig sand might be explained with the hypothesis that since the fine sand had a larger adsorbing capacity than the coarse sand, as a result it adsorbed the toxic substance and thus prevented its injurious effect.

As regards the barley, that grown in mixed cultures had the same dry matter and total nitrogen or even slightly less nitrogen than the barley in the corresponding control pots. Thus, as is perhaps not suprising since the charcoal had failed to stimulate fixation, once more the experiment failed to show any excretion. The hypothesis of Virtanen and Torniainen to the effect that by using very porous pots we could promote the excretion, failed to be verified.

As regards the growth of barley grown in mixtures with increasing quantities of activated charcoal, the growth decreased with increased quantities of charcoal in the mixture in both sands, suggesting once more that the activated charcoal acted as a competitor for the small available quantities of nitrogen in the medium.

The effect of the charcoal employed on the nodulation was in accordance with the previous experiments, and this time even more striking. Leaving aside the number of nodules in the large percentages where, for the coarse sand, the nodules were limited to a few large ones near the tap root, and considering only the number of nodules in the pots with

one per cent mixture, it can be seen that the number of nodules in 1% mixture of both sands was limited to one tenth of the number of nodules in sand alone. It thus appears that the beneficial effect of activated charcoal on genuine growth as shown in experiment II, is unconnected with the reduction in number and increase in size of nodules.

EXPERIMENT IV. This experiment was set up in the expectation that the strikingly beneficial effect of activated charcoal on growth of peas demonstrated in experiment II, would be reproducible in the following year. Experiment IV was intended to investigate whether the beneficial effect of charcoal was manifested only on nodulated legumes deriving their nitrogen from the atmosphere as was reported by Gukova and Butkevitch (1941), or whether the effect was extended also to non-nodulated peas grown in combined nitrogen. The experiment loses some of its point since the charcoal used in experiment III and in the present experiment failed to benefit inoculated peas. The results are however of some interest.

Twenty-four unglazed earthenware pots 8" in diameter, were filled with 7.5 kg. of medium consisting of yellow Bedfordshire sand or mixtures of the sand with 1 ,2 ,and 3% granular activated charcoal, sample No 195c. Special precautions by autoclaving the medium, covering the surface of the medium with sterilised gravel, and watering all the time with sterile solution were employed for all pots, in order to avoid chance inoculation of the peas supplied with combined

nitrogen. The arrangement employed proved satisfactory, since most of the plants were, at the end of the experiment, free from nodules, though occasional plants showed a few small nodules.

Nitrogen in the form of ammonium nitrate was added periodically every 8-10 days to 20 of the pots in two levels (total added nitrogen 1100 and 1650 mg. nitrogen per pot), whereas the remaining 4 pots, 2 with sand and 2 with sand plus 2% activated charcoal, were sown with inoculated peas and were used as control pots receiving the same sterile Rothamsted nitrogen-free solution strengthened with 1 cc. per litre A-Z minor elements solution plus molybdenum oxide.

Table IV gives the analytical results. The peas in both levels of nitrogen showed very good growth in sand alone. Unfortunately, the charcoal had the toxic effect on the peas in mixtures 2 and 3% as in experiment III, decreasing the growth of the peas considerably. This toxic effect was manifested in both peas growing in combined nitrogen and inoculated peas fixing nitrogen from the atmosphere, and the results show that the depressing effect is not exerted specifically on nodules.

EXPERIMENT V. Since the activated charcoal used in the early experiments in 1947 proved to be, in its effect on the peas, different from the one used in the experiments in 1946, despite the fact that it was provided by the same factory and had the same trade number, an experiment was carried out with four different samples, in order to find out if any of these

Table IV.

Data of experiment IV, May 15 - August 21 1947. 6 peas per unglazed pot containing 7.5 kg. of rooting medium. The plants treated with NH_4NO_3 received in total 1100 and 1650 mg. nitrogen in weekly doses. The inoculated peas were watered with the same nutrient solution minus nitrogen. The sand employed was Bedfordshire yellow coarse.

Rooting medium.	Treatment.	No of replicates.	Mean dry weight per pot gm.	Mean total nitrogen per pot mg.
Sand alone.	1100 mg N. added.	1	38.16	849
" "	1650 " " "	2	22.59	809
Sand plus 1% charc.	1100 " " "	2	26.88	772
" " " "	1650 " " "	2	27.65	969
" " 2% "	1100 " " "	2	18.32	582
" " " "	1650 " " "	1	15.08	571
" " 3% "	1100 " " "	1	16.90	537
" " " "	1650 " " "	1	12.95	511
Sand alone.	Inocul. No N.add.	2	17.53	549
Sand plus 2% charc.	" " " "	2	13.76	427

samples had the beneficial effect demonstrated in 1946 and possibly if it could induce any excretion. Small percentages of the charcoal were used and the charcoal was always a mixture 3:1 of different samples of granular charcoal No 193 and of the same sample of powdered activated charcoal No 189c, apart from one lot of pots in which powdered charcoal alone was employed in mixture with sand. The sand employed was yellow coarse Bedfordshire again.

Sixteen unglazed earthenware pots 8" in diameter, were filled with 7 kg. of medium, and six peas and six barley were sown in each pot for mixed cultures, the same day, whereas 12 barley were sown alone in the corresponding control pots. Nitrogen-free Rothamsted solution, plus 1 cc. per litre A-Z minor elements solution with molybdenum oxide was used for warering alternately with distilled water.

Table Va gives the analytical results for the peas. It will be noted that all the charcoals used gave a connsiderable increase in the dry matter and the fixed nitrogen of the peas, the increase for the fixed nitrogen being from 60% to 120% of the nitrogen fixed by peas grown in sand alone. This experiment was performed with dublicates and triplicates pots and the differences are statistically significant.

It is interesting to point out that whereas the granular sample No 193c in the experiments III and IV had no effect on the growth of peas in mixture of 1% ,(in experiments III and IV the granular charcoal was employed alone, i.e. without previous mixing with small quantities of powdered charcoal

Table Va.

Data of experiment V, July 8 - September 13 1947. 6 peas inoculated with NX strain and 6 barley per unglazed pot containing 7 kg. rooting medium. Sand Bedfordshire yellow coarse. The data are per pot, and for the peas only.

Charcoal semple.	Proportion of charcoal.	Dry wt. per pot gm.	Total* fixed N. mg.	Dry wt. of nod.mg.	Number of nodules.
Sand alone	-	6.17	154	193	970
" "	-	5.43	117	360	1090
" "	-	5.62	160	252	870
193b 189c	1.5%	8.72	257	307	1020
" "	2.0%	8.19	220	245	450
193c 189c	0.5%	10.35	355	287	350
" "	1.0%	8.64	285	263	220
" "	1.5%	9.54	304	262	155
193d 189c	0.5%	8.17	253	230	410
" "	1.0%	9.29	316	317	320
" "	1.5%	8.37	269	262	320
189c.	0.5%	8.29	258	262	330
"	1.0%	9.77	283	355	280

* The column total fixed nitrogen indicates the total nitrogen in the plants minus the nitrogen in the seeds.

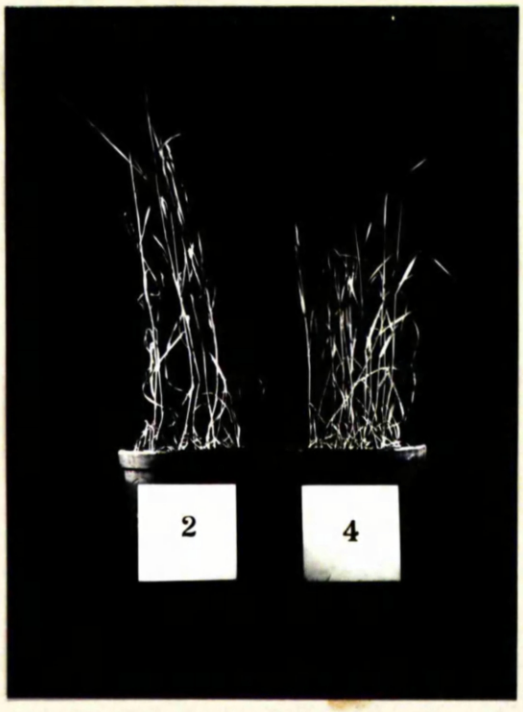
the same sample
in the present experiment employed in mixture with small quantities of powdered charcoal had a marked beneficial effect. Thus at least for this lot of charcoal the beneficial effect of the charcoal on the growth of peas might be attributed to the added small quantities of powdered activated charcoal in the medium.

The effect of charcoal on nodule numbers and size was as before.

Table Vb gives the results for the barley. There is an increase in the dry weight and the total nitrogen of barley grown in mixed cultures in comparison with the barley plants in the corresponding control pots (fig. 12, 13, 14). The increase in total nitrogen amounted to an average of 14.4 mg. per pot, suggesting occurrence of excretion. Unfortunately, as the appearance of barley in the control pot with sand alone, as well as the figures in dry matter and total nitrogen showed, the particular sample of Bedfordshire sand proved to be richer in available nitrogen than in previous experiments and this makes the interpretation of the results more complex, a matter considered in the discussion.

EXPERIMENT VI. Since, in the first experiment with the use of 6% animal charcoal in mixtute with Glenboig sand, the results suggested possible excretion, another experiment was carried out with similar sand and various mixtures of animal charcoal from 2 to 8% in glazed and unglazed earthenware pots, with the object of confirming previous results. Unfortunately the new sample of animal charcoal (obtained from the same

F i g u r e 12.

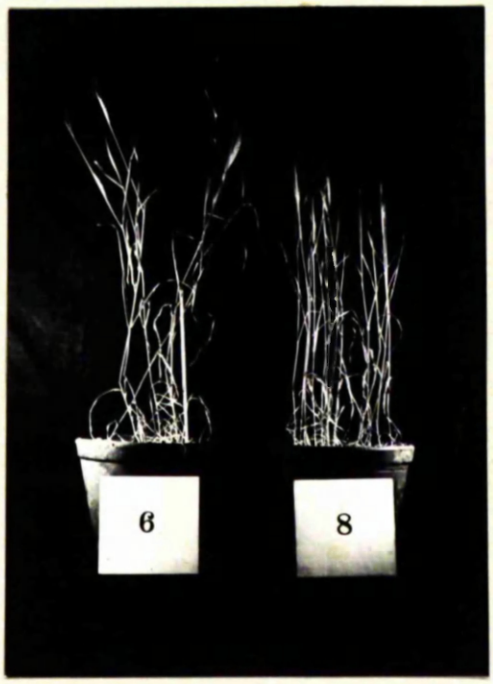


Experiment V. Pots containing sand alone. 2 barley in mixed cultures with peas, 4 barley alone.

F i g u r e 13.

Experiment V. Barley grown in sand with 1% mixture of granular and powdered activ. charc. 3 barley in mixed cultures with peas, 5 barley alone.

F i g u r e 14.



Experiment V. Barley grown in sand with 1% powdered activated charcoal. 6 barley in mixed cultures with peas , 8 barley alone.

Table Vb.

Data of experiment V. Six peas inoculated and six barley per pot of mixed cultures, twelve barley alone in control pots. The data are per pot for the barley only, except for the control pots for which half the actual yield of the pot is recorded.

Charcoal sample.		Proportion of charcoal.	Culture.	No. of pot.	Dry wt. in gm.	Total nitrogen minus N. seeds, mg.
Sand alone		-	Mixed cult.	221	3.03	53.5
"	"	-	" "	226	5.45	61.9
"	"	-	" "	231	5.92	64.8
			Mean		5.46	60.1
"	"	-	Control, barley alone	234	2.68	41.8
193b	189c	1.5%	Mixed cult.	222	4.47	59.7
"	"	2.0%	" "	227	3.11	53.0
193c	189c	0.5%	Mixed cult.	223	2.60	35.5
"	"	1.0%	" "	228	2.39	35.5
"	"	1.5%	" "	232	1.49	11.6
193d	189c	0.5%	Mixed cult.	224	3.99	55.8
"	"	1.0%	" "	229	2.90	43.2
"	"	1.5%	" "	233	3.05	52.9
"	"	1.0%	Control, barley alone	235	3.10	34.2
189c.		0.5%	Mixed cult.	225	4.03	59.1
"		1.0%	" "	230	3.54	51.1
"		1.0%	Control, barley alone	236	2.88	35.1

supplier as previously) proved extremely toxic for both peas and barley. The plants in the high percentages of charcoal died early in life as later on did most of those in the low percentages. As very few plants survived, the experiment was abandoned.

Another experiment was carried out, this time with the same animal charcoal that was used in the first experiment in 1946 already described. The only difference was that whilst in the 1946 experiment, granular animal charcoal was used, the charcoal used in this experiment was powdered prior to use and instead of glazed pots, unglazed earthenware pots 9" in diameter were used. The percentages of the animal charcoal used, were 2, 3, and 4 per cent.

Tables VIa, and VIb , contain the results. Although the results were not so regular and the growth of peas varied more than usual in the dublicates, it is quite clear that the animal charcoal employed, acted depressingly on the peas at the 4% percentage. The peas did not, however, display the conspicuous symptoms (very poor growth with small leaves red in margin and spots) as in 1946 experiment. The peas appeared normal with green leaves as did the peas in the pots with sand alone. There was a decrease in size of plants but much smaller than in the 1946 experiment.

The effect of the animal charcoal on the barley were unexpected. The barley grown with peas in pots containing animal charcoal showed similar growth and nitrogen content to the barley grown alone in sand, whereas the barley which

Table VIa.

Data of experiment VI, July 12 - September 14 1947. 6 peas inoculated and 6 barley per unglazed pot containing 8.6 kg. rooting medium. Sand Glenboig fine. The data are per pot and for peas only.

Rooting medium	Dry wt. gm.	Total fixed* N. mg.	Dry wt. of nod.mg.	Number of nodules.
Sand alone	7.67	254	520	3840
" "	9.43	332	499	3760
" "	9.65	359	471	3950
Mean	8.92	308	497	3820
Sand plus 2% animal chcl.	7.66	246	373	3240
" " 3% " "	9.27	317	366	2540
" " 4% " "	5.52	162	290	2360
" " 4% " "	5.46	156	255	1700

* The column total fixed nitrogen indicates the total nitrogen in the plants minus the nitrogen in the seeds.

Table VIb.

Data of experiment VI. 6 inoculated peas and 6 barley per pot in mixed cultures, 12 barley alone in control pots. The data are per pot and for the barley only except for the control pots for which the half of the yield is recorded.

Rooting medium.	Culture.	No. of pot.	Dry wt. gm.	Total nitrogen minus N. seeds, mg.
Sand alone	Mixed cult.	241	1.12	15.3
" "	" "	246	1.10	10.4
" "	" "	251	0.89	9.9
	Mean		1.04	11.2
" "	Control, barley alone	252	1.13	9.7
Sand plus 2% animal charc.	Mixed cult.	242	1.53	12.7
" " 3% "	" "	243	0.82	7.4
" " 4% "	" "	244	0.95	8.6
" " 4% "	" "	245	1.06	10.7
" " 3% "	Control, barley alone	253	3.41	41.2

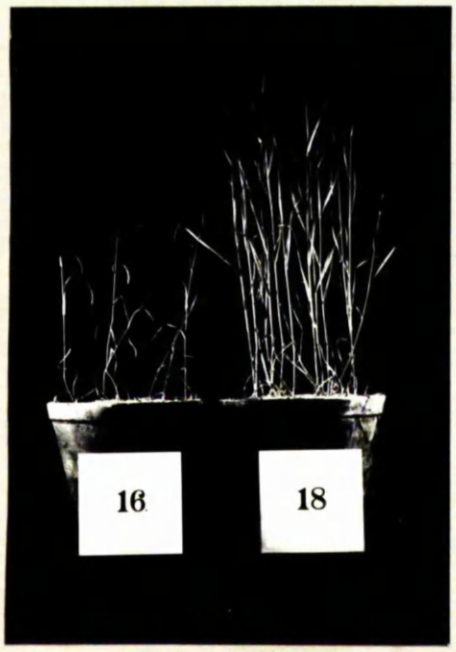
was grown alone in a pot with 3% animal charcoal exceeded the amount of growth and total nitrogen content of the barley grown in sand by 400%.(Fig. 15, 16, 17). It is difficult to explain why the charcoal had such a favourable influence on the barley in the control pot and, at the same time, had no influence on the corresponding barley grown with peas. At any rate the experiment failed to show any excretion at all, (fig. 15, 16), so that the result of experiment I was not confirmed. As pointed out, however, there were certain differences in the two experiments (percentage of charcoal, pots, etc.).

EXPERIMENT VII. The position in Spring 1948 was that a well-marked beneficial effect of activated charcoal on the growth of peas had been demonstrated in two experiments (II and V). It was decided to attempt some investigation of the reasons for the beneficial effect on peas. A certain similarity seemed to exist between the observations of Anderson and Oerttel (1946) on the effect of wood-ash on legume growth and those of the writer on activated charcoal. In both cases growth and fixation are promoted while the number of nodules is reduced and their size increased. Since the effect of wood-ash later was shown to be due to molybdenum, a posibility arose that the effect of charcoal was due also to molybdenum. Molybdenum in the form of oxide was supplied to all plants in experiments II and V, but since in this form it is only slightly soluble, it was decided to carry out an experiment with a soluble form (Sodium molybdate)

F i g u r e 15.

Experiment VI. Barley grown in sand alone. 12 barley in mixed cultures with peas, 13 barley alone.

F i g u r e 16.



Experiment VI. Barley grown in 3% animal powdered charcoal. 16 barley in mixed cultures, 18 barley alone.

Figure 17.

Experiment VI. Effect of animal charcoal on barley. 15 barley alone in sand plus 3% animal charcoal. 13 barley alone in sand without charcoal.

in order to clarify further the question whether the effect of activated charcoal on nodulated peas is due to any addition of molybdenum by the charcoal.

Two kinds of sand were employed, Bedfordshire white coarse and Glenboig fine sand, in order to obtain clearer results as regards any existing connection between the effectiveness of the charcoal on the growth and the coarseness of the medium. Forty new unglazed earthenware pots, 10" in diameter, were filled with 8.5 kg. sand or sand plus 1% activated charcoal (mixture one part to one part granular activated charcoal sample No 193d and powdered sample No 189d). Rothamsted solution, free from nitrogen, with A-Z minor elements solution, was again employed and to each of the pots treated with molybdenum was added a quantity of 2 mg Sodium Molybdate corresponding to a little more than 1/2 lb. addition of Sodium Molybdate per acre, suggested by Anderson and Thomas (1946). Six inoculated peas were left in each pot and no cereal was included.

Unfortunately the plants, which were grown out-of-doors on the usual site, were severely damaged by gales in early July. The plants did not recover properly, and only the observations made by eye on the growth will be presented.

The experiment began on the 8th of May and already on the 22nd of June the growth of peas in the Bedfordshire sand plus charcoal was markedly superior to that of the peas in the sand alone. With Glenboig sand the charcoal showed a smaller effect. Now as regards the effect of the molybdenum

on the peas, there was no sign of any response in either of the sands, the appearance of the plants being the same both in pots with, or without molybdenum. In another inspection of the plants on the 3rd of July similar conclusions with the above were drawn. Tests of the pH of the media showed a slight increase of the pH towards alkalinity in media with charcoal added, the increase being smaller with Glenboig fine sand. Thus the pH of the Bedfordshire sand increased from 6.6 in sand alone to 7.0-7.2 in mixture with charcoal, whereas the pH of the Glenboig sand was increased from 6.4 to 6.7 .

From the above observations on the growth of peas it can be concluded quite safely that the beneficial effect of charcoal was more pronounced in a medium with coarse sand and that the effect was not due to any addition of molybdenum by the charcoal, since the plants did not respond to added molybdenum.

EXPERIMENT VIII. Now that the beneficial effect of activated charcoal had beendemonstrated in a number of experiments with inoculated plants, it was decided to examine again the question whether the beneficial effect of charcoal was limited to inoculated legumes or if also extended to non-nodulated legumes grown in inorganic nitrogen. An experiment for this purpose was set up almost concurrently with experiment VII, with the same white Bedfordshire sand and the same mixture and percentage of activated charcoal, employed in the said experiment. Since from the appearance of the plants, in a preliminary experiment, suspicions were born that autoclaving

caused such alterations in the medium as prevented the manifestation of the beneficial effect of charcoal on the growth, the experiment was carried out with both autoclaved and non-autoclaved media.

Thirty-two unglazed earthenware pots 9" in diameter with 7 kg. medium were employed and while 16 pots were sown with inoculated peas, the remaining ones were sown with surface sterilised peas and received weekly doses of ammonium nitrate to the total amount of 1500 mg. nitrogen each. Rothamsted solution, free from nitrogen, plus A-Z minor elements solution, alternately with distilled water was employed for the watering of all the pots and for the sake of uniformity, autoclaved and non-autoclaved pots were covered with sterilised gravel and the solution employed for all the pots was autoclaved.

When the plants were one month old, the growth in the pots with charcoal was conspicuously superior than that in sand alone, especially in the non-inoculated peas which received ammonium nitrate. At this time the growth in the inoculated pots was about half of the growth in the pots with ammonium nitrate, and there was no apparent difference between the peas growing in sand or in sand plus charcoal. A difference however was obvious a week later.

Three out of the 32 pots proved to be extremely porous and the growth in these pots was far behind of the growth in the other corresponding pots, partly because the plants in these porous pots were kept actually in a lower percentage of

moisture and partly probably because the rooting medium was kept in a lower temperature due to greater evaporation through the pot. The three pots were discarded and this incident is merely reported in order to draw attention to the error that unglazed pots can introduce in an experiment when some of them differ substantially from the others as regards porosity.

The pH of the medium at the close of the experiment was as in the previous experiment about 0.5 of a unit of the pH scale more alkaline in pots with charcoal than in sand alone.

The roots of the non-inoculated peas had at the close of the experiment a considerable number of very small nodules scattered over the roots, but bearing in mind that the nodules were very small and that the plants were kept all the time in an abundance of ammonium nitrate, it appears reasonable to ignore the presence of these nodules.

Table VIII* gives the harvest data. From the table it is clear that the peas in all the lots with charcoal exceeded in growth the corresponding peas in sand alone to an amount of 22 - 25 per cent. This excess in growth although not so impressive as in previous experiments is still considerable and significant statistically above to point 0.01 .

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* The writer is indebted to Dr. R. A. Robb for his advise in connection with the statistical analysis of some of the experiments.

Table VIII.

Data of experiment VIII, May 16-August 2 1948.
Six New Zealand Maple peas per unglazed pot containing 7.2 kg. rooting medium. Bedfordshire white coarse sand with or without 1% mixture of activated charcoal. The nodulated peas inoculated with HX strain, the non inoculated received 1500 mg. nitrogen in the form of NH_4NO_3 in weekly doses.

Rooting medium and treatmt.	INOCULATED NO N. ADDED No. of pot.	Dry wt. per pot gm.	NON INOCULATED N. ADDED No. of pot.	Dry wt. per pot gm.
Sand alone	305	18.2	315	23.2
" "	313	17.1	331	22.6
" "	321	18.1		
" "	329	16.5		
	Mean	17.5	Mean	22.9
Sand plus 1% act. charc.	306	22.2	308	27.7
" " " " "	314	22.5	316	31.5
" " " " "	322	20.9	332	27.4
" " " " "	330	20.3		
	Mean	21.5	Mean	28.9
Sand alone, autoclaved.	301	18.0	303	24.1
" " "	309	19.7	311	26.5
" " "	317	18.0	319	24.1
" " "	325	17.7	327	25.0
	Mean	18.4	Mean	24.9
Sand plus 1% act. charc., autoclaved.	302	24.7	304	27.1
" " " " "	310	24.9	312	29.8
" " " " "	318	22.0	320	34.7
" " " " "	326	21.5	328	29.1
	Mean	23.3	Mean	30.2

Note: Standard error of a single pot 1.73 gm.

Required Minimum difference between the means of the yields of treatments in order the diff. to be regarded significant statistically.	Replicates	Signif. to point 0.05	0.01
	4 & 4	2.55	3.46
	4 & 3	2.75	3.74
	4 & 2	3.12	4.25
	3 & 2	3.27	4.44

The beneficial effect of thee charcoal on the growth of peas was slightly stronger on the inoculated peas than on the peas provided with ammonium nitrate (25 and 23 per cent correspondingly), and the results were but little affected by autoclaving.

From the results it can be concluded that the beneficial effect of charcoal on the growth of the peas is exerted on both inoculated peas and non-inoculated peas supplied with ammonium nitrate.

D I S C U S S I O N .

In the first place it will be helpful to summarise the results presented in the preceding section on the/of charcoals on the growth of peas. This may be done as follows.

Animal charcoal. In proportions of 4 - 6% markedly reduced growth and nitrogen fixation by peas.

Wood charcoal. In proportions 2 - 8% markedly increased growth and nitrogen fixation by peas but had little effect on the number of nodules.

Activated charcoal. The effect varied with different samples. When the sample employed was free from injurious substances for the peas, the effect was a marked increase in growth and nitrogen fixation by peas. Thus in experiments II and V, with addition of 0.5-2% charcoal the increase amounted to from 80 to 120%, while in experiment VIII it was 25%. It might be interesting to point out that in all the three mentioned experiments that powdered or mixture of powdered and granular charcoal was employed, the results thus giving the impression that from the tested samples, powedered charcoal had more beneficial effect than granular one. It appeared also as though the beneficial effect was more marked with coarse sand than with fine. The effect on nodulation was always the same, i.e., drastic reduction in the number of nodules and an increase in their size and concentration near the tap roots. Finally the effect of activated charcoal, contrary to the findings of the Russians Gukova and Butkevitch, was also extended to non-inoculated peas provided with an abundance of combined nitrogen.

Having now well estebliblished the beneficial effect on the growth and the fixation of nitrogen of peas, by the introduction in the sand of small quantities of special kinds of charcoal, the question arises as to from what source this beneficial effect is due.

Considering that the addition to the medium of such a complex substance as charcoal causes changes in the physical, mechanical and chemical properties of the medium, it is easily understood why the answer to the question is very difficult. An approach to the solution of the problem may be achieved by isolating one by one the possible factors by modification of which the charcoal may possibly act, discarding the apparently less important and giving more attention to others. It must be admitted that with this procedure the answer will not be necessarily clear since it is possible that the combined action, even of factors individually insignificant, might be entirely different from that which could be expected from the so-called additional effect.

Proceeding however in this way the first question is how much significance can be given to the probable change of the conditions of aeration of the medium.

Gukova and Butkevitch (1941), in explaining the effect of the use of 6 per cent charcoal in mixture with fine quartz sand on inoculated soya bean, give special significance to the loosening of the substratum and to better aeration. The above workers found that the effect of charcoal was limited only to inoculated beans deriving their nitrogen from the

atmosphere and later described this change as producing better conditions especially for the nitrogen fixation and not just for the growth of the roots.

Although the Russians reported that the sand used by them was "a rather dense substratum" and 6% mixture of granulated charcoal could be expected to improve the aeration still it is dificult to attribute the effect of charcoal in their experiments to the improvement of aeration, since the aeration of a sand medium is quite satisfactory in comparison with the aeration of a soil giving a good crop, even when the sand is fine sand.

As regards the present experiments the argument that the effect of charcoal is due to better aeration is further weakened when we have in mind that the best effect of charcoal was manifested in mixtures of small percentages (1 - 2%) of activated charcoal with coarse sand and when the plants were grown in unglazed, relatively small, porous earthenware pots. Under these conditions the aeration of the medium itself without charcoal was almost ideal, and it is very difficult to attach significance to any further small change in it. Since the aeration of the coarse sand is better than the aeration of a fine sand and since the aeration of a medium in unglazed earthenware pots is expected to be better than in glazed earthenware pots, the effect of charcoal if it was due to aeration ought to be more manifest in plants growing in glazed pots with fine sand than in plants growing in unglazed pots with coarse sand. The results in the present

experiments suggest the opposite since the maximum effect of the added charcoal was manifested with a medium of coarse sand in unglazed earthenware pots.

The influence of charcoal on the pH of the medium must now be examined.

Prianischnikow and Domontovitch (1926) attributed the striking results obtained by Janutskin with the use of mixtures of sand and powdered charcoal, to the probable adsorption of H and OH ions by the charcoal. Adsorbing these ions the charcoal didn't allow the shifting of the reaction of the culture solution either to acidity or alkalinity and consequently kept the medium at a more convenient pH for the growth of barley, oats and rye. Unfortunately in the paper measurements of the pH of the medium are not reported and the validity of their explanation cannot be discussed.

The pH of the medium is an essential factor for the growth of plants but at the same time it is well known that plants can adapt themselves in a range of pH between say pH 5-7.5, and any change of the pH between these two limits has very little effect on the growth of most of the plants. But any further change of the pH beyond these limits either to acidity or to alkalinity with its effect on the solubility of different ions, causes a great inconvenience for many plants and the effect of even slight changes of the pH is considerable for their growth.

On page 27 are measurements of the pH of the medium in the second experiment which was one of the experiments

where the favourable effect of the charcoal on the growth of peas was very intense. From this table can be seen that with the addition of two and four per cent of activated charcoal the pH of the medium was changed from 6.4 to 6.8. Since addition of small quantities of activated charcoal to the sand had always a similar effect on the pH, (an increase to the alkaline of a few decimals of one unit of the pH scale), it is very improbable that such an effect on the pH had any influence on the growth of the peas. The probable explanation of the effect of charcoal must be sought elsewhere.

Let us now consider the possibility that the charcoal added to the medium some of the essential macro-elements or micro-elements for the plants and first of all let us consider the possibility that it added nitrogen.

Analysis of the charcoal showed that the different samples contained a considerable amount of nitrogen, (0.4-1.0%) and although this nitrogen is chiefly unavailable for the plants, since the content in nitrogen in charcoal at the beginning and the close of the experiment was the same (see later), nevertheless it can be argued that the charcoal provided a very small quantity of available nitrogen to the medium. At the time that the inoculated peas are passing through the "hunger stage" earlier in their life, even a small supply of nitrogen may give to them an initial advantage over the peas grown in sand alone, an advantage that may steadily increase as the experiment is going on.

The results from the experiments show clearly that

this argument is on very unstable basis at least for the activated charcoal. Thus a) if the charcoal provided a small quantity of available nitrogen to the peas there is every reason to expect that barley grown with peas could have had their share; the result of analysis of barley grown with activated charcoal showed less nitrogen content than that of barley grown in sand alone. b) Uninoculated peas grown in sand and in two mixtures of sand with two samples of activated charcoal in an experiment carried out especially for the study of the availability of the nitrogen in charcoal, showed symptoms of extreme nitrogen starvation in all media. The analysis of the plants for total nitrogen had to be postponed but judging from the appearance of the peas and their dry weight which was practically the same in all the media, the conclusion is that the uninoculated peas were unable to assimilate nitrogen from that of charcoal. c) The fact that activated charcoal had a beneficial effect on peas grown in a medium with an abundance of combined nitrogen, strongly suggests that the cause of the beneficial effect of charcoal on the growth is not due to addition of nitrogen at the medium.

Apart from nitrogen it may also be argued that the charcoal provides the medium with considerable amounts of inorganic salts of other macro-elements or of micro-elements and to this supply the favourable effect is due. In answer to this argument it must be born in mind that the plants were kept all the time in an abundance of inorganic nutrients

(apart from nitrogen) since they were watered at least every 5-6 days with nutrient solution (Rothamsted), strengthened with A-Z minor elements solution.

As regards molybdenum to which special importance for the growth of inoculated legumes is attributed, approximate calculations of the molybdenum added in the culture solution (in the form of molybdenum oxide), showed that to each pot was added a quantity similar to the optimum quantity reported as necessary in the experiments of Anderson and Thomas (1946). The results also from experiment VII where the action of molybdenum was especially tested as Sodium Molybdate, showed fairly clearly that the effect of charcoal was not due to the addition of molybdenum and the hypothesis that the charcoal acted by adding molybdenum must be discarded.

The possibility that the effect of the charcoal is bound up with its adsorptive capacity has now to be considere

Bearing in mind a) that in all the experiments in which the beneficial effect of charcoal was manifested, Rothamsted solution had been employed as watering medium, -a solution not very well balanced as regards the concentration of the different ions -, b) that the plants had been watered alternately with Rothamsted solution and distilled water every 2 or 3 days, c) that the charcoal and especially the activated one is a strong adsorbing substance,-Tiselius (1941) reported that activated charcoal adsorbs amin♦acids and peptides -, and d) assuming that the adsorbing capacity of the charcoal is extended to various ions of the solution and

c) bearing in mind that the process of adsorption is an equilibrium (Gortner 1938), it could be argued that the charcoal acted as a regulator, maintaining with its selective adsorption of part of the ions, a better balance in the solution, and not allowing extreme oscillations of the concentration of the solution.

To such effect of the charcoal great significance cannot be attached, since the beneficial effect of charcoal was also manifested in the experiments carried out by Gukova and Butkevitch (1941) with the employment of diluted Helriegel's nutrient solution all the time.

Let us consider another possible effect of the charcoal; in the last few years special attention has been given to the fact that the plant absorbs the nutrients not only from the nutrient solution of the soil, but also through the direct exchange of ions between the root hair and the ionosphere of the colloidal particles of the soil. (Graham 1941, Hoagland and Arnon 1941, Albrecht, Graham and Shepard 1942, Overstreet, Broyer, Isaacs and Delwiche 1942.). Since the sand was lacking of colloids such an exchange of ions could not be performed in a medium with sand alone, whereas it was probably possible in a medium containing, apart from the sand, small quantities of charcoal. Whether such effect of the charcoal might have a significance for the nutrition and growth of plants in the present experiments is very doubtful, since the plants were kept all the time in a medium containing an abundance of nutrients in solution.

In the writer's opinion the most plausible explanation is that the charcoal with its adsorbing capacity adsorbs probable excreted products of the metabolism of the peas or of the micro-organisms inhabiting the medium, including the nodule bacteria in the case of inoculated peas, and thus prevents an accumulation of these substances which might be harmful to peas, or to symbiotic bacteria, or to both. In connection with this it is important to note that the medium in the present experiments was never watered to a point beyond its water-holding capacity, and that the pots were protected from the rain and consequently there was no drainage to remove soluble products of the metabolism.

This takes us back to the old theory expressed by Pickering (1905, 1917), that toxins are excreted by plants, a theory which has now been abandoned by most workers, Pickering's results being explained in terms of micro-elements deficiency etc. Varma(1938), and Papadakis(1940, 1941) tried recently to revive the theory by reporting experiments which suggest that plants excrete substances injurious to themselves and to other plants. Papadakis loc.cit. put forward the hypothesis that the colloids and the fine particles of the soil act as buffers adsorbing these toxins, and connected the low productivity of a coarse soil (even when provided with an abundance of nutrients and water) with its poverty in colloids and the subsequent rapid accumulation of toxins in it. In continuance he introduced the factor of space as essential for the productivity of the soil.

All the three workers mentioned, consider the toxins in the soil as products excreted by the roots of the higher plants and nobody considered the probability that they might be also products of the soil micro-organisms. In this connection it may be noted that Salisbury (1944) in an article about Antibiotics and Competition remarks " It may well be that water-soluble antibiotics are of widespread occurrence as one of the factors concerned in the competition of both lower and higher organisms occupying the same substratum ". Osborn(1944) also repobted that from 2300 green tested plants for antibacterial substances many of the tested plants gave positive results. It may be noticed that accumulation of toxic substances in a medium occupied by micro-organisms can be counteracted usually by use of heat or by treatment with charcoal, according to Waksman (1945).

It seems as though all the results of the present experiments were in close agreement with the hypothesis that the effect of charcoal on the growth was connected in some way with its adsorbing capacity.

With the assumption of this hypothesis it can be explained why in experiment II ordinary wood charcoal, to give strong beneficial effect on the growth, was needed in larger quantities than with activated charcoal, with its higher adsorptive capacity.

There is also the suggestion from experiments III and VII that the effect of the same sample and of course of the same percentage, of activated charcoal was stronger in a

medium with coarse sand than with fine or less coarse sand. Finally the fact that all the experiments which indicated the strong beneficial effect of the added charcoal were experiments with yellow coarse Bedfordshire sand, and the fact that the same sample of activated charcoal, employed in the same percentage with two different sands in experiments V and VIII, gave a stronger effect in experiment V with the coarser medium than in experiment VIII, with the less coarse medium, (the two experiments were carried out in different seasons and years and consequently comparison of their results is not fully valid), strongly suggest that the effect of charcoal is connected with its adsorbing capacity and is more intensely manifested with coarse medium.

Further experimentation is needed:

a) with even coarser sand than that employed already, to prove whether indeed the effect of the charcoal is stronger with the coarser medium;

b) with sterile cultures to test the effect of charcoal in the absence of micro-organisms and their products;

c) with ordinary sand cultures with increased population of micro-organisms in the medium through the addition of small quantities of sugar, to test the effect of charcoal in a medium with an abundance of micro-organisms;

d) with open sand cultures with coarse sand and frequent flushing of the medium, or with constant flow cultures, in order to investigate whether the probable products of metabolism are soluble or insoluble substances;

c) an attempt to isolate any toxins adsorbed by the charcoal must be made, by extracting the charcoal with water or organic solvents, since it is only if and when such substances are isolated that the problem will be elucidated.

A few words now about the possible practical applications of the results of the experiments described.

1) it is possible with a mixture of the appropriate kind and percentage of activated charcoal and sand, to increase the growth of crops in sand cultures. Further investigation is needed to answer whether such application of charcoal might give also results in coarse soils lacking large quantities of colloids if not in all soils as already suggested by Holynski (1928). 2) the results obtained by workers at the Woburn Experimental Farm (Rothamsted Report for 1946, p. 91) where with dressing with charcoal reduced the affection in clover sick soil, provide another example of possible application of charcoal and further provide arguments in support of the hypothesis that the effect of charcoal in the present experiments was connected with the adsorbing capacity of the charcoal.

Consideration will now be given to the striking influence of charcoal on the nodulation of peas. Looking through the tables of the results it can be seen that all the charcoals used led to a decrease in the number of nodules, the most effective being the activated charcoal. With increased quantities of charcoal in the mixture the number of nodules per pot decreased and in certain cases, as in experiment III,

the number decreased as much as 98% of the number of nodules in sand alone. At the same time the size of nodules increased and the nodules were more and more concentrated near the tap roots.

Table IX gives typical results of the effect of activated charcoal on the nodulation of peas for three of the preceding experiments. Apart from the number of nodules it is interesting to point out the figures showing the efficiency in nitrogen fixation of unit weight of the nodule tissue i.e. the figures showing how many mg. of nitrogen have been fixed by one mg. of dry weight of nodule tissue.* The results of experiment V are more reliable for this purpose since the peas in this experiment were harvested when they were 9 weeks old, just before flowering, whereas the peas in experiments I and II were harvested when 13 weeks old, with well-formed pods It is clear from the table that the charcoal increased the efficiency of the nodule tissue as much as 100%.

The effect of charcoal on the nodulation may be compar-

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* For determination of the efficiency in nitrogen fixation measurements of the active mass of bacterial tissue are needed, but since these measurements are tedious it seems satisfactory to use instead all the mass of the nodule. It is also realised that in calculating the efficiency on the basis of the dry weight of nodules and the total fixed nitrogen by the plant, once only at the close of the experiment, the findings have a limited value and that successive harvests and determinations in earlier stages of the growth will give more reliable picture (BOND 1936).

Table IX.

In the table are combined some of the data already recorded in the tables Ia, IIa, and Va in order to emphasise the effect of activated charcoal on the number of nodules as well as on the efficiency of nitrogen fixation by the nodule tissue. Data are per pot.

Expt.	Proportion of activat. charcoal.	N. fixed mg.	Dry wt. nodules mg.	Efficiency, mg. nitrogen fixed per mg. nod. tis.	Number of nodules.
I	Sand alone	721	316	2.28	2830
"	6%	752	260	2.89	830
II	Sand alone	594	535	1.11	5700
"	2%	1090	678	1.61	4640
"	4%	1007	542	1.86	3130
"	6%	933	455	2.05	1680
"	8%	713	300	2.38	810
V	Sand alone	145	228	0.65	980
"	1.5-2.0%	239	276	0.86	740
"	0.5-1.5%	315	271	1.16	240
"	0.5-1.5%	279	270	1.01	320
"	0.5-1.0%	261	309	0.84	860

Note: See the text for details about the sand and charcoal employed.

ed with the effect of molybdenum reported by Anderson and Thomas (1946). The authors in experiments with sand responding to this element, reported that addition of molybdenum caused a considerable decrease of the number of nodules and at the same time a remarkable increase in the fixed nitrogen. Unfortunately in the paper they do not give the dry matter of nodules from which could be drawn figures for the efficiency of the nodule tissue, but as they give details according to which " the effect of molybdenum was not due to an increase in the number or size of nodules but to more effective fixation per unit of bacteria tissue ", and in another paragraph " the plants which were not treated with molybdenum fixed little nitrogen, they were better nodulated than the treated plants; they had a greater number of equally well developed nodules", it can be concluded that the activated charcoal in the present experiments had the same effect on the number of nodules as had the molybdenum in the Australians' experiments, but it had less effect on the increase of the efficiency of the nodule tissue, since the molybdenum appears to have increased the efficiency up to 300%.

It may also be mentioned that Miss McGonagle (in press) working on sterile agar and sand tube cultures with peas, showed that with addition of sucrose the size of nodules increased and at the same time their number decreased.

The question now arises how can the beneficial effect of charcoal on the growth and the fixed nitrogen be connected with the effect on the number, size and location of nodules.

The theory of Allison (1935) that the carbohydrate supply is a primary factor in legume symbiosis may help in finding some explanation. Thus if we assume that for some or other reason the conditions for growth and nitrogen fixation are more convenient for plants grown in a mixture with charcoal than in sand alone we have to assume also that the process of fixation begins earlier and more vigorously in plants growing in a medium with charcoal added, (the early superior appearance of peas grown in charcoal supports this view), and that the place of the fixation is the first formed nodules near the tap roots. The next step is that these first formed nodules, by being on the way of the stream of carbohydrates running from tops to roots, have the opportunity of using the carbohydrates for fixation and the increase of their size and thus nodules located at some distance from the main roots are deprived of the necessary carbohydrates and are unable to be developed. For the same reason, the lack of carbohydrates, in remote roots nodules either do not initiate at all, or at least they cannot develop.

Let us consider now the results relating to excretion of nitrogenous substances from the roots of the inoculated legumes and let us begin with the observations on the detector plant.

Before proceeding with the examination of the results it is useful to mention again that the arrangement for the control pots was that suggested by Bond(1941) by growing in the control pots twice the number of barley as in the pots

with mixed cultures and comparing the dry matter and the total nitrogen of the barley in mixed cultures with half of the dry weight and total nitrogen of the barley in the control pots. Bond loc. cit. pointed out " that the arrangement of satisfactory controls for mixed culture experiments presents some difficulty ". It must be remarked that when the excretion is very extensive as it is in Helsinki, the problem has a rather theoretical interest, but it is a different matter when the excretion is very meagre, amounting to only a few mg. of nitrogen per pot, or non existent. Furthermore when the rooting medium is practically free from available nitrogen the solution of the problem is rather easy, but when, as in the most cases, the medium contains some available nitrogen the answer is more complicated.

Virtanen and his associates (1938, 1947) use as control pots mixed cultures with the same number of legume and detector plants as used in the main culture pots, the only difference being that the legume in the control pots is non-inoculated, and from the difference in the growth and nitrogen of the detector plant in the two lots, they draw up the figures for the excretion. The method looks quite satisfactory when the medium is free from available nitrogen and from non-symbiotic nitrogen-fixing micro-organisms. But when the medium contains a few mg. of available nitrogen the results are to some extent vitiated since it is not necessarily correct to assume that the detector plant in the two lots (control cultures with non-inoculated legume and mean cultures

with inoculated legume), has to share the same quantity of nitrogen, since in the control pots the non-inoculated legume competes with the detector plant for this nitrogen. The method needs special precautions to avoid chance inoculation of the legume in the control pots and is thus rather inconvenient for out-door open sand cultures.

Thornton and Nicol (1934a,1934b), Ludwig and Allison (1940), and most of the other investigators, use as control pots, pots with the same number of detector plants as those with mixed cultures and take as the excretion any difference in nitrogen in the detector plant grown in mixed cultures and control pots. The method ignores (1) the fact that even well inoculated legumes may draw, earlier in their life, small quantities of nitrogen from the medium and (2) that the detector plant in the mixed cultures is at some disadvantage in comparison with the detector in the control pots, since in the mixed cultures it is living in an overcrowded place.

Bond(loc.cit.) suggesting a double number of detector plants in the control pot than for the number of detector plants in the mixed cultures and comparing the dry weight and nitrogen of half of the control pot plants with the dry weight and nitrogen of the detector plants in mixed cultures, emphasizes the significance of maintaining approximately equal density of plants, and assumes evidently that the detector plant in the mixed cultures has for its share half of the available nitrogen in the pot. The last assumption is clearly very precarious and the whole position more

complicated. It seems to the writer that the detector plants in mixed cultures have for their share, more than half of the total available nitrogen in the medium, (assuming that the inoculated legumes, as soon as they develop nodules and start fixing nitrogen, stop competing with the detector plants for the remaining available nitrogen in the medium), being thus at some advantage when compared with half the detector plants of the control pot, but at the same time the conditions under which they live in the mixed cultures - competing with the strong legumes for the light, space, carbon dioxide, mineral elements - put them at a disadvantage in comparison with the detector plants in the control pot. The question arises whether we can assume that these two contrary conditions might neutralize each other.

From the above discussion it appears that none of these methods is ideal, and doubts about the accuracy of the methods grow stronger when the available nitrogen in the medium is appreciable. The above considerations must be borne in mind in considerinf results of experiments.

Looking at the results of all the described experiments it is apparent that, apart from the results of the pots treated with animal charcoal in experiment I, and also apart from the results of experiment V, which are dealt with below in detail, in all the other experiments barley growing in mixed cultures failed to give any appreciable increase in dry matter or in total nitrogen compared with the dry matter and total nitrogen of the half number of barley, of the corresponding control pot. Thus the experiments failed to give any sign

of excretion, the results being in full agreement with the negative results reported by Bond in several papers, with experiments carried out in the Glasgow environment. The addition of charcoal made no difference to the results. It may be interesting to point out that the percentage of nitrogen in peas responding to charcoal was similar to that in peas grown in sand alone. Judging from the percentage in nitrogen the charcoal failed to change the carbohydrate nitrogen relationship in the plants and according to this theory (which is fully discussed in section II) no excretion with the addition of charcoal ought to be anticipated.

Let us consider now, in detail, the results of the pots with animal charcoal in experiment I and the results of experiment V, since these results suggest probable excretion. From table Ib it can be seen that barley grown in mixed cultures in pots with 6% animal charcoal, contained - after the deduction of the nitrogen of the seeds - , 48.1 mg. nitrogen, (mean from 5 pots), while barley grown in the control pot contained 24.1 mg. (this is half of the total nitrogen of the plants in the control pot), and barley grown in the control pot with sand alone, contained correspondingly 16.6 mg. There is an excess of 24 mg. N. in the favour of barley grown in mixed cultures and the origin of this nitrogen must be sought. Let us make clear that had we followed the arrangement for the controls adopted by the other investigators this apparent excess of nitrogen would disappeared, since the nitrogen content of all the barley in the control pot

was actually equally large as the nitrogen content of the barley in the mixed cultures.

Although it is reasonable to admit that the possible inaccuracy of the method adopted for the controls is responsible for a part of this apparent excess of nitrogen, nevertheless it is difficult to attribute all the excess to this in view a) of the greatness of this excess, and b) of the fact that in all the other experiments (apart from expt. V) in spite of the employment of the same method and the introduction of a similar error, the nitrogen content of the barley in mixed cultures did not exceed the nitrogen content of the barley in the corresponding control pot. It seems that at least for a part of this nitrogen the source was the inoculated peas adjacent and since at the close of the expt. the nodules were apparently healthy, the only explanation is that excretion occurred in this experiment.

It is interesting to mention that the pH of the medium with animal charcoal was about 7.8 to 7.9, and the growth of the peas extremely poor or even pathological. Further investigation is needed to answer whether there is any connection between the change of the pH of the medium to alkaline, or the suppression of the growth of the legume and the induction of excretion. It appears reasonable to connect the occurrence of excretion in this expt. with the paper of Wilson and Wyss (1937) who, working in Madison (in an environment giving as a rule negative results on excretion) succeeded in inducing excretion by reducing the light and consequently by reducing

substantially the growth and fixation of peas. Thus whereas Virtanen and his co-workers in Helsinki, were able to detect excretion in cases with plants both having good and poor growth and fixation, it seems that in environments where the results on excretion are usually negative, positive results may be obtained by making the conditions for growth and fixation poor.

The results from experiment V recorded in table Vb, will now be considered. In this experiment in all the tested media, barley grown with peas exceeded the barley in the corresponding control pot in nitrogen content by an average of 14,4 mg per pot. It is unfortunate that the control pot for each medium was without duplicate, but since there were 3 control pots altogether and in all the media (with and without charcoal) the barley in the mixed cultures exceeded in nitrogen the barley in the corresponding control pot, combining the results for the 3 media it is possible to test the significance of the excess in nitrogen, of the barley grown in mixed cultures, for the 3 media as a whole. Application of the Student's "t" test (Fisher 1942) on the difference in nitrogen of the barley in mixed cultures and that in the control pots showed that the difference is statistically significant since t just exceeds the value needed for the 5% level of significance.

If the arrangement employed for the control pots could withstand criticisms about its accuracy, the present experiment might be thought to prove that under special conditions

in the Glasgow environment, the occurrence of excretion of nitrogenous substances from the roots of inoculated peas is possible. But bearing in mind all the arguments about the accuracy of the arrangement employed for the control pots and assuming that part of the apparent excess in nitrogen in barley grown in mixed cultures was due to an error introduced by the method employed, the remaining excess in nitrogen on behalf of the barley grown in mixed cultures is of doubtful significance and the position about the occurrence of excretion dubious.

It is interesting to note, that the semple of the sand employed in this experiment contained twice as much available nitrogen as the samples employed in previous experiments, and to connect the results of this experiment with the results reported by Thornton and Nicol (1934). These investigators, by adding small quantities of sodium nitrate to mixed cultures in sand succeeded in detecting as many as eleven times more excretion in the mixed cultures with sodium nitrate than in cultures without added nitrogen. In order to test whether sand medium with the addition of small quantities of nitrates is a more convenient medium for the occurrence and detection of excretion, an experiment was carried out the results of which are described in the second section of the thesis.

So far we have considered evidence for excretion provided by examination of the detector plants. The second method for the detection of excretion is by Kjeldahl analyses on the rooting medium, and the proper employment of the

method needs successive analyses of the medium at different stages of the growth in order to detect the maximum excretion, since according the findings of Virtanen (1938) it is possible for there to be a partial reabsorption by the legume of substances excreted in earlier stages. In the present experiments analyses of the rooting medium were carried out once at the close of the experiments and since the plants, in all the experiments but two (V and VI), were harvested at a late stage with well-formed pods, the results are of limited value. The results of analysis of the rooting medium in experiments V and VI in which the plants were harvested at the beginning of the blossom might be regarded more reliable.

Analysis of the total nitrogen in the sand in pots filled with sand alone and planted with mixed cultures or barley alone, gave in all the experiments an increase of a few mg. nitrogen per pot on behalf of the pots with mixed cultures, but since this slight increase even when significant statistically might easily be attributed to impurities from the fine parts of the roots which were not easily separated from the sand, the conclusion was that in pots with sand alone the phenomenon of excretion did not take place at all. The results were in full agreement with the results of experiments reported by Bond (1938, 1941a), Bond and Boyes (1939).

Analysis of the rooting medium sand plus charcoal was attempted but soon it was realised that small excretion could not be detected since the charcoal itself contained a high nitrogen content (0.4-0.5% for wood and activated charcoal,

1% for animal charcoal). Thus the nitrogen added in the medium with the charcoal amounted, for a pot containing 8 kg. rooting medium with 2% charcoal, to 680-850 mg. for the first two types of charcoal and to 1700 mg. for animal charcoal. Evidently with such large amounts of nitrogen, Kjeldahl analyses based on examination of relatively small samples (200 gm.) of the rooting medium are liable to a large sampling error, even though there is thorough mixing of the medium prior to analysis.

In order to reduce the sampling error, analyses on the separated particles of charcoal were carried out, with the idea that if any excretion and adsorption of nitrogenous substances had occurred during the expt., the adsorbed nitrogen ought mainly to be found in the powerful adsorbent, the charcoal, especially in the case of activated charcoal since Tiselius (1941)reported that activated charcoal adsorbs amino-acids and peptides. Unfortunately neither of these analyses gave any significant result, because as the chatcoal itself contained large quantities of nitrogen, small adsorbed quantities of nitrogen (if there were any) were masked by the experimental error. The detection of meagre-occurring excretion by the analysis of rooting medium containing a powerful adsorbing ingredient might be expected to give clear results only when the employed adsorbing substance is free or almost free from nitrogen.

The results of the analyses showed 1) that there was no extensive excretion, and 2) that there was no considerable decrease in nitrogen of the rooting medium - thus the benefit to peas was not due to extensive uptake of nitrogen from charcoal.

S U M M A R Y O F S E C T I O N I.

(1) A number of experiments with sand or mixtures of sand and charcoal as rooting medium and peas and barley as test plants, were carried out in order to investigate: a) whether addition of small quantities of charcoal to a sand medium gave as a consequence increase of growth and fixation of nitrogen to legumes, and b) whether increase in growth and fixed nitrogen by inoculated legumes was accompanied by nitrogen excretion.

(2) The addition to the medium of small quantities of activated charcoal and, in a lesser degree of wood charcoal, had as effect a considerable increase on the growth and the fixation of nitrogen of the inoculated peas.

(3) The beneficial effect of activated charcoal was exerted in a similar way both on inoculated peas deriving the nitrogen from the atmosphere and non-inoculated peas growing on medium supplied with ammonium nitrate.

(4) The effect of activated charcoal on the nodulation of inoculated peas gave a considerable decrease in the number of nodules accompanied by increase in the size and efficiency of the nodule tissue (mg. nitrogen fixed per mg. dry weight nodule tissue) and a close concentration of nodules near the roots.

(5) The beneficial effect of the added activated charcoal is not considered to be due to any addition to the medium of nitrogen, molybdenum, or another macro-element or

micro-element, nor to any improvement of aeration or of the pH of the medium.

(6) The effect of charcoal was stronger with coarse sand than with fine sand.

(7) The beneficial effect of charcoal on the peas is probably connected with its strong adsorbing capacity and the hypothesis that the charcoal acted by adsorbing injurious products of the metabolism of the peas or of the micro-organisms of the medium or of both, was accepted since this explanation fitted the results.

(8) The added activated charcoal had a depressing effect on the growth of barley, due possibly to its competition with the barley for the small quantities of available nitrogen in the medium.

(9) Animal charcoal in percentages 4-6% had a strong depressing effect on the growth of peas. This and the similar effect of some samples of activated charcoal was probably due to some toxic substance that the charcoals contained.

(10) In spite of the fact that with addition of charcoal to the medium a considerable increase in the growth and fixation of nitrogen by peas was obtained in a number of expt., there was no appreciable excretion. In fact all the experiments but two, failed to show any excretion at all. The results of these two experiments are considered in the light of certain reservations about the arrangement of control pots.

(11) The possibility of the employment of activated charcoal in farming in special cases was discussed.

Section II.

Further experiments on excretion of fixed nitrogen from nodules.

I N T R O D U C T I O N .

After numerous experiments on excretion of nitrogenous substances from nodulated legumes, carried out in various parts of the world, failed to confirm the results published by Virtanen and his co-workers in the late twenties, the cause for the discrepancy of the results was attributed successively to the different species or varieties of legume employed, strain of micro-organism, adsorbing capacity of medium, porosity or size of pots etc., until later on it was realised that the cause of the discrepency ought to be sought mainly in the effect of the climate, since experiments carried out in places away from Helsinki, with the same variety of legume, strain of micro-organism, and same rooting medium as those employed in Finland, failed to show any excretion. With a slight or drastic alteration of one or more factors of the climate, such as duration or intensity of light, temperature, etc., in one part of an experiment detection of excretion might be obtained, while the part of the experiment treated under natural conditions failed to give any excretion at all. Thus according to Wilson (1940) an experiment carried out by Wyss under low temperature and artificially prolonged day-light, showed significant excretion from peas detected with barley, while cultures treated with natural light failed to show excretion, and Strong and Trumble (1959) succeeded in detecting excretion in Australia (where the results were normally negative) by shading the cultures of peas and oats.

It appears that the majority of the investigators now

agree that "although several factors (strain of organism, type of sand, species of plant) may influence the results quantitatively, it is the relation between the rates of photosynthesis and nitrogen fixation that controls the occurrence of the phenomenon". (Wilson 1940). An indispensable prerequisite for the occurrence of excretion is the existence of convenient conditions that enable the plant to fix more nitrogen than the photosynthesised carbohydrates can "tie up" and it is this excess of nitrogen which is excreted by the nodule.

Thus the occurrence of excretion was connected with the more general phenomenon of fixation and the theories emphasising the importance of the available quantity of carbohydrates (Allison 1935), or of the carbohydrate-nitrogen relationship (Wilson 1940), for the occurrence of fixation found also their application in the explanation of the conditions under which the phenomenon of excretion occurs.

Since under the Glasgow environment Bond (1938, 1941a) Bond and Boyes (1939), reported negative or negligible excretion, in spite of the fact that they had taken into consideration all the mentioned factors and they had employed different media, strains of micro-organisms, pots different in size and material etc., further experimentation on excretion, as has already been pointed out in Section I, was considered as useful only under artificially modified conditions which could possibly bring about a change in the carbohydrate-nitrogen relationship in the legume and consequently induce excretion.

In the first section of the thesis experiments have been described with mixures of sand and charcoal as rooting medium and it has been shown that the experiments failed to show excretion in spite of the considerable increase in fixed nitrogen. On the above hypothesis this ought to be anticipated since no actual change in the carbohydrate-nitrogen relationship was obtained, the increase in fixed N. being followed by increase in the total dry matter of the plant.

In the experiments now to be described the following modifications were applied: a) reduction of daylight, b) reduction of the assimilating surface of the legume by clipping the plant at a time when the fixation was vigorous, and c)addition of small quantities of combined nitrogen to the medium in order to suppress the growth of the legume, by means of the competition exerted by the detector plant with better growth. Altering by the above modifications the photosynthesis of the legume, it was hoped to bring about an abrupt change in the carbohydrate-nitrogen ratio and in this way to induce excretion. The study of excretion under the reduction of daylight especially in the way applied in the present experiments has purely theoretical interest, while the study of the alteration of clipping has both theoretical and practical interest (in the case of pastures), and the study of excretion with added nitrogen in sand cultures theoretical and practical interest since the growth of plants is more like to that of mixed cultures growing in soil under natural conditions.

Experiments founded on the same ideas were carried out

by other investigators. Thus a moderate reduction of daylight by shading the plants was applied in experiments in Wisconsin by Wilson and Wyss (1937), and in Australia by Strong and Trumble (1939), the effect of clipping of lucerne on ryegrass was studied by Thornton and Nicol (1934b), and the effect of added combined nitrogen in mixed cultures was studied by Thornton and Nicol(1934), Ludwig and Allison (1940), Wyss and Wilson (1941).

In all the experiments described in the first section barley was employed as detector plant, since barley was used by many investigators for this purpose and since with barley investigators including Virtanen reported detection of excretion. In view (a) of the statement of Virtanen(1947) according to which wheat and barley cannot use aspartic and glutamic acid while oat does, (b) that even in open sand cultures the conditions are not ideal for rich population of micro-organisms splitting compound organic substances to simpler inorganic ones, and (c) that usually in experiments of excretion the plants are not kept for a long time, in the present experiments it was considered useful, in addition to barley, to attempt to detect excretion with other plants in the hope that these new detectors being possibly more able to use excreted nitrogenous compounds would detect small excretion where barley failed. Legumes belonging to different cross-inoculation group than the inoculated legume appeared as promising detectors. For the above reasons 3 detector plants were employed in the first two experiments, namely, barley, soybean and lupin, while oat was employed in the third.

M E T H O D S.

The experiments were carried out in open sand cultures as in the experiments described in the first section of the thesis. Glenboig fine sand was employed in the first two experiments and Bedfordshire white coarse sand for the third, with unglazed earthenware pots in all the three experiments. In order to avoid chance nodulation of the non-inoculated detector legumes, the media with the pots in the first two experiments were autoclaved and other precautions were taken, namely, covering the surface of the pot with sterilised gravel and watering the plants with sterile solution through glass cylinders. (Fig. 18).

New Zealand Maple peas inoculated with HX strain of nodule bacteria were employed in all the experiments and barley (Spratt Archer variety) again was used. The surface sterilisation of peas and barley was done as in the first section, while the seeds of Soyabean (var. Manchu) and Lupin (sweet) were surface-sterilised by immersing for ten seconds in absolute alcohol and afterwards burning the alcohol on the surface by passing the seeds above a gentle flame. Rothamsted solution free from nitrogen strengthened with A-Z minor elements solution plus molybdenum oxide was employed in all the experiments. As regards the arrangement for the control pots the method of keeping twice the number of detector plants in the control pots than in pots containing mixed cultures and and comparing the growth of half of them with that in mixed cultures, was applied in the first two experiments, while in

the third experiment in which combined nitrogen was added, an equal number of detector plants in mixed cultures and control pots was sown, and the yieldsof the detector plants in mixed cultures and control pots were compared.

The harvest, drying and analysis of plants for total nitrogen as well the analysis of sand, were carried out as described in the first section.

D A T A.

Although the investigation of the effect of the two factors limitation of day-light and clipping of legume was carried out in one combined experiment, for convenience the data are presented separately for the effect of each of the two factors as experiment I and II respectively. Forty-five unglazed earthenware pots 7" in diameter with 8.5 pounds Glenboig fine sand were employed all together. 30 pots were employed with mixed cultures (4 inoculated peas and 4 detector plants in each) while the remaining 15 pots were employed as controls with 8 plants of one of the three detector plants, barley, soyabean or lupin.

Despite the fact that the germination and the growth of lupins at an early stage was very good, from the fourth week they started wilting and with the progress of time they died one after the other. It seems that either the pH of the medium or the calcium content of the solution was unsuitable for the lupins. This incident consequently decreased the value of the results for the part of the experiments with lupins and although the peas in mixed cultures with lupins were

harvested and the results recorded, little significance can be given to them.

EXPERIMENT I. Limitation of daylight. From the 21st of July, when the plants entered their 8th week of growth and the appearance of peas indicated rapid fixation of nitrogen, 3 pots from each of the mixed cultures and 2 control pots from each lot, selected at random, were transferred for some hours every day into an adjacent outbuilding transformed into a dark room, while the remaining pots continued receiving all the daylight. It was arranged for a single pot from every lot to be kept 6, 9, and 11 hours respectively in darkness during daytime in the first fortnight, 6, 8, 10 hours in the second fortnight and 5, 7, and 9 hours from the third fortnight till harvest. The number of hours in darkness was reduced in this way because of the natural shortening of the length of day at this season. For simplicity in the results the hours that the plants were kept in the darkness during daytime are reported as 6, 8, 10 respectively.

The symptoms of the treatment were soon manifest, the colour of the peas being changed to pale-green yellow and the top of the plant becoming fragile and twining.

Table Ia, gives the results for the peas and table Ib, for the detector plants. It can be seen from the table Ia that the growth and the total fixed nitrogen by peas were very satisfactory, and also that the differences in both, due to applied treatment, well marked. Thus by keeping the plants for 10 hours in darkness during daytime, the growth and

Table Ia.

Data of experiment I, May 29- September 6 1947.
Four peas inoculated with NX strain and 4 detector plants per unglazed pot containing 8.5 pounds fine Glenboig sand. The data are for peas only (per pot). Curtailment of light commenced 21st July.

Culture	Treatment	No. of pot.	Dry weight gm.	Total N. minus N. of seeds mg.	Number of nodules.
Peas Barley	Uncurtailed day light	183	14.1	456	2750
" "	" "	184	13.2	388	-
" "	" "	202	17.4	478	3200
	Means		14.9	441	2980
" "	6 hours in dark during day-time.	186	8.2	257	-
" "	8 " "	187	5.5	137	3220
" "	10 " "	205	5.5	140	-
Peas Soya	Uncurtailed day light	182	10.1	294	-
" "	" "	183	10.5	295	1700
" "	" "	201	11.5	305	1900
	Means		10.7	298	1800
" "	6 hours in dark.	185	6.4	174	-
" "	8 " "	186	4.3	102	-
" "	10 " "	204	3.8	86	-
Peas Lupin	Uncurtailed day light	181	15.3	454	-
" "	" "	182	13.9	409	-
" "	" "	200	18.6	558	-
	Means		15.9	474	-
" "	6 hours in dark	184	7.6	214	-
" "	8 " "	185	5.1	129	-
" "	10 " "	203	5.0	124	-

Table Ib.

Data of experiment I for the detector plants. Mixed cultures 4 inoculated peas plus 4 barley or 4 soyabean or 4 lupin, control pots 8 barley or 8 soyabean or 8 lupin. The data shown for the control pots are the half of the yield of the pot, corresponding to the growth of 4 detector plants.

Culture	Treatment	No. of pot.	Dry wt. gm.	Total N. mg. minus N. of seeds.	Total N. in sand mg.
Peas Barley	Uncurtailed day light.	183	1.02	13.7	45
" "	" "	184	1.43	24.0	45
" "	" "	202	0.66	3.6	46
	Means		1.04	13.8	45
Barley alone	" "	175	0.96	6.7	30
" "	" "	196	0.99	7.7	31
	Means		0.98	7.2	31
Peas Barley	6 hours in dark during day-time	186	0.69	-	-
" "	8 " "	187	0.50	3.4	37
" "	10 " "	205	0.45	2.2	-
Barley alone	6 " "	178	2.00	22.9	33
" "	10 " "	199	0.63	6.0	33
Peas Soya	Uncurtailed	182	1.96	1.3	-
" "	"	183	2.28	6.2	51
" "	"	201	1.91	-1.6	50
	Means		2.05	2.0	51
Soya alone	"	174	4.25	17.7	41
" "	"	195	4.21	17.0	-
	Means		4.23	17.4	41
Peas Soya	6 hours in dark	185	2.22	8.9	47
" "	8 " "	186	1.93	14.9	46
" "	10 " "	204	2.11	10.5	-
Soya alone	6 " "	177	3.77	11.8	38
" "	10 " "	198	3.30	5.9	-

nitrogen fixed were limited to less than one third of the growth and nitrogen fixed of the peas receiving all daylight. It is interesting to note that the total dry matter and the total nitrogen fixed decreased almost to the same percentage, and that at the close of the experiment the percentage of nitrogen was almost the same in all plants, whether or not subject to limited light. Therefore, judging from the figures of the final analysis of the plants, the applied reduction in light did not induce any alteration at all in the carbohydrate-nitrogen relationship in the peas.

From table Ib for the detector plants, it can be seen that the results for barley were unusually irregular among the replicates, and had time permitted the experiment would have been repeated. From the beginning of the experiment the growth in one of the control pots with barley - pot No 178 - was conspicuously superior to that in any of the others; this was probably due to some impurity in the sand, and the concerned pot consequently must be discarded. The results now in detail showed a) that barley grown in mixed cultures with peas in pots receiving uncurtailed daylight exceeded by few mg. in nitrogen content the corresponding plants in the control pots. Thus on the basis of the applied arrangement of comparing the dry matter and nitrogen of the detector plants in mixed cultures with that of the half in the control pots, barley in mixed cultures treated with uncurtailed daylight exceeded in nitrogen the barley in the control pots by 6.6 mg per pot, (mean for 5 pots). Since however the differences

in growth from pot to pot were very large (the barley in the third replicate pot actually contained less nitrogen than the barley in the control), this apparent excess of nitrogen is not significant statistically, and the apparent excretion suggested by some of the figures is entirely fortuitous.

b) that barley grown in mixed cultures with peas in pots treated with curtailed daylight not only failed to show any improvement from the presence of the peas with the applied treatment but rather the opposite for they contained less nitrogen than the corresponding plants in the control pots.

The results for the soya bean were more regular and conclusive. Soya beans grown with peas and receiving uncurtailed daylight, had only half the dry matter of the corresponding soya beans in the control pots, while their total nitrogen did not exceed appreciably the total nitrogen contained in the seeds. It seems that soya beans grown in mixed cultures not only did not show any improvement by the presence of the peas but that, on the contrary, they suffered possibly owing to crowding and, particularly, to competition for light.

Soya beans grown with peas in pots treated with curtailed light contained a few mg. of nitrogen more than the seeds but, at the same time, did not exceed in nitrogen the corresponding soya beans in the control pots (fig. 18). The experiment failed to show any improvement in either of the lots as regards soya beans grown in mixed cultures with peas.

Analysis of the total nitrogen in the sand at the close of the experiment (table Ib) showed a gain in the total

Figure 18.

Experiment I., Section II.
Left soyabean in mixed cultures with peas treated with curtailed day-light, right soyabean alone received the same curtailed day-light.

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nitrogen in all the pots with mixed cultures, but it is doubtful whether this gain is due to any excretion or to small fine roots which were difficult to separate from the sand. In any case, this excess in nitrogen was a little larger in the pots kept in uncurtailed daylight than in the pots treated with curtailed daylight.

EXPERIMENT II. Effect of clipping the legume. When the plants were 8 weeks old and the growth of the peas showed a vigorous fixation, the tops of the peas from 2 pots from each of the 3 mixed cultures, selected at random, were cut at about 10-12" above the surface of the sand, the part cut representing half to two thirds of the total above the ground part of the plant. Few days after the clipping the peas responded by producing new shoots. The growth of the detector plants was followed carefully but there was no sign of any improvement.

Tables IIa, and IIb , give the analytical results for the peas and the detector plants respectively. Since it was necessary to compare the growth in the pots with the clipped peas with that in pots with untouched plants, and since this experiment was combined with the first one, the pots from the first experiment, presented under the treatment "uncurtailed day light", were employed for this purpose, but on this occasion under the treatment of "non-clipped". Table IIa shows that both, total dry matter and nitrogen fixed were reduced in the clipped peas to two-thirds of that of the non-clipped peas; but as both decreased at the same rate, at the close of the expt. the percentage of nitrogen was almost the

Table IIa.

Data of experiment II, May 29 - September 6 1947.
Four peas inoculated with HX strain and four detector plants per unglazed pot containing 8.5 pounds fine Glenboig sand.
The data are per pot and for peas only, and include the clippings. Clipping of peas 22nd July.

Culture		Treatment	No. of pot.	Dry weight gm.	Total N. minus N. of seeds, mg.	Number of nodules.
Peas	Barley	Non-clipped	163	14.1	456	2750
"	"	" "	184	13.2	388	-
"	"	" "	202	17.4	478	3200
		Means		14.9	441	2980
"	"	Clipped	172	10.4	334	4930
"	"	"	193	8.9	297	4500
		Means		9.7	316	4720
Peas	Soya	Non-clipped	162	10.1	294	-
"	"	" "	183	10.5	295	1700
"	"	" "	201	11.5	305	1900
		Means		10.7	298	1800
"	"	Clipped	171	8.2	241	-
"	"	"	192	7.4	215	4060
		Means		7.8	228	4060
Peas	Lupin	Non-clipped	161	15.3	454	-
"	"	" "	182	13.9	409	-
"	"	" "	200	18.6	558	-
		Means		15.9	474	-
"	"	Clipped	170	11.4	366	-
"	"	"	191	11.8	371	-
		Means		11.6	369	-

Table IIb.

Data of experiment II for the detector plants. Mixed cultures 4 inoculated peas plus 4 barley or 4 soya bean or 4 lupin, control pots 8 barley or 8 soya bean or 8 lupin. The data shown for the control pots are the half of the yield of the pot, corresponding to the growth of 4 detector plants.

Culture	Treatment	No. of pot.	Dry weight gm.	Total N. mg. minus N. of seeds.	Total N. in sand mg.
Peas Barley	Non-clipped	165	1.02	13.7	45
" "	" "	184	1.43	24.0	45
" "	" "	202	0.66	3.6	46
	Means		1.04	13.8	45
" "	Clipped	172	1.42	16.9	37
" "	"	195	1.17	16.0	38
	Means		1.30	16.5	38
Barley alone		175	0.96	6.7	30
" "		196	0.99	7.7	31
	Means		0.98	7.2	31
Peas Soya	Non-clipped	162	1.96	1.3	-
" "	" "	183	2.28	6.2	51
" "	" "	201	1.91	-1.6	50
	Means		2.05	2.0	51
" "	Clipped	171	3.17	5.6	43
" "	"	192	3.02	2.8	-
	Means		3.10	4.2	43
Soyabean alone		174	4.25	17.7	41
" "		195	4.21	17.0	-
	Means		4.23	17.4	41

same in both, clipped and non-clipped peas.

As regards the detector plants, from table IIb, it can be seen a) that barley grown in all mixed cultures but one, exceeded in nitrogen content the corresponding barley in the control pots by 6.5 to 16.8 mg. per pot. This excess however -as was already mentioned in the first experiment- is not significant statistically for the barley grown with non-clipped peas, while it is significant for the barley grown with clipped ones. Thus on the basis of the applied arrangement of comparing the half growth of the control pot to the growth of the detector plant in mixed cultures, barley grown with clipped peas showed a detection of excretion of 9.3 mg. nitrogen per pot (mean from 2 pots). In spite of the fact that the excess in nitrogen in barley grown with clipped peas is significant statistically, in view of the large variation of the growth of barley in pots with non-clipped peas, and in view also of the small number of replicates, the results are considered inadequate and further confirmation is needed, before to reach the conclusion that indeed with the applied treatment of clipping the peas the occurrence of excretion is induced. b) that soyabean grown in both mixed cultures with clipped or non-clipped peas failed in exceeding in growth or nitrogen content the corresponding soyabean in the control pots. On the opposite soyabean in mixed cultures had a less growth and less nitrogen content than soyabean in the control pots and thus the clipping treatment failed to give any profit to the soyabean. (Fig. 19).

F i g u r e 19.

Expeeiment II, Section II.
Left soyabean in mixed cultures with clipped peas, right soyabean grown alone.

Analysis of the sand for total nitrogen showed an excess of few mg. of nitrogen per pot on behalf of the pots with mixed cultures (table IIb). Since the excess was very small it can easily be attributed to fine fragments of roots, and in any case this excess was smaller in pots with clipped peas than with non-clipped peas.

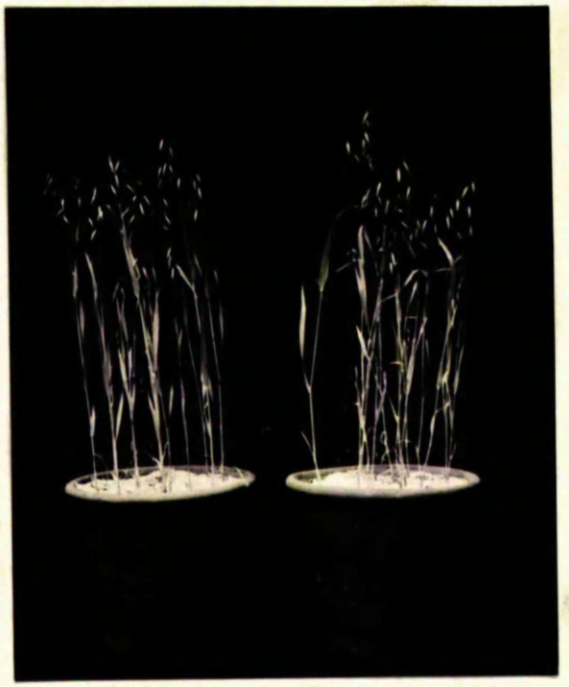
Finally since the clipped peas , by the time of harvest showed a considerably greater number of nodules than the non-clipped peas, the effect of clipping on the nodulation is further dealt in the discussion.

EXPERIMENT III Addition of small quantities of combined nitrogen to the medium. Twenty-seven earthenware unglazed pots 10" in diameter were employed, each filled with 23 pounds Bedfordshire white sand. 15 pots were employed as mixed cultures with 7 inoculated peas and 14 oats in each while 12 pots were employed as controls with 14 oats alone in each. A third of the pots were kept without any addition of nitrogen whereas to each of the other pots were added 55 or 110 mg. nitrogen in the form of ammonium nitrate, in two doses, the first at sowing and the second one month later. Rothamsted solution free from nitrogen plus A-Z minor elements solution, was again employed for watering, alternately with distilled water. Records of the growth of the plants were kept all the time and the impression from the appearance of the plants was that although the oats in the pots with added nitrogen made substantial growth, nevertheless the growth of the peas was only slightly retarded and as regards the excretion there was no sign of it. (Fig. 20, 21, 22.).

F i g u r e 20.

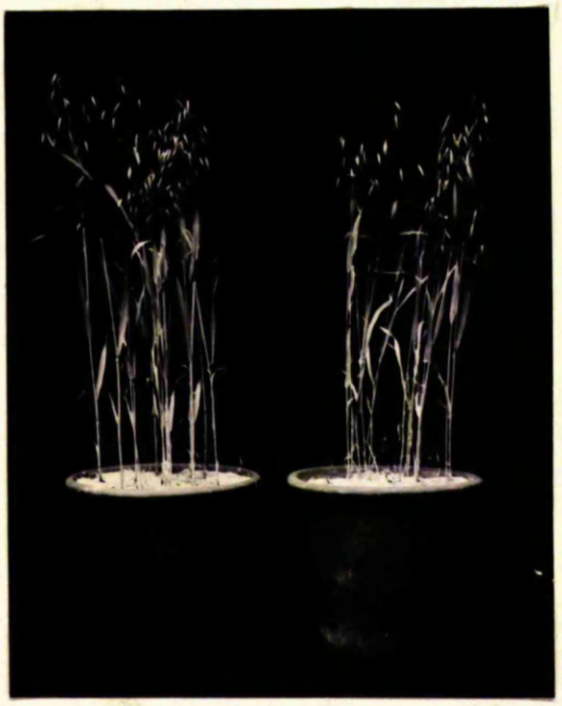
Effect of addition of combined nitrogen on excretion. Expr. III. Left control pot oat alone, right oat in mixed cultures with peas. No nitrogen added.

F i g u r e 21.



Effect of addition of combined nitrogen on excretion. Expert. III. Left control pot oat alone, right oat in mixed cultures with peas. 55 mg. nitrogen added per pot.

Figure 22.



Effect of addition of combined nitrogen on excretion. Expt. III. Left control pot oat alone, right oat in mixed cultures with peas (peas removed). 110 mg. nitrogen added per pot.

Table III gives the harvest results.

Table III.

Data of experiment III, 16 May - August 13, 1948. Seven inoculated peas with HX strain and fourteen oats, or fourteen oats alone, per unglazed pot containing 23 pounds White Bedfordshire coarse sand. The data are per pot and represent means of five replicates in the case of mixed cultures, or four replicates in the case of control pots.

Treatment.	Dry weight in gm. Peas.	Oats in mix. cultures.	Oats alone in controls.
Without added nitrogen.	21.4	2.14	2.42
With 55 mg. added nitrog.	19.3	4.98	6.65
With 110 mg. added nitrg.	17.6	7.08	9.75

Minimum difference required in gm. for the peas in order the differences in growth to be regarded significant statistically:

to point 0.05	to point 0.01
2.73	3.85

It is unfortunate that due to lack of time the analysis of plants for total nitrogen has had to be postponed and the table contains only the dry weight of the plants. It is clear however from the table a) that the growth of peas treated with added nitrogen was depressed, probably due to competition exerted by the oats, but the depression of the growth was not very large even with the high added quantity of nitrogen, (actually the depression due to small added quantity of nitrogen was not significant statistically whereas the depression due to large added quantity of nitrogen, although significant statistically, was not larger than 18% of the dry matter of the growth of peas without added nitrogen)., and b) that the experiment failed to give any excretion at all since the growth of oats in mixed cultures with peas was less than that of oats in the control pots (oats alone).

D I S C U S S I O N.

An attempt was made in two of the experiments described, by reducing the photosynthesis of peas, to reduce abruptly the carbohydrate-nitrogen ratio and, according to the carbohydrate-nitrogen relationship theory of Wilson and his co-workers (1940), to induce excretion of the excess of the nitrogen. The term carbohydrate-nitrogen relationship is very wide and includes all the possible ratios, viz., a) total carbohydrate to total nitrogen, b) total carbohydrate to soluble nitrogen, c) soluble carbohydrate to total nitrogen, and d) soluble carbohydrate to soluble nitrogen. For the present all of these ratios are used in the literature, but it seems that if the theory has sound bases further work will reveal which one of these four ratios is the important one for the process of fixation itself as well as for the process of excretion. Inaddition to these ratios, Wilson (1940), reviewing many experiments, employs the percentage of nitrogen, although he points out, that the percentage of nitrogen is not as accurate measure of the chemical constituents believed to be concerned in the process of fixation and excretion, and emphasises the need for more accurate measurements. In this connection the statement of Nightingale quoted by Wilson (loc. cit.), according to which " in view of the various functions of carbohydrates and the many forms of nitrogen found in the plant, it seems highly improbable that a ratio of significance can ever be obtained by dividing the total carbohydrate content of a plant by its nitrogen content", is interesting.

Since in the present experiments more accurate determinations for the ratio of carbohydrates to nitrogen were not attempted and the only available figure is the percentage of nitrogen, the above reservations of the inadequacy of the percentage of nitrogen figure must be kept in mind during the examination of the results.

Let us consider first the results obtained by other investigators in experiments related to the present. Thornton and Nicol (1934a) studying the effect of clipping the tops in lucerne, on the development of the roots reported (a) that the total number of nodules as well their size and activity was not affected by the clipping, the total nitrogen of the plants being the same in clipped and non-clipped plants; (b) that the clipping increased slightly the dry weight of the tops, decreased considerably the dry weight of the roots and, decreased slightly the total dry weight of the clipped plants, and (c) that rye grass growing in mixed cultures with lucerne failed to show any extra profit in nitrogen due to the clipping of lucerne (Thornton and Nicol 1934b).

Leonard (1926), investigating the carbohydrate synthesis and the nodule formation, clipped to half 14-day-old soya bean and continued clipping weekly the same plants for 5 weeks leaving only one leaf on each occasion; examination for nodules at the close of the experiment revealed that the clipped plants had practically no nodules while the non-clipped plants had in average 30 nodules each. Eaton (1931), by altering the length of daylight and clipping young soyabeans

reported (a) that the amount of growth and nodule development was in direct proportion to the length of day and in indirect proportion to the severity of clipping, (b) that the nodule development was correlated with the percentage of acid-hydrolysable carbohydrates of the plant, the correlation being greater with the content of the tops than with the content of the roots. Hopkins (1935), studying the effect of long and short day and shading on nodule development and composition of the soyabean concluded (a) that inoculated legumes without nitrogen decrease sharply in available carbohydrates (total of sugars, starch and dextrins), soon after the hunger period since the supply of carbohydrates is drawn for the synthesis of proteins, (b) in general the plants which yielded the larger percentage weight of nodules, contained the higher percentage of sugars and starch in the roots, and (c) that the nodule efficiency does not always coicide with the nodule development since, although the non-shaded plants exceeded the shaded in percentage of nodules - calculated on the base of the weight of the whole plant-, the shaded plants fixed considerably more nitrogen.

Virtanen and Nurmia (1956), studying the effect of cutting on the carbohydrate reserve in red clover, reported that cutting causes an abrupt decrease in the carbohydrate content of the roots, the decrease being more pronounced in the insoluble carbohydrates than in the soluble, and also that the sugar content of the roots falls to the minimum 12 to 15 days after the cutting. Wilson and Wyss (1937), by

screening the plants reported excretion, and so did Strong and Trumble (1939), by reducing the daylight from the normal 11 hours to 2 hours.

Of the experiments mentioned, especially interesting is the experiment carried out by Thornton and Nicol (1934a, 1934b), since in this experiment the clipping was performed at a late stage when the fixation was already well established and the results are comparable with the present results of the clipping of peas. They are many differences in the result in the two experiments; thus while the clipping of lucerne had no effect on the number, the size, or the efficiency of nodules and had only slight effect on the total growth, in the case of the clipped peas the effect was considerable both on the number and efficiency of nodules as well as on the total growth of the plant. The cause of the different responses to the clipping must mainly to be sought in the existing peculiarities of the two legumes employed.

Lucerne, a perennial plant, has an extensive root system, whose dry matter in an adult plant is twice or thrice larger than the dry matter of the shoot, and the root system is employed as a storage organ for the carbohydrates and proteins, while in the pea the root system is less extensive, its dry weight being only one fifth to one tenth of the dry matter of the shoot. In the case of lucerne, in spite of the fact that the plant, with the severe clipping was deprived of almost all its green parts, the fixation of nitrogen was not affected, since the roots provided the necessary carbohydrates for the fixation. The same source provided the necessary

carbohydrates for the building of the new shoots and in the building of these new shoots probably was used the nitrogen fixed in the time immediately after the clipping. The plant quickly formed new shoots and soon started photosynthesising vigorously carbohydrates and thus the total growth of the clipped lucerne was only 9-15% less than the total growth of the non-clipped plants. In view of this explanation it is doubtful whether with the clipping of the lucerne there was any accumulation of fixed nitrogen and this is perhaps the reason why the clipping was not followed by excretion. It appears that perennial legumes with extensive roots as store organs are not convenient plants for the induction of excretion through the employment of clipping.

Let us turn now in the examination of the present results with the peas where both treatments of clipping and curtailing of daylight had a pronounced effect on efficiency of nitrogen fixation and total growth of plant but where also the phenomenon of excretion was absent or very meagre. It is unfortunate that the data from these experiments are incomplete since they do not contain analyses of the carbohydrate-nitrogen ratio in the peas at different dates after the clipping or the curtailement of daylight. We do not know to what extent and for how long with the treatments employed we altered the carbohydrate nitrogen ratio in the peas. The percentage of nitrogen at the close of the experiment suggests that the peas in spite of the drastic alteration of the environment and the abrupt decrease in photosynthesis, restored in a few weeks

time almost identical carbohydrate-nitrogen ratio in the plants under the various tretments. The mechanism by which the plant restored the carbohydrate-nitrogen ratio is simply that the nitrogen fixation is dependent on the constant flow of carbohydrates to the nodule. Thus with the employed treatments and the abrupt decrease of photosynthesis the nodules were deprived of carbohydrates and the fixation of nitrogen was slowed down or even stopped for a certain time. In view of this explanation the employed treatments failed to create an excess of nitrogen in the treated peas and to this failure must be sought the non-occurrence of substantial excretion. With experiments however similar to that described but with more moderate treatments (slight clipping of the leaves, a few hours curtailement of daylight), it is probable that a decrease of photosynthesis without severe interference in the nitrogen fixation might be achieved, leading to an excess of nitrogen fixed and occurrence of excretion.

Let us comment now the experiment III with the addition of combined nitrogen at the medium. The results of the expt. V described in the first part of the thesis, where the sand employed was richer in available nitrogen than the sand employed in previous experiments, suggested that a medium with small quantities of nitrogen was probably a more convenient medium for the occurrence of excretion than a sand practically free from nitrogen. Inspection of the literature from this point revealed that the attention of some investigators was already drawn to this point but the results obtained as regards the excretion were contradictory. Thus

while Thornton and Nicol (1934), by adding combined nitrogen to sand cultures reported strong excretion, Ludwig and Allison (1940), on the opposite reported negative results. Wyss and Wilson (1941) reviewing experiments on excretion stated that there " is definite evidence that excretion does not occur even under specialised green house environments when any approach to normal field conditions obtain, e.g., presence of combined nitrogen or poor aeration".

Let us examine in detail the experiment by Thornton and Nicol mentioned above. Adding 55 or 163 mg. sodium nitrate all at ounce at the sowing to sand cultures containing 20 lucerne plants or 20 lucerne plus 20 rye grass plants per pot, they studied the effect of the combined nitrogen on the growth and nitrogen content of the mixture of these two plants. The data were presented in graphs and the following table gives approximate figures from the results 4 months after the commencement of the experiment.

Data from Thornton and Nicol (1934).

		Pots with lucerne aln. mg. nitrogen added per pot		Pots with lucerne and rye grass. mg. nitrogen added per pot	
Nitrogen content of lucerne, mg. per pot.		55	163	55	163
	Tops	760	820	660	550
	Roots	980	960	650	340
	Total	1740	1780	1310	890
N. content of rye grass, mg/pot		-	-	300	390

It is unfortunate that the experiment did not contain pots without addition of nitrogen, nor pots with rye grass alone, and due to this incompleteness the results regarding the excretion were accepted with strong reservations by Trumble

and Strong (1937), but since Thornton and Nicol in another paper (1934b), in an experiment carried out under the same environment with similar pots and presumably similar sand, gave the total nitrogen of rye grass alone and in mixed cultures with lucerne, at the end of the fourth month, as 17 and 57 mg. respectively, we can assume that more or less similar figures would have been obtained had the experiment contained pots without added nitrogen and also pots with grass alone. Thus while rye grass grown in mixed cultures with lucerne in sand without added nitrogen gave a slight increase in nitrogen, rye grass grown in mixed cultures in sand with added nitrogen gave after 4 months growth a content of nitrogen 2 to 6 times more than the added nitrogen. Since the authors reported that no decay of the lucerne roots was apparent and since in plants harvested in a younger stage the nitrogen content of the rye grass exceeded considerably the added nitrogen, it appears that in this experiment excretion amounted to hundreds of mg. nitrogen occurred.

Whether now as the cause of this considerable excretion we will accept the suggestion given by Thornton and Nicol according to which "small doses of nitrate, by encouraging the early root development of the grass, increased its capacity for absorbing nitrogen compounds from the lucerne", or whether we will seek the explanation in the complex interaction of many factors (due to early and strong development of the grass and the consequent alteration of the whole environment), is a problem for further investigation. The present writer gives emphasis particularly to the strong

competition that followed the promoted growth of the grass since the figures for the decreased content in nitrogen in lucerne, especially for the content in roots, are very remarkable. The writer is inclined to believe that Thornton and Nicol in their experiment produced conditions for the growth of lucerne close to conditions existing in mixed cultures in nature, where as a rule the growth of the legume is depressed by the growth of the grass or cereal, and that these conditions of growth and competition are probably essential for the occurrence of excretion.

Since experiments with mixed cultures in sand poor in nitrogen, apart from Helsinki, have practically failed to reveal excretion, further investigation must be sought with media giving closer approximation to conditions for the growth to those existing in nature and the employment of sand with added nitrogen appears as promising. Addition of combined nitrogen to the medium has of course as consequence an inconvenience in the proper arrangement of the control pots, as was already pointed out in the first part of the thesis, but in spite of this handicap if results similar to those obtained by Thornton and Nicol could be produced, the inaccuracy of the measurement of the excretion will be a minor trouble since the most important point at issue is the solution of the controversial problem of whether excretion is a common and important phenomenon in nature or not.

The experiment carried out by the writer with peas and oats has many similarities with the experiment of Thornton and Nicol but at the same time it also differs widely. Thus

apart from the difference in the plants employed, ammonium nitrate was employed instead of sodium nitrate, and the application was done in two doses instead of in one. Whether these differences had any special significance is not known. The experiment failed to give any evidence of excretion and this failure might be connected with the failure of the oats to decrease considerably the growth of peas even in the pots treated with the 110 mg of nitrogen. (The decrease in dry matter in peas with the addition of 55 mg nitrogen per pot was not significant statistically, while the decrease with 110 mg. nitrogen added was significant statistically but no larger than 18% of the dry matter of peas without added N.).

The negative results reported by Ludwig and Allison can be explained in a similar way since these authors with the addition of 10 to 30 mg. nitrogen per pot induced actually an increase in the growth of the legume.

In spite of the negative results obtained by the present experiment the writer is of the opinion that the experiment of Thornton and Nicol with the striking results that it produced, is worthy of further consideration and of repetition of the experiment with the same plants and same quantities of nitrogen plus the inclusion of pots without added nitrogen and pots with rye grass alone. With such experiments if comparable results with those of Thornton and Nicol could be obtained, an important step to the answer of the controversial subject as to the existence and the significance of excretion will be achieved.

Consideration now will be given to the effect of clipping on the number of nodules; at the close of the expt. the clipped peas had a considerably larger number of nodules than the non-clipped, while the size of the nodules was smaller, and the nodules were scattered all over the roots giving the appearance of roots infected with inefficient strain. It must be recalled that the clipping took place when the peas were 8 weeks old and they probably had formed by this time the maximum number of nodules, which is limited by the mass of the root system according to the investigation by Chen (1941). When exactly the extra nodulation occurred and why such abundant nodulation followed the operation of the clipping it is not known. A part of the extra nodulation probably occurred following the development of new roots; (as was already mentioned the clipped peas responded with the development of new shoots and it is probable that the growth of these shoots was accompanied by the growth of new roots).

Professor Virtanen in a lecture in University College, London, in December 1946, reported that small quantities of b-alanine (2 mg. per litre of culture solution) inhibit the uptake of aspartic acid by the legumes and even smaller amounts exert a definite restraining effect. Excretion, he suggested, can be detected only when b-alanine is present in the rooting medium, since, otherwise the legume itself reabsorbs the excreted aspartic acid.

According to this statement addition of small quantities of b-alanine to the medium in which inoculated legumes are

growing might have as result the accumulation of the excreted nitrogenous substances allowing the detection of excretion. The proper test of this hypothesis needs a sterile medium lacking micro-organisms able to decompose the added b-alanine. Although open-sand cultures with the micro-organisms that they contain are not a very convenient medium, nevertheless a few pots in the combined experiment I and II were devoted to this test, by adding to them, with the nutrient solution, a small quantity of b-alanine. Since the added quantity of b-alanine was larger than the reported necessary quantity for inhibiting reabsorption of the aspartic acid, and since the addition of b-alanine was repeated every 3 days, it can be argued that in spite of the probable disintegration of it by micro-organisms, the solution of the medium always contained a small quantity of b-alanine and the inhibiting effect of the added b-alanine to the reabsorption of the excreted nitrogenous substances ought to be anticipated.

When the plants of the combined experiment I and II were 9 weeks old, the addition of small quantities of b-alanine was commenced in the case of two pots of mixed cultures of each type (peas plus barley, peas plus soya bean, peas plus lupin). Each of these pots received, by the end of the experiment, a total of about 12 mg. of b-alanine. Since both the detector plant and the sandfailed to show any extra nitrogen, the conclusion was that the phenomenon of excretion per se was absent in the experiment.

S U M M A R Y.

(1) Experiments on excretion of nitrogenous substances from nodulated pea plants grown in sand culture were carried out with barley, oat, soya bean and lupin as detector plants. Analysis for total nitrogen of the rooting medium for the detection of excretion was also employed.

(2) The effect of an abrupt reduction of photosynthesis in vigorously-growing peas, by means of shortening the hours of daylight, or by severe clipping, was studied, in connection with the possibility of induction of excretion under altered growth conditions.

(3) By frequent addition of small quantities of b-alanine into the medium, it was sought to prevent reabsorption of any excreted nitrogenous substance in a late stage by the peas.

(4) By adding combined nitrogen and altering the growth of the plants in mixed cultures the possibility of excretion was studied.

(5) There was no evidence of appreciable excretion by examination of the detector plant or by analysis of the medium, either under normal conditions or as result of the various treatments listed above.

(6) Both the legumes employed for the detection of excretion were proved unsuitable plants since, the lupin died early, and the soya bean suffered from shading in mixed cultures.

(7) In view of the results obtained the suitability of the various methods employed for the induction of excretion

is discussed and the conclusion is reached that in spite of the failure of the present experiments to induce excretion, the alterations employed *per se* appear promising tools for the study of excretion, and special significance is given to the method of adding combined nitrogen to the medium since, it is in this way possible to reproduce conditions for the growth of the legume and detector plant more like to the existing growth conditions for mixed cultures in Nature.

Section III.

Nitrogen nutrition of non-nodulated clover plants.

I N T R O D U C T I O N .

Although, for practical purposes, the answer to the problem of which is the best form of nitrogenous fertilisers for the growth of higher plants has been given long ago, and in general it is considered that the best forms are inorganic salts of nitrates and ammonium, and further although it is admitted that under field conditions any form of added organic nitrogen is eventually available to the higher plants through the action of soil micro-organisms in the above forms of nitrate and ammonium salts, nevertheless from the theoretical point of view it is interesting to know whether higher plants apart from inorganic nitrogen are also able directly to utilise organic nitrogenous compounds such as amino-acids and amides.

As soon as the humus theory of nutrition had been contradicted and the theory of mineral nutrition of plants had been established by the growing of plants in water cultures with the addition of mineral salts only, a tendency arose for the possibility that organic nitrogenous compounds could be directly absorbed by the plants to be overlooked.

Actually, by the early part of the present century, experimental evidence, suggestive of the use of organic nitrogenous compounds, existed. The exact value of most of these earlier experiments is however doubtful since, as Miller (1938) has pointed out, in many cases the plants were not grown under conditions that would prevent bacterial action. The investigation of the direct absorption and utilisation of organic nitrogenous compounds by higher plants needs

the employment of cultures under sterile conditions, and since the appearance in 1912 of a paper by Hutchinson and Miller, employing sterile cultures, a considerable amount of reliable data has been accumulated as a result of the work of a number of investigators. The results proved definitely that some of the green plants tested were able to absorb directly various organic nitrogenous compounds and to utilise these substances as a source of nitrogen, and at the same time that there existed great differences among various plants in respect of the extent of this ability. As regards the relative nutrient value of the organic nitrogenous compounds investigated in comparison with the inorganic salts of nitrate and ammonium, the results were entirely contradictory. For instance: Hutchinson and Miller (1912) growing peas in sterile culture solutions found that peas could assimilate a number of organic nitrogenous substances, among which were urea, acetamide, glycine, sodium aspartate and peptone, and also that the growth was better in some organic nitrogenous compounds as urea and acetamide than in ammonium sulphate. Tanaka (1951) working with sterile agar and water cultures and employing Sisyringium (Iridaceae), and Plantago as test plants, found that urea and asparagine were equally good sources of nitrogen for Sisyringium as was calcium nitrate, that Plantago absorbed and utilised urea (although to a less extent than calcium nitrate) but could not use asparagine, and that both Sisyringium and Plantagoassimilated to a small extent acetamide.

Virtanen and v. Hausen (1931), employing sterile water cultures concluded that even plants belonging to two species of the same genus might differ as regards the ability to utilise organic nitrogen. Thus while red clover gave the best growth with hydrolysed casein and aspartic acid, white clover, although it absorbed and utilised to some extent these organic substances, grew far better in inorganic nitrogen.

Knudson (1933), employing orchid embryos as plant material and glycine, leucine and aspartic acid as organic nitrogenous substances, reported that the orchids failed to utilise any of these compounds. Macht (1934), measuring the growth of young seedlings of Lupinus albus, growing in full Shive's solution with or without addition of small quantities of amino-acids, reported that the same amino-acids in different concentrations had a diametrically opposite effect on the growth, (from inhibiting to stimulative) and that in general the laevo-isomers of amino-acids exerted a greater physiological activity on the growth of the plants than the dextro-isomers. Virtanen et al (1933), growing legumes and cereals in pots with sterile quartz sand, showed that there was a fundamental difference between legumes and cereals as regards the ability to absorb and utilise aspartic acid. Thus while red clover and peas grew well in a medium with aspartic acid, wheat and barley failed entirely to use it. There was also a difference between red clover and peas, since aspartic acid proved a better source of nitrogen for red clover than for peas, the growth of red clover in cultures with aspartic acid

being almost double the growth in cultures with potassium nitrate, and the growth of peas being equal in both organic and inorganic media.

Virtanen (1938, 1947), from the results of experiments on excretion, suggested that b-alanine, probably deriving from decarboxylation of aspartic acid through the action of nodule bacteria, might be absorbed and utilised by barley but not by peas and further that the presence of small quantities of b-alanine in the medium inhibited the absorption of aspartic acid by the peas. Virtanen (1947) suggested that oats differ from wheat and barley since they can utilise aspartic and glutamic acid.

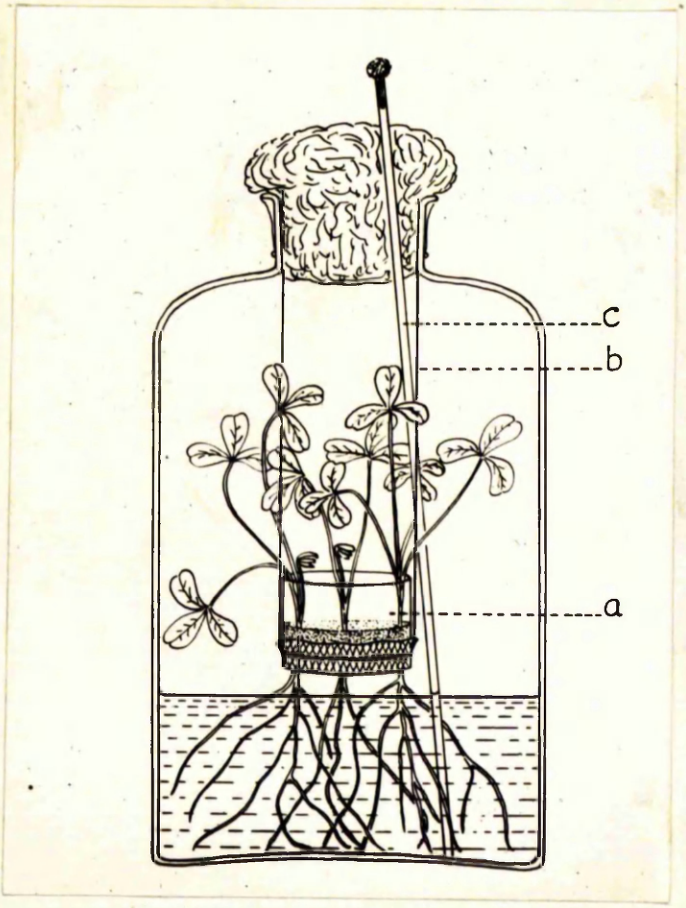
Virtanen and Linkola (1946), growing peas in sterile water cultures containing aspartic acid and inorganic nitrogen together, reported that aspartic acid competed succesfully with nitrates and ammonium salts for nitrogen nutrition of non-inoculated peas.

In view of the interest of the finding of Virtanen and his collaborators that some non-nodulated legumes can make extensive utilisation of organic nitrogen, the writer considered it worthwhile (1) to attempt to confirm these findings, (2) to investigate further the difference in the ability to use organic nitrogen which Virtanen and v. Hausen reported between red and white clover, (3) to test whether the hypothesis that b-alanine in small quantities in a medium containing aspartic acid impairs the absorption and utilisation of aspartic acid by the legume, may be extended to other legumes than peas.

M E T H O D S.

In preliminary experiments the technique of Virtanen and von Hausen (1931), of aseptic water cultures was employed (Fig. 23). Two and a half litre, wide mouthed bottles, filled to one third with nutrient solution, were employed. The basal nutrient solution was Hiltner's free from nitrogen, and four sources of nitrogen, ammonium sulphate, potassium nitrate, asparagine and l-aspartic acid in the quantity 170 mg. N. per litre were tested. A glss tube 2" in diam. x 2" high with one end closed with mosquito net cloth and with a few gm. sand at the bottom, was suspended by thread ½" above the surface of the nutrient solution. pH was adjusted initially to 6.5. Any alteration of the pH of the solution in the direction of greater acidity was followed by observing any change in the colour of the culture solution to which was added a few cc. bromocresol purple. Any necessary adjustment of pH was carried out by adding N/10 sodium hydroxide through a glass tube which projected through the cotton plug of the mouth of the bottle. After the sterilisation of the bottle and medium, surface-sterile red clover seeds were transferred aceptically to the sand of the glass tube; within a few days the roots made contact with the solution while the stems were grown, supported by the glass tube, in the empty space of the bottle. This arrangement supplied the plants with satisfactory conditions for growth but unfortunately it proved inadequate to keep the solution sterile for a long time, the sterile cultures at the close of the experiment being a small

F i g u r e 23.



The photograph shows the applied technique of Virtanen and von Hausen for aseptic water cultures.
(a) small pot with few gr. sand,
(b) thread
(c) side tube for readjustment of the pH.

The writer is indebted to Dr. Bond for the above drawing.

percentage of the initial number of cultures. After two trials, this method was abandoned and instead the experimentation was continued with the following sterile agar culture method.

For the purpose of agar culture 1" by 6" and 1½" by 8" test tubes were employed with 16 and 37 cc. nutrient medium respectively, the basal nutrient medium beig the following used by Chen and Thornton (1940):

K_2SO_4.	0.9 g.	$FeCl_3$	0.02 g.
K_2HPO_4.	0.5 g.	Boric acid.	0.02 g.
$CaH_4(PO_4)_2.2H_2O$.	0.5 g.	$MnSO_4$.	0.02 g.
$MgSO_4.7H_2O$.	0.5 g.	Agar.	15.0 g.
NaCl.	0.5 g.	Glass distilled water.	1 litre

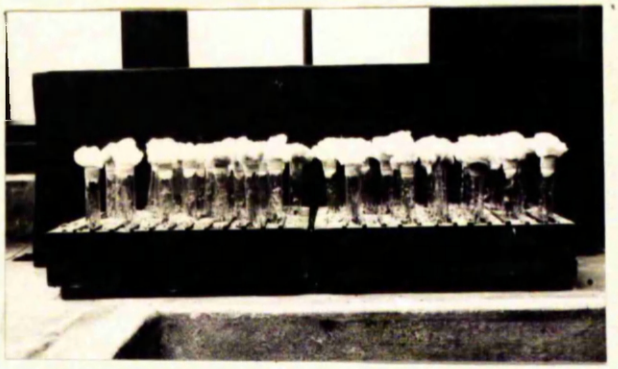
The above basal nutrient medium was employed for all the treatments except for the tubes receiving KNO_3, in which case the K_2SO_4 was substituted with equimolecular quantity of Na_2SO_4. The nitrogenous substances employed were potassium nitrate, ammonium nitrate, ammonium sulphate, asparagine, aspartic acid, and aspartic acid plus small quantities of b-alanine. The amounts of nitrogen supplied per litre were similar to those used by Von Hausen (1936), viz. 127.5, 170, and 255 mg. nitrogen per litre of medium. To the tubes recorded as control, no nitrogen was added. The tubes of agar were sterilised by autoclaving for 20 mins. at 15 lb. pressure, or by steaming for 30 mins. for three successive days. Before the sterilisation, appropriate amounts of N/10 or N/100 sodium hydroxide or sulphuric acid were added to the media,

to make the pH about 6.5 (in most tubes) or 4.8 at the end of sterilization. This was obtained by carrying out preliminary trials with spare medium. Since the agar tube culture method was not convenient for readjustment of the pH, the alterations were only followed with the change of the colour of the medium in some tubes to which bromocresol purple indicator was added, or with determination of the pH in some tubes at intervals.

Undamaged seeds of red clover (permanent pasture Montgomery), and white clover (New Zealand), selected with the help of a 10 x 1 magnifying lens, were surface sterilised by immersion for 3 mins. in absolute alcohol, for another 4 mins. in 0.1% $HgCl_2$, and by six subsequent washings with sterile water. An approximately equal number of seeds was planted in each tube with a flamed platinum loop. In experiment II a uniform number of plants was secured in each tube by thinning. In the other experiments in order to avoid contamination, the thinning was omitted and instead tubes with an extreme number of plants were discarded. Soon after germination the tubes were transferred to the green-house supported in shallow boxes with sawdust, or in blocks of wood drilled with suitable holes of such depth as to keep the root system shaded. (Fig. 24, 25.).

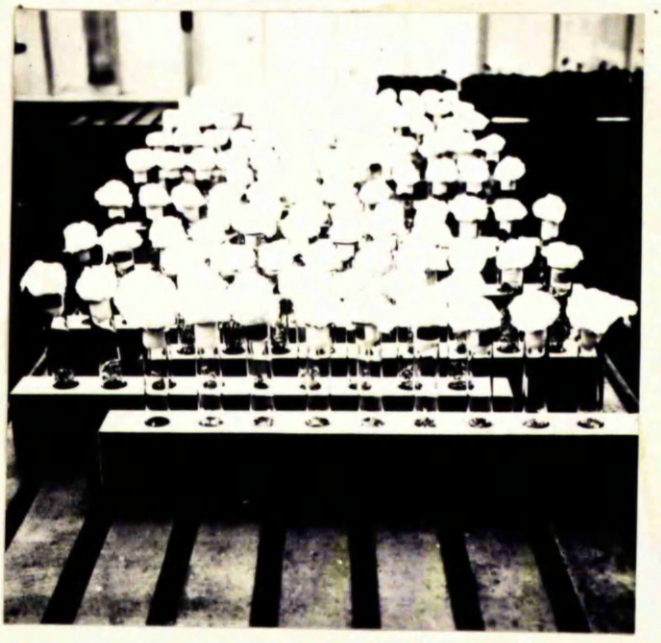
Records of the appearance of the plants were kept during the growth. Any obviously contaminated tube was immediately discarded. At the conclusion of the experiment the plants were carefully separated from the medium by washing

F i g u r e 24.



Sterile agar tube cultures supported in boxes with saw-dust.

F i g u r e 25.



Sterile agar tube cultures supported in blocks of wood.

of the roots with water, then were dried at 97° C. and were analysed for total nitrogen with the Kjeldahl method modified by Ranker (1925, 1926).

Using the Nessler analysis the media were examined for the presence of ammonium and, in some cases, the sterility of the medium was tested by deep and surface cultures. The final pH of the medium was also determined either colorimetrically or electrometrically, and the measurement was carried out with the extract prepared by leaving the chopped agar medium from the tubes in two and half times its volume of distilled water, for an hour.

D A T A.

Experiment I. Forty test tubes 1½" by 8" were one third filled with 37 cc. basal nutrient medium containing 127.5, 170 and 255 mg. nitrogen per litre, of one of the following nitrogenous substances: potassium nitrate, ammonium sulphate, aspartic acid and asparagine and thus to each tube were added respectively 4.7, 6.3, and 9.4 mg. nitrogen. Another seven tubes received the same basal nutrient medium without any addition of nitrogen and served as controls. The pH of the medium was adjusted in all treatments to about 6.5 and all tubes were sown with approximately twenty-two surface-sterile seeds of Red Clover.

Table I gives the analytical results. Due to the lateness of the season and to some degree of overcrowding, growth was rather poor and differences between the control plants (no nitrogen) and those receiving nitrogen are small in terms of dry weight. The results therefore are regarded as being of a preliminary nature. At any rate, judging from the dry weights it appears that the best growth was obtained with the addition of organic nitrogen - particularly aspartic acid -, while the clovers treated with the addition of inorganic nitrogen failed to exceed the control plants in growth. (See fig. 26, 27 - by oversight no control included). Of the three concentrations of nitrogen it would seem that the greatest concentration, (255 mg. nitrogen per litre), had a slightly depressing effect on the dry matter, the effect being stronger and significant statistically in the tubes treated with ammonium sulphate, but

Table I.

Data of experiment I, 16th August - 1st December 1946. Tubes 8 by 1½" containing 37 cc. agar medium, pH initially 6.5 in all media. Test plant Red clover. Number of plants per tube 12-18. The data are per tube.

Form of N.	Treatment.	No of tubes harvested.	Mean Dry wt. of plants. mg.-tube.	Mean N. content of plants, mg. per tube.	Nitrogen per cent.
KNO_3	127.5 mg.N. per l.	2	67.0	2.62	3.9
"	170.0 " "	5	60.4	2.00	3.1
"	255.0 " "	2	67.5	2.80	4.2
$(NH_4)_2SO_4$	127.5 " "	2	65.5	2.76	4.2
"	170.0 " "	5	69.8	3.09	4.4
"	255.0 " "	2	43.0	2.04	4.7
Asparagine	127.5 " "	2	75.5	4.15	5.5
"	170.0 " "	4	77.5	4.50	6.0
"	255.0 " "	1	70.0	4.67	6.7
Aspartic acid	127.5 " "	2	85.5	3.91	4.6
" "	170.0 " "	4	81.7	3.47	4.2
" "	255.0 " "	1	76.0	3.70	4.8
Control	No added N.	4	68.2	1.67	2.4

Note: Standard error of a single tube, regarding dry wt. 8.4m
" " " " " " " N. 0.48 mg.

	Replicates.	For dry wt.mg. Signif. to point. 0.05	0.01	For N. cont.(mg) Signif.to point 0.05	0.01
Minimum difference required between the means of the yields of treatmts in order the diff. to be regarded sign. statistically.	5&5	11.1	15.0	0.63	0.85
	5&4	11.7	15.9	0.67	0.90
	4&4	12.4	16.8	0.70	0.95
	5&2	14.6	19.7	0.83	1.12
	4&2	15.1	20.4	0.86	1.17

FIGURE 26.

Experiment I. Red clover growing in organic nitrogen. Effect of various concentrations. Left to right: Aspartic acid 255, 170, 127.5 mg. N. per litre, Asparagine 255, 170, 127.5 mg. N. per litre.

FIGURE 27.

Experiment I. Red clover growing in inorganic nitrogen.in various concentrations. Left to right: Potassium Nitrate 255, 170, 127.5 mg. N. per litre, Ammonium Sulphate 255, 170, 127.5 mg. N. per litre.

since the replicates for the extreme concentrations were very few, the results must be taken with reservations.

The figures for the total nitrogen of the plants are more interesting, since the absolute amount of absorbed nitrogen was considerably larger in the plants treated with added organic nitrogen than with inorganic, and the column which gives the percentage of nitrogen shows that the plants growing in medium with asparagine contained a larger percentage of nitrogen than plants in the other media.

EXPERIMENT II. 180 test tubes 1" in diameter by 6", were used with 16 cc nutrient medium containing 2.7 or 4.1 mg. nitrogen (170 or 255 mg. nitrogen per litre). Three forms of inorganic nitrogen: ammonium nitrate, ammonium sulphate, and potassium nitrate, as well as aspartic acid, asparagine, and aspartic acid plus small quantities of b-alanine (1.2 to 12 mg. b-alanine per litre of medium), were tested with Red and White clover. The pH of the medium was adjusted to about 6.5 and this time with careful thinning soon after the germination, five to six plants were left in each tube. By thinning, a more uniform growth was obtained, the only drawback being that a considerable number of tubes became contaminated and subsequently to be discarded.

Four weeks after the germination there were marked differences in the appearance of both clovers, in connection with the form of nitrogen supplied, the growth of both clovers being superior in the tubes with inorganic nitrogen, while the plants supplied with organic nitrogen were smaller with

yellow or even white leaves. At this time the plants with the best apparent growth were those supplied with ammonium nitrate. With the progress of time, the plants in organic nitrogen recovered and aquired a greener colour although all through the experiment they appeared smaller than the plants in inorganic nitrogen.

The alterations of the pH in the media were followed by the change of the colour of bromocresol purple which had been added initially to a few of the tubes. The first tubes to show acid pH were the tubes with ammonium nitrate and ammonium sulphate, the change in the tubes with ammonium nitrate being even more rapid than the change in the tubes with ammonium sulphate.

It is interesting to give some details about the appearance of the roots in the different media. The roots of both clovers in the medium with aspartic acid were very small stunted, and red in colour with an obvious tendency to avoid the medium and grow between the tube and the medium, suggesting that the aspartic acid had some toxic effect on them, while the roots in asparagine altough comparatively much longer, were still redish and shorter than the roots of the plants receiving inorganic nitrogen. The longest roots were seen in the tubes with ammonium nitrate and in the control tubes.

Tables IIa and IIb give the analytical results. It can be seen from the tables that the two clovers responded almost identically to various treatments. (See fig. 28, 29). In both clovers the best growth, on the dry weight basis, was obtained

Table IIa.

Data of experiment II for Red Clover. Growth period 6th July - 30 September, 1947. Tubes 6" by 1" containing 16 cc agar medium, pH initially 6.5 in all tubes. Two concentrations of added nitrogen into the medium indicated as low and high; low signifies that 170 mg N. was originally added per litreof medium (or 2.7 mg. per tube), high 255 (or 4.1 mg. per tube). 5-6 plants per tube.

Treatment.		No.of tubes harvested.	Mean Dry wt., mg. per tube.	Mean Nitrogen content mg.per tube.	Final pH of medium.
NH_4NO_3.	Low	4	53.6	1.97	4.3 - 4.7
"	High	6	52.9	2.25	" - "
KNO_3.	Low	5	47.7	1.34	6.1 - 6.5
"	High	4	48.2	1.52	6.2 - 6.3
$(NH_4)_2SO_4$.	Low	6	44.5	1.51	4.2 - 4.3
"	High	4	42.8	1.51	4.0
Asparagine.	Low	4	48.8	2.29	6.2
"	High	3	43.6	2.25	6.4 - 6.7
Aspartic acid	Low	4	37.9	1.25	6.3 - 6.8
" "	High	2	30.8	1.33	" - "
Aspartic* acid	Low	5	42.2	1.33	6.2 - 6.4
" "	High	5	32.7	1.22	" - "
Control,no added N		6	34.8	0.59	5.6

Replicates.	Minimum Difference Required For dry weight. Significant to point. 0.05	0.01	For nitrogen content. Significant to point 0.05	0.01
6 & 6	6.4	8.7	0.23	0.31
6 & 5	6.7	9.1	0.24	0.33
5 & 5	7.1	9.6	0.25	0.34
6 & 4	7.2	9.7	0.26	0.35
5 & 4	7.4	10.0	0.27	0.36
4 & 4	8.1	10.9	0.28	0.38
4 & 3	8.5	11.5	0.31	0.41
4 & 2	9.6	13.0	0.35	0.47
3 & 2	10.2	13.7	0.37	0.49

* Plus b-alanine. See text.

Table IIb.

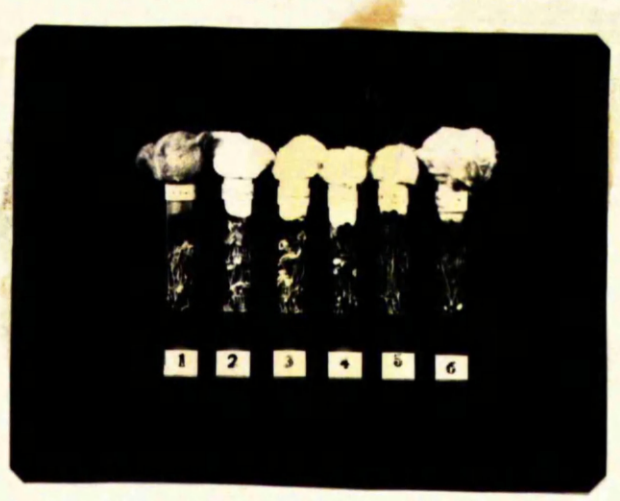
Data of experiment II for White Clover. Growth period 6th July - 30 September, 1947. Tubes 6" by 1" containing 16 cc agar medium, pH initially 6.5 in all tubes. Two concentrations of added nitrogen into the medium indicated as low and high; low signifies that 170 mg. N. was originally added per litre of medium (or 2.7 mg. per tube), high 255 (or 4.1 mg. per tube). 5-6 plants per tube.

Treatment.		No. of tubes harvest.	Mean Dry wt., mg. per tube.	Mean Nitrogen content mg. per tube.	Final pH of medium.
NH_4NO_3.	Low	4	59.4	1.57	4.3 - 4.7
"	High	7	37.9	1.75	" - "
KNO_3.	Low	4	56.6	1.11	6.1 - 6.5
"	High	4	35.9	1.15	6.2 - 6.3
$(NH_4)_2SO_4$.	Low	5	55.2	1.22	4.2 - 4.3
"	High	6	54.1	1.28	4.0
Asparagine.	Low	4	50.5	1.73	6.2
"	High	5	29.9	1.78	6.4 - 6.7
Aspartic acid.	Low	4	57.0	1.07	6.3 - 6.8
" "	High	1	18.0	0.63	" - "
Aspartic acid.*	Low	3	29.8	0.88	6.2 - 6.4
" "	High	2	25.8	0.96	" - "
Control, no added N.		6	18.2	0.19	5.6

Replicates	Minimum Difference Required For dry weight. Significant to point. 0.05	0.01	For nitrogen content. Significant to point 0.05	0.01
7 & 6	5.9	8.0	0.27	0.37
7 & 5	6.2	8.4	0.29	0.39
6 & 5	6.4	8.6	0.30	0.41
7 & 4	6.7	9.0	0.31	0.42
6 & 4	6.9	9.3	0.32	0.43
5 & 4	7.1	9.6	0.33	0.44
4 & 4	7.4	10.0	0.35	0.47
4 & 3	8.0	11.0	0.37	0.51
4 & 2	9.2	12.4	0.43	0.56
3 & 2	9.7	13.1	0.45	0.61

* Plus b-alanine. See text.

F i g u r e 28.



Experiment II. Red clover growing in different media. In all media but the control 170 mg N. added per litre.
1 control, 2 aspartic acid plus b-alanine, 3 aspartic acid, 4 asparagine, 5 ammonium nitrate, 6 potassium nitrate.

F i g u r e 29.

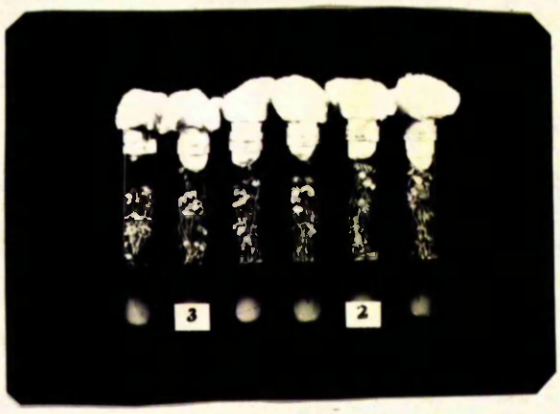
Experiment II, White clover (170 mg N. added per litre). A control, B aspartic acid plus b-alanine, C aspartic acid, D asparagine, E ammonium nitrate, F potassium nitrate.

with ammonium nitrate. In the case of red clover, the growth in asparagine (low concentration) was close to that of ammonium nitrate, while the growth in aspartic acid was smaller than that obtained with any other treatment at all. From the table IIa it can be seen that the dry weight in tubes with aspartic acid was inferior to that in tubes with ammonium nitrate or asparagine beyond the error of the experiment. As regards the white clover -table IIb- the growth on the whole was better in the three inorganic nitrogenous compounds than in the organic ones but the differences expressed on the basis of the dry weight were rather small. It is interesting to note that in the case of the white clover and on the basis of dry weight aspartic acid was proved an equally good, if not better, nutrient than asparagine.

The growth of the two clovers in aspartic acid and aspartic acid plus small quantities of b-alanine, was similar and no impairment of the growth due to the addition of b-alanine was detected. (See also fig. 50, 51). As regards the two tested concentrations of nitrogen it appears as though the higher one (255 mg. N. per litre)had some depressing effect on the dry matter in the case of the organic nitrogenous compounds. Finally, in this experiment again plants of both clovers growing in asparagine absorbed the largest amount of nitrogen and the percentage of nitrogen in these plants was considerably higher than that in any other treatment.

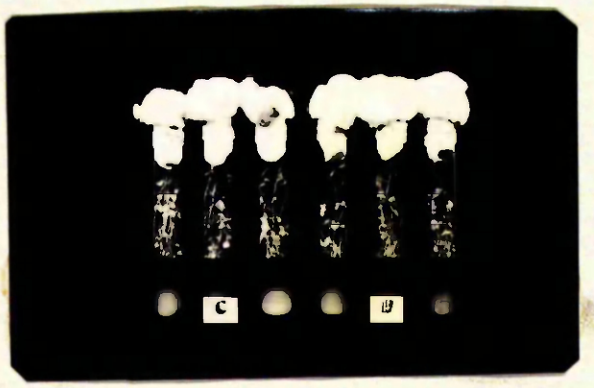
EXPERIMENT III. In order to confirm the findings of the second experiment with the use of larger test tubes and to investigate further the availability of inorganic nitrogen in

F i g u r e 30.



Experiment II, Red clover in aspartic acid with or without addition of b-alanine.
3 without addition of b-alanine, 2 with addition of b-alanine.

F i g u r e 31.



Experiment II, White clover in aspartic acid with or without addition of b-alanine.
C without addition of b-alanine,
B with addition of b-alanine.

a more suitable pH than that of 6.5, experiment III was carried out. 144 test tubes 1" by 8" with 37 cc. basal nutrient medium were employed, the concentration of added nitrogen being 170 mg. per litre (6.3 mg. N. per tube). As sources of nitrogen, ammonium nitrate, ammonium sulphate, potassium nitrate, aspartic acid and asparagine, were tested. Red and White clover were once more used. The pH was again adjusted to about 6.5 for all the media except for a second lot of tubes with potassium nitrate where the pH was adjusted to 4.8. At this low pH the medium in a few tubes was semi-liquid so that the growing plants sank and these tubes were eventually discarded. A second lot of tubes with ammonium sulphate was also employed, this time with the addition of small quantities of calcium carbonate corresponding to 0.5 gm per litre of medium. Ten to eleven surface sterile clover seeds were planted in each tube and instead of thinning, relative uniformity was obtained by discarding at a later stage, all the tubes with an extreme number of plants and harvesting only the tubes with medium numbers (4-7 plants for tubes with red clover and 6-9 for white).

The plants were kept in a new green house and since the conditions for light were very good the growth was rapid. When the plants were 32 days old there were marked differences in their appearance in the various media, the growth of both clovers being better in inorganic than in organic nitrogen. The growth of red clover in all the inorganic media was particularly good with a slight suspicion of superiority in the medium with ammonium nitrate, while the plants in the organic

media had stunted brownish roots and small shoots with yellow leaves. The appearance of the plants, especially in aspartic acid, was so poor that even the control plants appeared superior to them. As regards the growth in the medium with ammonium sulphate, with or without calcium carbonate, it appeared that the plants of red clover at this stage were slightly better in the medium without calcium carbonate. The differences in the growth of white clover were less intence and the plants in asparagine, although they had a few yellow leaves, had rather good roots and tall shoots.

A fortnight later the red clover in asparagine had large leaves and the appearance of the plants showed marks of recovery although there were still some yellow leaves. The plants in aspartic acid with pale yellow to white leaves had the smallest growth of all the plants supplied with nitrogen. The plants growing in inorganic nitrogen were the best and at this stage there were no distinct differences in the growth in the various inorganic forms or the otherwise different treatments. As regards the white clover plants, the growth in asparagine was similar if not superior to the growth in inorganic nitrogen, while the growth in aspartic acid, although still behind that in asparagine or inorganic nitrogen, showed that the plants were assimilating nitrogen. At this stage white clover in potassium nitrate with pH adjusted at the beginning to 4.8 appeared better in growth than the corresponding plants at pH 6.5.

With the progress of time further changes in the

appearance of the growth were noticed, the most interesting being that in some of the tubes with red clover and aspartic acid the plants showed a marked recovery while in others the growth was still very poor. (See fig. 34). Regarding root growth, roots in aspartic acid in both clovers were now red in colour and stunted as they were in experiment II. (See fig 39). Tables IIIa and IIIb give the harvest results of the plants at the age of 69 days.

It is unfortunate that due to lack of time the analysis of plants for total nitrogen has been postponed and in the tables only the dry weights of the plants are presented. However, it appears from the results that asparagine was the best source of nitrogen for both clovers while aspartic acid was an equally good source for white clover but a poor source of nitrogen for red clover. The growth of both clovers in inorganic nitrogen was slightly inferior to that in asparagine. (See also fig. 32, 33, 34, 35, 36, 37, and 38). There were no significant differences in growth between the various inorganic salts employed or in the treatments apart from the growth of white clover in potassium nitrate with high and low pH, the growth at pH 4.8 being considerably larger than at pH 6.5. The tables also give details of the alteration of the pH of the medium during the growth as well as at the close of the experiment and comment on this point follows in the discussion.

Table IIIa.

Data of experiment III, for Red Clover. Growth period 4th June - 12 August, 1948. Tubes 8" by 1½" containing 37 cc agar medium with 170 mg nitrogen added per litre, (6.3 mg N. per tube). 4 - 7 plants in each tube.

Treatment.	pH of medium 4/6	19/7	12/8	No. of tubes harvest.	Mean Dry wt., mg. per tube.
NH_4NO_3.	6.5	4.3	4.2	10	125
KNO_3.	4.8	5.2	5.3	5	129
"	6.5	6.6	6.4	7	125
$(NH_4)_2SO_4$.	6.5	4.4	4.0	8	127
$CaCO_3$*	6.5	5.0	4.5	8	134
Asparagine.	6.5	6.3	4.9	8	139
Aspartic acid.	6.5	5.3	6.0	7	88
Control, no added N.	6.5	6.1	5.9	9	61

Replicates.	Minimum difference required in mg. in order the differences in dry weight to be regarded significant statistically: to point 0.05	to point 0.01
10 & 9	12.8	17.1
10 & 8	13.3	17.7
9 & 8	13.6	18.2
8 & 8	13.9	18.6
9 & 7	14.2	18.9
7 & 8	14.4	19.2
8 & 5	15.9	21.2
7 & 5	16.3	21.7

* 0.5 gram. $CaCO_3$ per litre of medium.

Table IIIb.

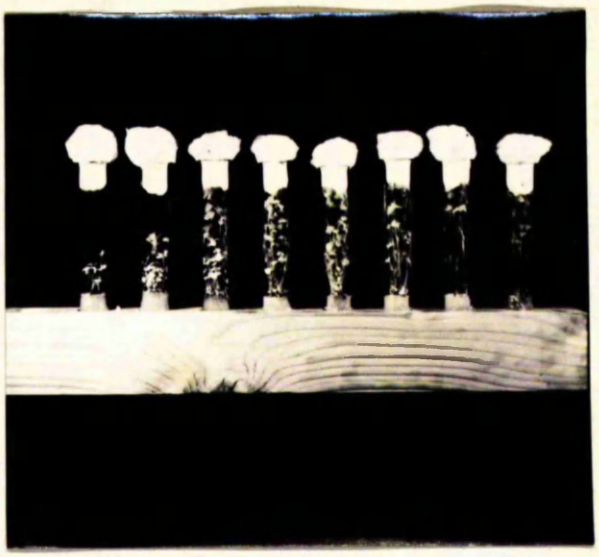
Data of experiment III. for White Clover. Growth period 4th June - 12 August, 1948. Tubes 8" by 1½" containing 37 cc agar medium with 170 mg nitrogen added per litre, (6.3 mg N. per tube). 6 - 9 plants in each tube.

Treatment.	pH of medium 4/6	10/7	12/8	No. of tubes harvested.	Mean Dry wt., mg. per tube.
NH_4NO_3.	6.5	4.3	4.0	8	114
KNO_3.	4.8	5.6	6.4	6	116
"	6.5	6.8	6.7	9	88
$(NH_4)_2SO_4$.	6.5	5.2	3.7	9	113
$(NH_4)_2SO_4$. $CaCO_3$*	6.5	5.6	4.5	8	111
Asparagine.	6.5	-	5.3	9	125
Aspartic acid.	6.5	6.2	6.3	7	125
Control, no added N.	6.5	6.3	6.1	9	35

Replicates.	Minimum difference required in mg. in order the differences in dry weight to be regarded significant statistically: to point 0.05	to point 0.01
9 & 9	9.2	12.2
9 & 8	9.9	13.1
8 & 8	10.1	13.5
9 & 7	10.2	13.6
8 & 7	10.5	14.0
9 & 6	10.7	14.2
8 & 6	11.0	14.7
7 & 6	11.3	14.9

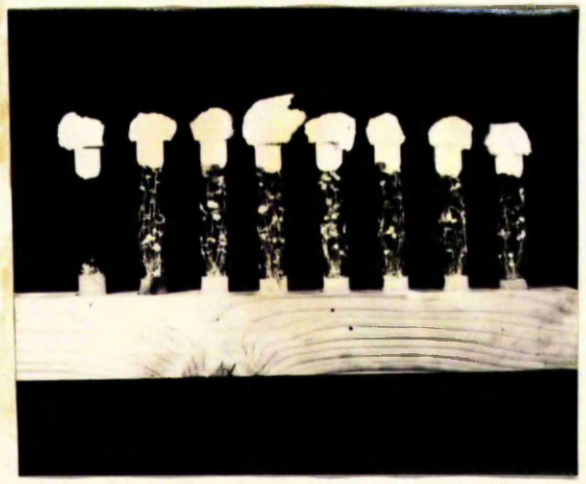
* 0.5 gram. $CaCO_3$ per litre of medium.

F i g u r e 32.



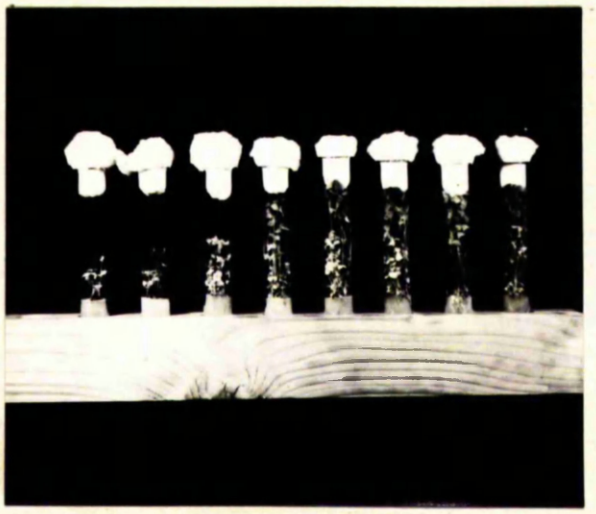
Experiment III. Growth of Red clover in various media. Left to right: Control, aspartic acid, asparagine, potassium nitrate (pH at the beginning 4.8), potassium nitrate (pH at the beginning 6.5), ammonium sulphate, ammonium sulphate plus calcium carbonate, ammonium nitrate.

F i g u r e 33.



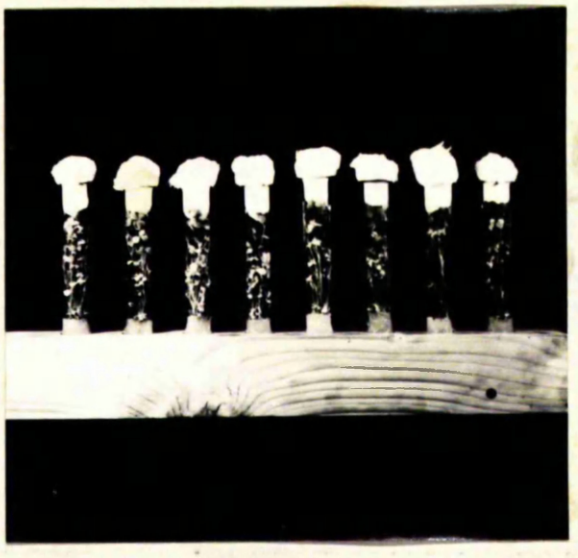
Experiment III. Growth of White clover in various media. Left to right: Control, asp. acid, asparagine, potassium nitrate (pH 4.8), potassium nitrate (pH 6.5), ammonium sulphate, ammonium sulphate plus calcium carbonate, ammonium nitrate.

F i g u r e 34



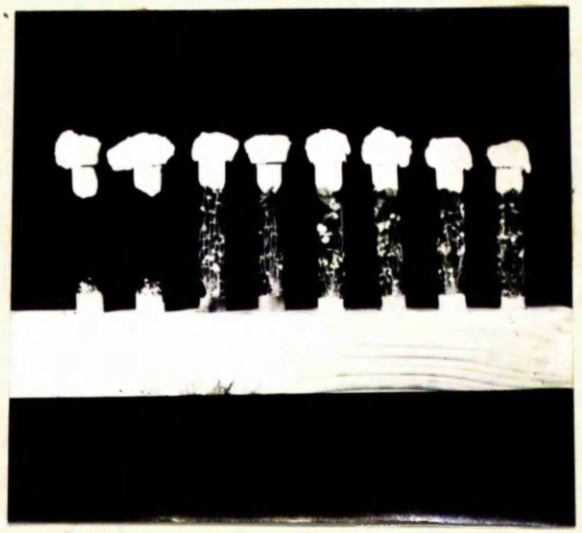
Experiment III, Red clover. Left to right in pair of tubes:
Control, Aspartic acid, Asparagine, Ammonium Nitrate.

F i g u r e 35.



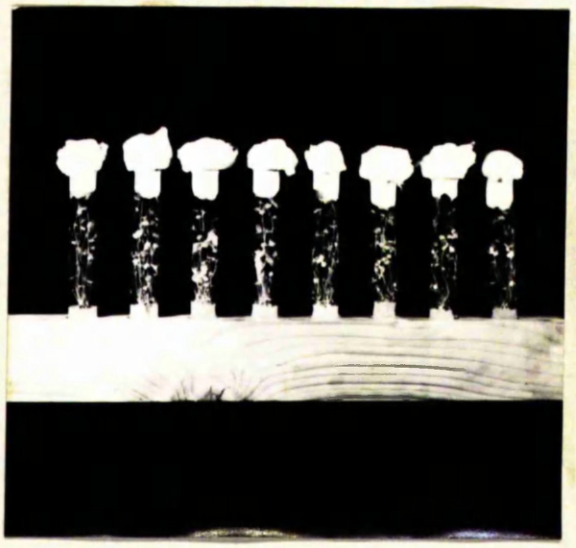
Experiment III, Red clover. Left to right in pair of tubes:
Potassium nitrate (pH 4.8), Potassium nitrate (pH 6.5), Ammonium sulphate, Ammonium sulphate plus calcium carbonate.

F i g u r e 36.



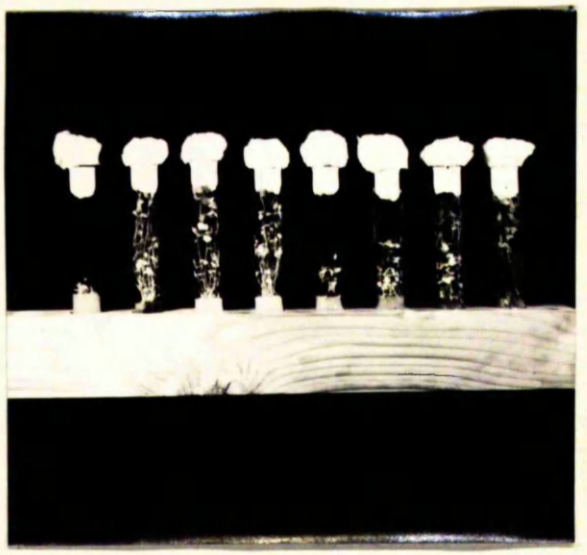
Experiment III, White clover. Left to right in pair of tubes:
Control, Aspartic acid, Asparagine, Ammonium Nitrate.

F i g u r e 37.



Experiment III, White clover. Left to right in pair of tubes:
Potassium nitrate (pH 4.8), Potassium nitrate (pH 6.5), Ammonium sulphate, Ammonium sulphate plus calcium carbonate.

F i g u r e 38.



Experiment III, comparative growth of White and Red clover in various media. Left to right: Control, Aspartic acid, Asparagine, Ammonium nitrate, White clover. The next to the right in the same order Red clover.

F i g u r e 39.

Experiment III, typical roots of White clover in various media. Left to right: plants in aspartic acid, asparagine, ammonium nitrate. Note the small stunted roots in aspartic acid.

D I S C U S S I O N .

Since investigation on the availability of organic nitrogenous compounds needs the employment of an artificial environment to secure sterility of the medium it is inevitable that the results, however conclusive they may be, are of value only for the special conditions under which the investigation was carried out; obviously arrangements imposing as little departure as possible from normal conditions of growth are preferable. Of all the known methods for sterile culture that devised by the Helsinki investigators, of transplanting sterile seedlings into sterile vessels containing sand or solution and allowing the growing of the shoots in the open, is by far the most preferable when the experiments are carried out with large seeds developing strong seedlings convenient for handling and wrapping in sterile cotton wool. Since this method was inconvenient for clover a second method in sterile water cultures described by Virtanen and Von Hausen (1931), was chosen, but was as already noted soon abandoned as inadequate to secure sterility of the medium for a long time. Instead the method of growing plants in sterile agar test tubes was followed. It is obvious that the growth conditions in the adopted method are entirely artificial, since apart from the fact that the roots are growing in agar instead of in soil or sand, the space for the growth of both roots and shoots is extremely small, the light inside the tube is limited, due especially to a layer of condenced water vapour present for most of the time and finally to the concentration

of CO_2 in the inside the tube which soon becomes smaller than in the surrounding atmosphere since according to Knudsen (1917), the diffusion of CO_2 through the cotton wool of the tube is considerably slower than in open tubes. Bearing in mind all these alterations in the environment implied by the method employed, let us proceed in the commentation of the present experiments.

Sterility of medium.

An indispensable prerequisite for reliable results was the securing of sterility of the medium. The use of agar rooting medium has the advantage that the development of contaminating organisms can usually be readily observed. All tubes included in the harvests were free of any obvious contamination. Particular attention was paid to the sterility of tubes with organic nitrogen since the plants in aspartic acid and in a lesser degree in asparagine, had peculiar stunted roots brownish or red in colour. Also, as recorded, since at early stages they showed little benefit from the presence of organic nitrogen, while they later recovered and even exceeded in growth the plants supplied with inorganic nitrogen, the suspicion was born that the tubes might have been contaminated with micro-organisms producing no apparent growth and that the recovery of the plants might have been due to absorption of the decomposed products of the organic compounds. On the conclusion of the second experiment, Dr. S. A. Hutchinson kindly tested the sterility of the medium by culturing it in deep and surface cultures and the conclusion was that the

medium was sterile. Repeated tests for ammonia (with Nessler's reagent) at the conclusion of second and third experiments, carried out by chopping and extracting with glass distilled water for an hour the medium, showed no or negligible traces of NH_3 in tubes originally supplied with organic nitrogen. These results may be considered as additional evidence of the sterility of the medium since tests with tubes with obvious contamination gave a strong reaction for ammonia.

pH of the medium.

Since the experiments of Virtanen and Von Hausen (1931 were carried out at the constant pH of 6.5, the intention was to carry out the present experiments at the same constant pH. However, since it is not possible to readjust pH in the agar method, the experiments were set up at pH 6.5 or 4.8 and alterations in the pH which accompanied the growth were recorded. Tables IIIa and IIIb illustrate this point since the pH of the medium was recorded twice, when the plants were 45 and 69 days old.

It can be seen that the alteration was gradual, and that apart from the medium with potassium nitrate which was slightly changed towards the alkalinity, in all the other media the change was to the acidity. The biggest change occurred in medium with ammonium sulphate where the pH dropped at the end to 3.65 to 3.95 and an almost equally strong change occurred in medium with ammonium nitrate, confirming the findings of Prianischnicow (1926) and other investigators that ammonium nitrate is physiologically an acid nutrient due to preferential absorption of ammonium ion by the roots.

It is interesting in this connection to notice that test for ammonia with Nessler's reagent at the end of the third experiment in tubes with ammonium nitrate, showed complete absence of ammonia suggesting total removal of ammonium ion by the roots when the medium was still containing nitrogen as nitrate. The change in pH in the medium with asparagine although less than the change in the media with ammonium salts, was considerable, since it dropped to 4.9 - 5.5, while the change in the medium with aspartic acid and control was very small amounting to few decimals of one unit in the pH scale.

As was already mentioned the writer is not aware of any method for the adjustment of the pH in sterile agar tube cultures. One method of partial control is to add to the medium small quantities of calcium carbonate in order to prevent or lessen the change of the pH to the acidity, and this method was employed in few tubes with ammonium sulphate in the third experiment. The added quantity of calcium carbonate was 0.5 gm. per litre of medium and the effect was to lessen the drop of the pH a little less than one unit of the pH scale. When the growing plants in the tubes are able to stand larger concentrations of calcium carbonate (clover will not tolerate a large concentration), with the employment of this method it will be possible to keep the pH fairly constant. It has, however, to be remembered that carbon dioxide is released as the calcium carbonate is decomposed and as the air in the tube is relatively poor in carbon dioxide, even a small release of it might have an effect on the growth.

So much for the difficulty of keeping the pH of the medium constant.

Another problem in experiments investigating the relative nutritive value of different nitrogenous substances is the choice of the pH under which the experiments will be carried out. In the present experiments the pH of the medium was adjusted to 6.5 since that was the pH employed by Virtanen and his co-workers, but the question arises as to whether at this pH, the clover could make the best growth in each of the tested nitrogenous compounds. There is extensive literature on the effect of the reaction of the medium on the absorption of ammonium and nitrate salts by numerous plants (unfortunately mostly non-legumes), but there are very few papers regarding this effect on organic nitrogenous compounds. The investigations on the first point showed clearly that when in experiments carried out in water or sand cultures, salts of ammonium as a source of nitrogen were employed, the best growth was obtained at a pH not much differing from neutral, while in medium with nitrates the best growth was in acid medium 4 - 4.5 pH. (Clark and Shives, 1934, Davidson and Shives, 1934). Nightingale (1937) reviewing the literature for the nitrogen nutrition of green plants summarises that "there is no one best pH value for a given nutrient solution for all plants nor for the same kind of plants under different environmental conditions". In this connection may it be mentioned (1) that in the third experiment, the pH in the medium with potassium nitrate was adjusted initially to two levels 6.5 and 4.8 and the results showed that the white

clover, particularly, grew considerably better at the lower pH than at 6.5 pH. (2) that the relative superiority of ammonium nitrate as a source of nitrogen might be connected with the composition of the salt and the changes of the pH. Thus at the beginning with pH 6.5 the plant absorbs ammonium ions and later on when the pH is low and inconvenient for absorption of ammonium, the absorption of nitrate follows.

In papers dealing with the nutritive value of organic nitrogenous compounds, usually, apart from the record of the pH of the medium, there is no further comment. In view of the findings on the effect of the reaction of the medium on the absorption of ammonium and nitrate salts, it appears necessary to investigate the effect of the pH on the availability of organic nitrogenous compounds. It might be possible that differences exist among the various organic nitrogenous substances as regards this point, and that experiments carried out under different pH of the medium might change the order of the tested nitrogenous substances from the point of view of their nutritive value.

Concentration of nitrogen in the medium.

Three concentrations of nitrogen were tested: 127.5, 170, and 255 mg. nitrogen per litre of medium, the concentrations being similar to those employed by Von Hausen (1936). Since the concentration of 127.5 mg. was employed only in a few tubes in the first experiment the information from its employment was not conclusive. The high concentration of 255 mg. showed a slight depressing effect on the dry matter in

the first and second experiment and consequently was omitted in the third experiment and only the concentration of 170 mg nitrogen per litre was employed in the three experiments. Since it is possible that the optimum availability of the various nitrogenous compounds tested might occur in different concentrations of nitrogen in the medium, further investigation on this point is considered necessary. Experiments carried out with smaller concentrations of nitrogen, especially in the case of medium with aspartic acid, would reveal whether the somewhat toxic effect of the medium on the growth of the roots in the present experiments, (roots stunted, red in colour with tendency for avoiding the mass of medium and instead growing between the tube and agar), was due to aspartic acid itself, or to some unsuitability of the concentrations of aspartic acid employed.

The conclusions that might be drawn from the experiments are:

(a) that both Red and White clover were able to absorb and utilise extensively organic nitrogen in the forms of asparagine and aspartic acid.

(b) Asparagine was proved in all experiments with both clovers to be an equally good source of nitrogen as the best of the tested inorganic salts (ammonium nitrate), since the dry matter of plants growing in medium with asparagine at the close of the experiments, was equal to, or larger than, that obtained in the medium with ammonium nitrate; and the percentage of nitrogen was always higher in plants in asparagine

than in plants supplied with ammonium nitrate. This last result is in agreement with that of Von Hausen (1936), who reported that the percentage of nitrogen in peas grown in water or sand cultures with asparagine was double that in plants supplied with inorganic nitrogen, and may be connected with the larger percentage of nitrogen in plants receiving nitrogen exclusively as ammonium salts in comparison with the percentage of nitrogen in plants supplied with nitrates, (Miller 1938). The higher concentration of nitrogen in plants supplied with asparagine might be considered as supporting the assumption that the whole molecule of asparagine is absorbed by the roots, or at least that the absorption does not take place in the form of ammonium or nitrate ions, (following the splitting of the asparagine to these forms through the action of enzymes excreted by the roots), because otherwise it would be reasonable to expect that the percentage of nitrogen in plants supplied with asparagine could not exceed that in plants supplied with salts of ammonium or nitrate. It might be mentioned in connection with this that Tanaka (1931), with microchemical analysis in plant tissue of plants supplied with urea, showed needles of urea in the tissue, and Virtanen and Linkola (1946), by determining the ratio carbon to nitrogen in medium with aspartic acid before and after the absorption of part of aspartic acid by peas, reached the conclusion that the aspartic acid was absorbed by the roots as a whole molecule.

(c) Aspartic acid was also absorbed and utilised by both

clovers, but the extent of its availability varied considerably in the three experiments. Thus while in the first experiment red clover plants supplied with aspartic acid exceeded in dry matter plants supplied with other media, in the second experiment it proved a relatively poor source of nitrogen for both clovers. In the third experiment it was proved a good source of nitrogen for the white but not for the red clover, the growth of red clover being extremely irregular in the replicate tubes. In view of the fact that the three experiments were carried out in different seasons, with different size of tubes, different mass of nutrient etc, dissimilarities in the relative availability of a nutrient ought to be expected as a result of the influence of diverse environments. Judging from the appearance of the plants at early stages, it appeared as though aspartic acid and in a lesser degree asparagine, were for a time unavailable to the young plants. Finally the appearance of roots of plants supplied with aspartic acid suggested that the young roots were injured and further experiments are necessary to show whether with smaller concentrations of aspartic acid, better development of roots and better growth of the whole plant would be obtained. In this connection it is interesting to note that Tanaka (1931), using 0.01% asparagine in agar cultures with Sisyringium reported that the roots had brown tips and were inhibited.

(d) Of the inorganic salts tested it appeared as though in early stages ammonium nitrate, and in a lesser degree ammonium sulphate, were better sources of nitrogen than potassium

nitrate. This can be explained by the difference in availability of ammonium and nitrate ions at the particular pH employed. The differences in dry matter had lessened or disappeared at the close of the experiment. Bearing in mind the limited space in the tube culture it is reasonable to expect that small differences in the availability of the nutrients tested would be masked at a later stage, since a limit is placed on the growth and plants with initially less growth by continuing growing, catch up in growth with those which were more advanced.

The method of agar tube culture could be employed to give more accurate information about the relative availability of various nitrogenous compounds, by carrying out a more elaborate experiment with combinations of different pH and concentrations of nitrogen in the medium, also with successive harvests of the plants to be made at short intervals, beginning as soon as the control plants show exhaustion of the reserve nitrogen in seeds. With such experiments, apart from reliable information concerning the relative availability of different nitrogenous substances, information would be obtained about the necessary time between the process of absorption of nitrogenous compounds by the roots and the actual utilisation for the growth and building of new tissues by the plant.

(e) The experiments failed to confirm the findings of Virtanen and his co-workers, that red and white clover differ as regards the ability to use organic and inorganic nitrogen,

since in the present experiments red and white clover behaved similarly in connection with the absorption and utilisation of different forms of nitrogen.

(f) The investigation of the effect of the presence of small quantities of b-alanine in medium with aspartic acid, on the availability of aspartic acid, showed that with clover plants there was no effect at all since both red and white clover were able to absorb and utilise aspartic acid to the same extent with or without addition of b-alanine.

S U M M A R Y.

(1) Experiments were carried out by growing non-nodulated clover in agar medium in test tubes under sterile conditions, in order to investigate (a) whether red and white clover can use organic nitrogen in the form of asparagine and aspartic acid, (b) whether differences exist between red and white clover as regards the ability of using organic and inorganic nitrogen, and (c) whether small added quantities of b-alanine in the medium with aspartic acid, inhibit the absorption and utilisation of aspartic acid by clover plants. pH was adjusted initially -in the most of the treatments - to 6.5.

(2) The experiments showed that both asparagine and aspartic acid were used by the clovers.

(3) Asparagine as source of nitrogen was proved equally good as or even superior to, inorganic nitrogen for both clovers, since plants supplied with asparagine grew equally well and at the same time exceeded in total nitrogen plants supplied with inorganic nitrogen.

(4) Aspartic acid, although it was absorbed and utilised by both clovers, was rather an inferior source of nitrogen in comparison with inorganic nitrogen.

(5) There were no differences between red and white clover as regards the ability of using organic and inorganic nitrogen.

(6) Small quantities of b-alanine added to the medium

with aspartic acid, had no effect at all on the availability of aspartic acid, since both clovers absorbed and utilised similar quantities from media with or without added b-alanine.

(7) The results were discussed in relation to the effect of the pH on the availability of various nitrogenous substances.

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