THE PRESENCE OF A NITRATE REDUCING ENZYME IN GREEN PLANTS

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INTRODUCTION

The question as to the form in which nitrogen is most easily assimilated by the green plant has long been under debate, and very conflicting statements have been made by various investigators :—

Thus Boussingault's¹ observations showed that nitrates seemed the most suitable form for the absorption of nitrogen by the plant.

Treboux² found that—

1. Nitrites are probably useful to the plant in alkaline solution, but poisonous in acid solution.
2. Nitrates have the same, if not a greater value than nitrites.
3. Ammonium salts are still better than nitrates or nitrites.
4. Amino acids and amides can be used but their nutritive value is much less.

He suggests that amino acids are decomposed by enzymes with liberation of ammonia.

Mazé³ thinks that nitrates and ammonium salts are of equal value in metabolism.

1. Boussingault, *Agron.*, Tome I, pp. 69, 130, 1860.

2. Treboux, *Chem. Centr.*, p. 1619, 1905, from *Ber. deut. bot. Ges.* XXII, p. 570-572.

3. Mazé, *Compt. rend.*, 1899, pp. 128, 185-187.

Godlewski¹ found that higher plants when kept in darkness could produce proteids from nitrates and from the decomposition products of proteids, but in the case of the higher plants the assimilation of these substances is restricted in the absence of light. The necessary energy for nitrate assimilation is supplied by metabolism and respiration.

Laurent, Marchal and Carpiaux² found that plants kept in distilled water containing ammonium sulphate and saccharose respectively were able to assimilate these substances when placed in the light.

Hansteen³ showed that nitrates were assimilated to a small extent in darkness.

Laurent⁴ maintains that neither ammonium salts nor nitrates are assimilated in the dark.

Zuleski's⁵ results show the possibility of proteid formation in the dark.

Suzuki⁶ found that if plants were fed on 1 to 10 per cent. sugar solution, assimilation of nitrate and subsequent formation of proteid took place in the dark, as well as in the light. Plants containing a large amount of sugar were able even in the dark to form proteids from nitrates without the addition of sugar solution.

The observations of Suzuki would seem to suggest that in the case of Godlewski's experiments, and in those of Laurent, the plants had not contained any reserve carbohydrate, and were, therefore, dependent for their sugar upon the supplies formed during their exposure to the light.

The same explanation might hold for the other conflicting statements upon this point, as for example, those of Hansteen. From our point of view it is interesting to note that there are statements

1. Godlewski, *Bul. Acad. Cracow*, Vol. VI, p. 313, 1903.
2. Laurent, Marchal and Carpiaux, *Bied. Centr.*, Vol. XXVII, 1898, pp. 821-823; from *Bul. Acad. Belg.*, Vol. XXXI, pp. 815-865, 1896, and *Bot. Centr.*, Vol. LXX, p. 232, 1897.
3. Hansteen, *Ber. deut. bot. Ges.*, Vol. XIV, p. 368, 1896.
4. Laurent, *Bul. Acad. rag. Belg.*, *J. C. S. Abstracts*, Vol. II, p. 323.
5. Zuleski, *Ber. deut. bot. Ges.*, Vol. XV, p. 336, 1897.
6. Suzuki, *Bul. Coll. Agr., Imp. Univ.*, Tokyo, Vol. III, pp. 488-507, 1898.

by various investigators pointing to nitrates as the best source of nitrogenous food for the green plant, and there are indications to show that this may be correlated with the presence of carbohydrates formed in photo-synthesis.

As the nitrogen is usually regarded as present in the proteid molecule chiefly in the form of NH_2 groups, there must obviously be a very efficient reducing apparatus in the green plant capable of converting the received grouping NO_3 into the necessary NH_2 form.

There are very few statements in the literature of plant physiology suggesting that such is the case, but one or two cases of the existence of a nitrate reducing enzyme have been recorded :—

Abelous and Aloy¹ showed that an enzyme capable of reducing nitrates to nitrites and nitrobenzene to aniline, is found in animal structures. They also demonstrated the presence of a similar enzyme in potato tubers.

Kastle and Elvolve² confirmed its presence in the potato, and showed that it was also present in the fruit of the egg plant (*Solanum melongina*).

Weehuizen³ found that nitrous acid was present in the leaves of *Erythrina*, and concluded it was set free from a glucoside by the action of an enzyme; because if the leaf were killed by immersion in boiling water for thirty seconds, no nitrous acid was formed.

It seems very probable that Weehuizen's enzyme was the nitrate reducing enzyme which has formed the subject of this paper. There are also records of nitrate reducing bacteria.

Thus Burri and Stutzer⁴ found that certain bacteria decomposed nitrate with liberation of free nitrogen, and that the action was increased in absence of air.

Also very recently Mattio Spica⁵ has found that under anaerobic conditions yeast was able to reduce nitrates.

1. Abelous and Aloy, *Compt. rend. Soc. Biol.*, Vol. LV, p. 1080, 1903.

2. Kastle and Elvolve, *American Chemical Journal*, Vol. XXXI, pp. 606-641, 1904.

3. Weehuizen, *Pharm. Weekblad*, Vol. XLIV, pp. 1229-1232, 1907.

4. Burri and Stutzer, *Ann. Agron.*, Vol. XXII, pp. 491-494, 1896, from *Centr. Bact.*, Par. 1895, Vol. I, p. 2, Abt. 257, 350., pp. 392-422.

5. Mattio Spica, *J. C. S. Abstracts*, October, 1907.

It is clear that a more general distribution of such an enzyme is to be expected if nitrates are utilised in the formation of proteids. The present paper is the outcome of work carried out upon this hypothesis.

EXPERIMENTAL

Water plants (*e.g.*, *Elodea*, *Vallisneria*, etc.) were used in the first experiments on account of the greater facilities which these plants offered for collecting and examining any gases which might be liberated.

The following experiments were set up :—

A.—*Elodea* was placed in boiled tap water containing 0.5 gramme of asparagin and 1 gramme of potassium nitrate per litre. The plant was placed under an inverted funnel in the solution, and a test tube filled with water was put over the stem of the funnel to collect any gases which might be given off during the experiment. Thymol was added for antiseptic purposes to E and F. This was left in the light for two days.

B, C, D, E and F were set up in the same way.

B.—*Elodea* in a solution containing asparagin and potassium nitrate in the same proportion as in A but placed in the dark.

C.—*Elodea* boiled for some time, before putting it into a solution containing asparagin and potassium nitrate, and then placed in the light.

D.—Boiled *Elodea* put into a solution of asparagin and potassium nitrate and then placed in the dark.

E.—Chloroformed *Elodea* in a solution of asparagin and KNO_3 in the dark.

In this case the protoplasm would be killed, but many of the operative ferments present in the plant might remain.

F.—Chloroformed *Elodea* in a solution of asparagin and potassium nitrate placed in the light.

No gas was evolved on the first day, but on the second it was found that gas had been given off from the *Elodea* in A, B, E and F, but not in C and D. The gas was collected in a Winkler Hempel apparatus and analysed.

A.—Total volume = 24.6 c.c.

1. Passed through KOH, bulbs = 24.6 c.c.

2. Treated with KOH and pyrogallol, vol. = 24.6.

3. Sparked with oxygen and treated again with pyrogallol to absorb the oxygen = 24.6.

∴ Gas = nitrogen.

B.—Total volume = 25·6 c.c. This, treated in a similar manner, also proved to consist solely of nitrogen.

E.—Total volume = 37·5 c.c., which proved on analysis to consist of 37·5 c.c. nitrogen, 1·2 c.c. carbon dioxide.

F.—Total volume = 19 c.c. On analysis, only nitrogen present.

All analyses were carried out as described for A. These experiments were carefully repeated, and in all cases nitrogen was found to be given out by normal and chloroformed plants, but not by the boiled ones.

The solutions of asparagin in which the *Elodea* had been placed were examined: A, B, E and F were found to contain nitrites by the starch and potassium iodide test. Before the experiment they contained only nitrates. Therefore, during the experiment the nitrates were reduced to nitrites.

Substitution of Ammonium Salts for Nitrates.—Experiments were set up with an equivalent amount of ammonium sulphate in the place of nitrate, but no gas was given off, either in the light or in the dark, when *Elodea* and asparagin were placed in the solution.

The following Explanation is suggested for the Evolution of the Nitrogen:—

The nitrite formed by the reduction of the nitrate is converted into nitrous acid by the slightly acid cell sap. This in turn acts upon the asparagin giving malic acid and nitrogen.

Owing to the nature of the reducing action the oxygen is not liberated as a gas, but retained in some form.

The reaction came to an end after a few days in the dark, and the *Elodea* was then found to contain no starch.

The necessary acid medium for the reaction is probably provided by the cell sap. The oxidation of the carbohydrates may provide the energy necessary for the reaction, and its cessation may be due to the exhaustion of the supply of carbohydrates in the plant.

This is suggested by the fact that the reaction is accelerated, or restarted when it has stopped, by the addition of cane sugar, or glucose, to the solution.

Support is given to this theory by the fact that very little sugar, if any, was present in the plant which had been kept in the dark, whereas in the ordinary plant there was an appreciable supply of carbohydrates. An estimation of total sugars and starches in normal *Elodea* gave as a result 1.268 per cent. of dry weight as sugars and starch, while some of the same crop of *Elodea*, after placing in a solution of nitrate and asparagin in the dark, until no gas was liberated, yielded upon estimation merely a trace of carbohydrates.

From these experiments it thus seems probable that malic acid is formed in the plant in proportion to the nitrogen evolved as gas. It ought, therefore, to be possible to detect the malic acid in the plant itself.

Elodea was treated as described for A in the previous series of experiments. The plant was then finely ground, and shaken with a little water for two hours, and left overnight to extract. The solution was then filtered and treated with lead nitrate. A precipitate, presumably lead malate, was thrown down. The solution and precipitate were then heated, when the precipitate partially re-dissolved. The solution was then boiled to coagulate the proteids, and afterwards filtered, a clear solution being thus obtained. On concentrating and cooling small colourless crystals separated out. These were examined microscopically and found to have a similar structure to those of lead malate. They were re-dissolved in water, treated with a solution of calcium nitrate, which brought down a precipitate of calcium malate. This was filtered off, and the filtrate concentrated. No crystals came out, so it was concluded that all the malic acid had been removed as calcium malate.

This process involves the formation of nitrate as an intermediate product in metabolism, and nitrite is supposed to be poisonous to the plant. Experiment seems to suggest that in dilute solutions the protoplasm is not killed, and in stronger solutions ferment action is not arrested by the presence of nitrite.

Sprigs of *Elodea* were placed in solutions of nitrites of different strengths ranging from '001 per cent. to 10 per cent. It was found that an enzyme present in all of them catalysed hydrogen peroxide, even after an immersion of three days in the nitrite solution. Trials made to plasmolyse the leaf cells after this immersion in solutions of potassium nitrite, indicated that in all the solutions used that were above 0.1 per cent. in concentration, the protoplasm was killed even after twenty-four hours.

But the amount of nitrite formed by the plant under normal conditions would probably be very minute at any moment, and would be removed almost immediately.

Extraction of an Enzyme capable of Reducing Nitrate to Nitrite.—Grass was dried at the temperature of the air, powdered, treated with water and left overnight to extract at 30° C. Chloroform was added for antiseptic purposes.

The solution so obtained was filtered, and treated with alcohol to precipitate the enzyme. This was filtered off, washed and dried.

The following experiments were then set up :—

- 1.—Enzyme in a solution of glucose, asparagin and potassium nitrate in water.
- 2.—Enzyme in a solution of cane sugar, asparagin and potassium nitrate.
- 3 and 4.—Controls to 1 and 2 in which the enzyme had been boiled for some time.
- 5.—Enzyme in a solution of asparagin and glucose but with no potassium nitrate present.
- 6.—Control to 5 ; containing the enzyme after prolonged boiling.

After twenty-four hours gas was being liberated by 1 and 2, but not by 3, 4, 5 or 6.

The glucose and cane sugar experiments had approximately the same volume of gas in each, but only about 1.5 c.c. was obtained in

either case. No analysis was made, but from its small solubility it was concluded that it was not carbon dioxide. The enzyme obtained in a similar way from *Elodea* gave the same results. At a later date this experiment was repeated upon a larger scale, and after two unsuccessful attempts, sufficient gas was obtained to make quantitative analysis quite possible.

By extraction from a very large bulk of the dried plants 0.5 gramme of the dried and powdered enzyme was obtained, though, of course, still in a very impure state.

This was placed in water containing respectively 2 per cent. of potassium nitrate, asparagin and dextrose.

The enzyme, which only partially re-dissolved after drying, was placed in a test tube containing the solution; over this was inverted a slightly larger test-tube, and both these were placed in a larger dish of the solution.

In this way owing to the slowness of the outward diffusion of the enzyme, very little gas was lost as a result of its liberation taking place outside the walls of the larger test-tube.

The reaction proceeded in an incubator kept at 30° C. from November 28th until December 11th; thymol was used as an antiseptic, and on the later date when the experiment ceased, there was not the slightest indication of bacterial activity in the solution.

During the first week the liberation of gas was very slow, but latterly it collected more rapidly, and at the end of the experiment 6.2 c.c. were available for analysis.

This gas underwent no change in volume, either over strong caustic potash or upon treatment with pyrogallol and potash (solutions made up according to Clowes¹ formula); it was therefore concluded that the only gas present was nitrogen.

A nitrate reducing enzyme has also been found to be present in the following plants:—*Potamogeton*, *Vallisneria*, *Iris*, *Vicia faba*, various Gramineae.

1. Clowes, *Brit. Association Report*, 1896, p. 74.

In the case of *Vicia faba*, it was found in all parts of the plant, in root, stem, and leaves; but the reaction was longer in starting, and slower in progress in the case of the roots when placed in the nitrate and asparagin solution. As far as our experiments go, there seems no reason to doubt its very general distribution in plants.

CONCLUSIONS

The presence of a reducing ferment in green plants seems to have been established by means of this reaction with asparagin. It is not intended to suggest that this actual reaction occurs normally to any great extent in green plants, as the asparagin occurring in such plants is presumably to be regarded as an upgrade stage in the synthesis of proteids.

Further, as asparagin occurs to a considerable extent in such plants, it seems essential that the centres of nitrate reduction and of proteid formation must be quite distinct.

The reaction is to be regarded as abnormally wasteful in the plant economy, and not occurring in nature to any appreciable extent. Its occurrence under the experimental conditions has to be regarded as being due to the excess of both nitrate and asparagin in the solutions in which the plants were placed.

Possibly, under the conditions existing in ensilage, and in similar cases, the loss of nitrogen that takes place in the slowly decomposing heaps of grass may be due in part to the evolution of gaseous nitrogen, owing to the distribution of the enzyme becoming, as it naturally would, less localised.¹

In the normal plant the only conditions necessary for nitrate reduction seem to be the presence of the enzyme, found in roots, stems, and leaves, and a suitable carbohydrate. The latter condition suggests the green leaf as the centre of reduction, and this agrees with the distribution of nitrates in the plant.

Our results seem to show that any hexose or polysaccharide is suitable for the supply of energy for nitrate reduction; not as in

1. Evolution of gaseous ammonia takes place at the same time, and probably accounts to a large extent for the loss of nitrogen that occurs.

later stages of proteid synthesis where, according to Borodin¹ and Hansteen,² glucose is the only carbohydrate which, together with asparagin, can provide the necessary basis for construction of these bodies.

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1. Borodin, *J. C. S., Abstracts*, Vol. II, p. 323, 1899.

2. Hansteen, *Chem. Centr.*, Vol. I, p. 295, 1897, from *Ber. deut. Ges.*, Vol. XIV, p. 362-371