CCXIX. LOSSES OF NITROGEN FROM GREEN PLANTS

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THE possibility that the nitrogen metabolism of plants may involve large losses of nitrogen appears to have been overlooked. We drew attention to this possibility in a preliminary note [1936], Eggleton [1935] has also suggested it, and this paper presents data which shows that such losses may exist and suggests indeed that they may be of frequent occurrence. The data are drawn from experiments on two widely different problems, viz. growth in *Chlorella* and nitrogen metabolism in *Narcissus* leaves. They are adequate for the present purpose though not primarily designed for it. In each case the cells or tissues were kept under aseptic conditions in a medium containing glucose and a source of nitrogen. Thus gains of nitrogen by the cells and losses by the medium could be estimated and a nitrogen balance sheet constructed.

Analytical methods

The tissues were rapidly dried by heating to 95° for 30 min. and then to constant weight at 65°. Both species are suitable for this method and show no discoloration after drying. Total N was estimated by the Kjeldahl-Gunning method as described by Ranker [1928]. The micro-apparatus of Pregl [1930] was employed with exact standardization of all procedure. The dry material was finely powdered and 10–15 mg. were used in each estimation. These were done in triplicate.

Ammonia-N and nitrate in the external solution were estimated as follows. An aliquot of the solution in the micro-distillation flask was made alkaline (3 ml. of *N* NaOH) and ammonia distilled off with steam into standard acid. The flask was then cooled, 0.2 g. Devarda's alloy added, the outlet closed with an acid trap and the solution heated just to boiling for 15 min. The ammonia formed was then swept off with steam as before into standard acid. These estimates were done in triplicate and with routine blanks on reagents only. The total N of the external solution was also estimated by the Kjeldahl-Gunning method. This normally gave, in the *Narcissus* experiments, a somewhat lower value than the sum of ammonia-N and nitrate-N, though the difference was not significant. This is probably because (as shown by Ranker [1928]) large proportions of nitrate-N are not easily reduced when sugar is abundant. We have, therefore, used the higher estimates.

The technique was not primarily designed for the examination of soluble constituents in the cells, but estimates of nitrate-N, ammonia-N and total soluble N were made. The soluble constituents were extracted with cold 60% alcohol, the alcohol evaporated and the extract cleared with lead acetate, using Na₂HPO₄ as deleading agent. The method described above for ammonia and nitrate was used, but the blank for nitrate was taken as the extract, with ammonia removed, heated without Devarda's alloy as described above. This was

to allow for the possibility of organic N being decomposed by the alkali used. Data are also given below for protein-N and organic N. The former was estimated as total N minus soluble N. The organic N was taken as total N minus ammonia and nitrate-N.

(1) *Chlorella*

In work on *C. vulgaris*, the alga (pure culture) is usually grown aseptically in mixtures containing equal volumes of the following solutions:

A. 0.4 g. MgSO_4 (anhyd.), 1.5 g. NH_4NO_3 , 0.4 g. sodium citrate, 20 g. glucose, made up to 1 l. with distilled water; 3 drops of saturated FeSO_4 added.

B. 14.528 g. KH_2PO_4 , 4.646 g. K_2HPO_4 per l. (pH 6.0).

Each culture flask contains 50 ml. of medium and, therefore, 13.1 mg. of N. The cultures are grown at 23°. Details of the culture and cropping methods have been given by Pearsall & Loose [1937]. When NH_4NO_3 is used as the source of N, nearly all the N supplied can be accounted for in the crop and culture medium. There is, however, evidence of a small loss of N of about 0.5 mg. per culture under these conditions [1937]. Bristol & Page [1923] have also shown that "luxuriantly growing" cultures of various algal species exhibit a small N loss of a similar order of magnitude when grown on glucose-inorganic N media. According to their data more slowly growing cultures did not show this loss, and it appeared to be more marked in nitrate cultures than when only ammonium salts were used. Further, Leeds cultures of *Chlorella* when grown on other inorganic N sources, such as $\text{Ca}(\text{NO}_3)_2$ and NH_4Cl , showed small N losses of a similar order of magnitude. All these cultures would be "luxuriantly growing". Generally, when these cultures approach their maximum dry weight (250–300 mg.) about 8 mg. of N may be present in the cells and 3.5–4.5 mg. in the medium. Miss F. M. Gatenby observed, however, that when NaNO_3 was used as a source of N in the dark, all the nitrate-N might disappear from the medium even before the cultures had reached maximum dry weight. It was evident from her results that either the cells must be exceptionally rich in N or else that abnormally large losses of N had taken place.

Hence additional cultures were grown in the usual glucose media but with NaNO_3 (13 mg. of N per flask) instead of NH_4NO_3 . Cultures were kept both in the light and in the dark at 23°, and to make them comparable they were harvested when they possessed similar weights. The mean dry weight per culture (average of six cultures) was: light, 152 mg. in 11 days; dark, 141 mg. in 28 days. The N balance sheets for these cultures are given below.

N recovered and lost in Chlorella cultures (as mg. per culture)

	N supplied*	N in culture medium		N in cells	N lost
		Total N	Nitrate-N		
Dark	13.0	1.15	0.62	3.40	8.45
Light	13.0	8.12	7.67	4.07	0.81

* Estimated.

It is evident from these results that very large losses of N may take place in NaNO_3 media in the dark, almost two-thirds of the N supplied being unaccounted for in the analysis. The N unaccounted for in the light is possibly somewhat greater than when NH_4NO_3 is used but of a similar order of magnitude. The experimental error is not more than 0.1 mg. on any estimation, that is, a N loss of 0.2 mg. would not be significant. The results have been checked by determining total N of medium + cells without separating them.

For short-period experiments, Warburg & Negelein [1920] have shown for *Chlorella* that the rate of nitrate reduction in the light is 4–5 times greater than in the dark, while, for cultures comparable with those above (in NH_4NO_3), Loose & Pearsall [1933] found the rate of protein synthesis to be 4–5 times greater in light. It is therefore remarkable to find much more nitrate utilized in the dark in the above experiments. Thus 12.38 mg. of nitrate-N were converted in the dark and only 5.33 mg. in the light. The cultures were, however, exposed for very different times.

We assume that the curves of growth in weight of the cultures are of similar form in the light and the dark but differ only in rate. Unpublished results show that this is sufficiently true for our present purpose. As the final weights are similar in the above experiments, the relative rates of N utilization per unit of cell weight must be proportional to time and they will be of the following orders of size: dark, 0.442 (12.38/28), and light, 0.45 (5.33/11).

Allowance should be made for the fact that the dry weight of the illuminated cultures was slightly higher than that of the cultures in the dark (152 : 141), that is, that their time for growing to 141 mg. should be slightly less than 11 days (about 10.5 days). Even so, it is clearly improbable that the rate of nitrate utilization differs very greatly in the light and in the dark, while in contrast, and on the same basis, the relative rates of N loss are of quite different orders of magnitude, viz. dark, 0.301 (8.45/28) and light, 0.074 (0.81/11), and are clearly much higher in the dark. This effect, which has only been observed with NaNO_3 , may possibly be due to the special effects of the Na ion, such as, for example, an increase in protoplasmic permeability. Whatever the nature of this effect may be however, it appears to be largely removed by light.

Thus the data for *Chlorella* appear to indicate that N losses are normally small but may be very large when the cultures are grown in NaNO_3 in the dark.

(2) *Narcissus leaves*

The data for leaves were obtained when leaves of *N. pseudonarcissus* ("single" flowered) were floated on nutrient media. The standard medium contained 3 % glucose, 0.2 % NH_4NO_3 and a phosphate buffer at pH 5.4 (8.7168 g. KH_2PO_4 and 0.4648 g. K_2HPO_4 per l.). The medium was used in a depth of 1.5 cm. in large conical flasks. These were, when necessary, illuminated from below by 100 W. daylight lamps, the temperature being controlled to within 1° by a surrounding water-bath.

Twenty similar leaves were used in each series. They were divided into four segments: (1) the white basal meristematic portion, (2) the extending tissue just above, also becoming coloured, (3) the lower part of the fully green tissue, (4) the apical and oldest segment. One type of segment was taken for each determination, and each experiment consisted of four different types of leaf segment in four separate lots of medium. Before starting the experiment, each segment was split longitudinally and one-half was taken for analysis to represent the original condition of the segments. Samples were weighed and all data can be given as % of the original fresh weight.

The technique used was devised to permit estimates of changes in the leaves and the external medium. The leaves were sterilized by washing in calcium hypochlorite for 10 min. [Wilson, 1915]. The danger of bacterial infection was reduced by using a medium at pH 5.4. This was sterilized by autoclaving and aseptic methods were employed throughout. The experiments normally ran for 60 hr., but the medium and tissue could be maintained without visible

(microscopic) infection for 6–7 days. The medium rapidly becomes turbid on infection, and infection can normally be detected a day after it occurs. The material is not exposed again to contamination after it is set up, and hence its purity or otherwise can easily be seen after 60 hr.

Typical results are given below showing the fate of the N in the medium during experiments with NH_4NO_3 . The first series deals with young but mature leaves and the second with older leaves from plants in full flower. In each case, one set of experiments was done in the light and a comparable series in the dark; using leaves of similar size and appearance.

Table I					
Segment		Original fresh wt. g.	mg. N		
			Gained by tissue	Lost by solution	Unaccounted for
Series I. Young leaves— $16 \pm 1^\circ$					
Light	1 Base	13.27	14.55	19.25	4.70
	2	16.52	24.63	31.29	6.66
	3	12.85	20.40	36.49	16.09
	4 Apex	6.93	6.07	22.99	16.92
Dark	1 Base	9.31	7.35	10.48	3.13
	2	13.92	7.90	12.87	4.97
	3	13.01	13.05	19.50	6.45
	4 Apex	6.40	5.11	10.40	5.29
Series II. Older leaves—fully mature— $22 \pm 1.0^\circ$					
Light	1 Base	16.00	10.70	19.30	8.60
	2	17.47	28.76	36.55	7.79
	3	12.50	16.79	34.32	17.53
	4 Apex	9.27	1.82	27.27	25.45
Dark	1 Base	16.380	6.32	17.63	11.31
	2	16.497	8.06	21.47	13.41
	3	12.031	1.80	17.26	15.46
	4 Apex	6.780	-6.17	8.36	14.53

These results show that a considerable part of the NH_4NO_3 absorbed cannot be accounted for in the analyses. As one criterion of the accuracy of the determinations we use similar results by this method in which organic sources of N were employed (urea, alanine, asparagine). With these sources of N, the amounts gained by the tissue agree with those lost by the medium, to within 1 mg. A similar variation was obtained in solutions of nitrate alone (see below). We conclude, therefore, that when NH_4NO_3 is used, a real and relatively large loss of N from medium and tissue can be demonstrated.

The possibility exists that NH_4NO_3 might be decomposed in the presence of organic matter, especially if light is supplied. To test this possibility, sterile media without leaf tissue were prepared and exposed in the usual manner, three samples in the dark and three in the light. Each sample contained originally 69 mg. of ammonia-N and an equal amount of nitrate-N calculated on the weighed dry salt, and estimated for each form of N to within 1 mg. by analysis. The ammonia-N and nitrate-N were again estimated after 3 days' exposure. Again the amounts of N recovered after this time lay within 1 mg. of the original calculated values.

It is not probable that bacteria were present in sufficient numbers to cause these relatively large losses of N. When the tissue was removed aseptically, after being in the medium for a day, the medium showed (within the stated limits of experimental error) no further loss of N. The possibility is not precluded that some bacteria exist on or in the tissue, but scraping the tissue with

a sterilized platinum wire and transferring the scrapings to nutrient media gave only occasional infection and suggested that the tissues were, if not always bacteria-free, certainly not always infected and apparently never heavily infected. Hence we think that the N losses are due to the tissues of the leaves.

In regard to the causes of the N losses, the following table is of interest, in which the results are recalculated as amounts lost by a constant weight of tissue.

Table II. *Mg. N absorbed from the medium and lost in the experiment (by 10 g. of tissue)*

Segment	Series I				Series II			
	Dark		Light		Dark		Light	
	N absorbed	N lost	N absorbed	N lost	N absorbed	N lost	N absorbed	N lost
1 Base	9.76	2.91	14.48	3.54	10.74	6.0	12.07	5.38
2	9.25	3.57	18.93	4.03	13.01	8.12	20.88	4.45
3	14.99	4.97	28.42	12.52	14.63	13.10	27.43	14.01
4 Apex	16.24	8.27	33.15	24.40	12.16	21.13	29.44	27.47

These results show that the largest losses are observed in the light, the difference being most marked in the younger series of leaf segments (I). No doubt this is due in part to the fact that the illuminated leaves absorb much greater relative amounts of N from the solution.

The age of the leaf segment, however, has also a pronounced influence on the N loss. The losses are heaviest in the oldest segments (apical). These are mature and green and, on the whole, the light effects tend to be greatest in them. In the colourless basal segments (youngest) light has comparatively little effect.

The results suggest then that the N loss is in some way dependent upon the amount of N absorbed and also upon the internal metabolism of the leaf segment as reflected by its age. In leaf segments under these conditions, protein synthesis is confined to the two basal segments, while the apical ones may show protein hydrolysis (results in course of publication). The N losses are, therefore, greater in segments showing protein hydrolysis and presumably depend also on some product of protein hydrolysis. Examples are given below.

We have, in all, twelve series of results, each of four samples, giving data for losses of N from media containing inorganic N. Data for KNO_3 , NH_4Cl and ammonium tartrate solutions containing a similar weight of N are given below.

Table III. *Absorption and losses of N (in mg. per 10 g. leaf tissue) in the dark*

Segment	KNO_3		NH_4Cl		Ammonium tartrate	
	N absorbed	N lost	N absorbed	N lost	N absorbed	N lost
1 Base	13.51	3.54	12.08	6.80	13.16	5.23
2	8.23	2.68	10.94	5.63	7.04	1.51
3	9.53	3.75	13.98	5.88	14.13	5.27
4 Apex	12.92	8.02	16.68	13.31	21.79	11.55

All of these show losses of a similar order of magnitude. No losses are detected when using organic sources of N, urea, asparagine and alanine. It appears, therefore, that the mechanism of N loss is associated in some way with the transformation of inorganic N.

While analytical evidence as to the fate of the N absorbed is available, it usually throws little light on the causes of the N loss. We shall consider, therefore, simply a few typical examples which may be informative in this respect. These include the two extremes (a) where proteolysis and large N losses occurred, (b) where marked protein synthesis was observed. The data are calculated on 10 g. of tissue for convenience and the experimental error is indicated. For protein and total soluble N, estimates on replicate half-leaf samples agree to within 3%. This on the weight of protein-N estimated is given as the error for gains or losses of protein-N. The internal ammonia and nitrate estimations are subject to an error of 2% (from replicate samples). As no nitrate is usually present in the original leaf, gains of nitrate are subject to the single error.

Example A. (Apical segments in the dark in solutions containing KNO_3 as source of N, Table III.) In this case, 12.9 ± 0.5 mg. of nitrate-N were removed from the medium, of which 9.2 ± 0.2 mg. were found in the plant tissue. Hence 3.7 ± 0.7 mg. of nitrate-N were converted into something else. There was no significant change in ammonia-N.

The tissue lost 7.4 ± 2.0 mg. of protein-N and 4.3 ± 2.0 mg. of organic N. The total loss of N by medium and tissue was 8.0 mg. Approximately half of this appears to be derived from the inorganic source and half from the tissue, the latter being presumably derived from products of protein hydrolysis.

Example B. (Taken from Series II above, using apical (oldest) segments in the dark and in NH_4NO_3 .) The total inorganic N absorbed by 10 g. of original fresh tissue was 12.2 ± 0.5 mg. (3.8 ± 0.2 mg. ammonia-N and 8.4 ± 0.3 mg. nitrate-N). Of this amount, the leaves contained 1.9 ± 0.3 mg. (1.3 ± 0.2 mg. ammonia-N and 0.6 mg. nitrate-N). Hence 10.2 ± 0.8 mg. of inorganic N were converted or lost.

The total loss of N by medium and tissue was 21.1 ± 1.0 mg. and 10.9 ± 1.6 mg. of organic N were lost by the leaf tissues, that is to say that once again about half the loss was derived from the inorganic N absorbed and half from the organic N in the leaf. As in example A, protein hydrolysis occurred, the loss of protein-N being estimated as 8.5 ± 1.5 mg. Here again therefore the loss of organic N might fall mainly on products of protein hydrolysis.

Example C. (Apical segments, in the dark and with NH_4Cl as the source of N, Table III.) The ammonia-N absorbed from the medium was 16.7 ± 0.5 mg., of which 2.5 ± 0.2 mg. remained as such in the tissue. The latter also gained 0.9 ± 0.02 mg. of nitrate-N, the original tissue having (as is usual) none of this fraction. (The production of nitrate from ammonia was observed in all samples of daffodil leaves in NH_4Cl .) Thus 13.3 ± 0.7 mg. of inorganic N were converted into other forms. The total loss of N from the medium and tissue was 13.0 ± 0.5 mg., that is, about the same as the inorganic N converted. Hence we have either to assume that the inorganic N was wholly lost, or else to argue from examples A and B that one-half of the N loss (6.5 mg.) came from products of protein hydrolysis, and half from the inorganic N.

In agreement with this latter assumption is the fact that the protein-N decreased by 7.5 ± 1.7 mg. In this case about 6.8 mg. of inorganic N must have been built up into forms of organic N such as amides. The total organic N remained unchanged.

Example D. (Taken from Series II, second segments (zone of elongation) in ammonium nitrate and in the light.) While the preceding examples represent cases where N losses and protein hydrolysis predominate, the present one is the example giving least N loss and most protein synthesis of those examined. In this case, 10 g. of tissue absorbed 20.9 ± 0.4 mg. N from the solution (8.3 mg. of

ammonia-N and 12.6 mg. of nitrate-N). No nitrate was present in the tissue but 5.5 ± 0.2 mg. of ammonia-N were gained. (The facts presumably demonstrate the rapidity with which nitrate is converted in young tissues in the light.) The tissue gained 16.4 ± 1.1 mg. of total N, showing a gain of 10.9 mg. of organic N. The gain of protein-N amounted to 5.4 ± 0.9 mg. The total N loss by tissue and medium was 4.45 ± 1.0 mg. In this case, then, we may say approximately that of four parts of inorganic N absorbed, one remained as such in the tissue, one was lost, one became protein and one was present as some other form of organic N. While such examples as this are of interest in showing the considerable N losses in an actively synthetic tissue, they throw little light on the source of the N losses and hence no further example will be given.

DISCUSSION

As no N losses are observed when organic sources of N are used, the mechanism of N loss must involve one of the stages of conversion of inorganic N. Warburg & Negelein [1920] have shown with *Chlorella* that nitrate is reduced via nitrite to ammonia. The existence of a similar process has been demonstrated in higher plants though details as to ammonia production are less complete. In particular, Eggleton [1935] has shown the presence of nitrites in leaves engaged in nitrate reduction and has, incidentally, also suggested that their presence may lead to losses of N in the manner described below. We assume, therefore, that nitrate reduction in acid media proceeds

Our experiments with NH_4Cl show that the process may be reversible.

The presence of nitrous acid in tissues containing monoamino-N should lead to the production of free N:

Van Slyke [1912] has pointed out that, *in vitro*, this reaction is extremely rapid, whilst by contrast the reaction between nitrous acid and ammonia or asparagine amide-N is extremely slow.

The fact that nitrous acid may be formed in suitable quantities was shown some years ago by Irving & Hankinson [1907]. They immersed *Elodea* in solutions of nitrate and asparagine (as a source of monoamino-N) and proved the production of gaseous N. They interpreted their results simply as showing the presence of a nitrate-reducing enzyme and the production of nitrite. Clearly, however, the nitrite in the tissues must be present as nitrous acid and there seems no reason why it should not react with any amino-N present in the tissues. Our own and the other experimental results mentioned could all be combined in the following scheme:

In this, equal amounts of amino-N and nitrite-N would be degraded when N is lost.

It will be observed that in older tissues, tending towards protein hydrolysis, an abundant supply of nitrates (undergoing reduction) will tend to increase the rate of N loss (cf. Table II and examples A and B). Further, the existence of

N losses when ammonium salts are supplied, would, on this hypothesis, be a proof of the oxidation of ammonia apart from the evidence given in example C. Finally, when upgrade processes predominate (as in example D) all of the N loss might fall upon freshly synthesized amino-N and nitrites accumulating during reduction.

The question arises as to whether the plant tissues we used are sufficiently acid to permit the presence of free nitrous acid. The work of Barritt [1931] bears upon this question. He found that the process of N loss here postulated may be brought about by bacteria and that it occurs in soils where amino-acids and nitrites are produced simultaneously by bacterial action. According to Barritt no such reaction appears to take place if the *pH* of the medium is above 6.0. In this connexion, it may be recalled that the nutrient medium in our experiments was buffered at *pH* 5.4 for daffodil leaves, and at *pH* 6 for *Chlorella*, in which large N losses were only observed in one type of medium. The expressed sap of the daffodil leaves used has a reaction of *pH* 5.2–5.6. The internal *pH* of *Chlorella* appears to lie in a similar range. The available data on our interpretation thus agree with Barritt's results.

Our results would agree equally well with the assumption that N losses originate from the decomposition of ammonium nitrite. It would be necessary merely to assume in examples A and B that amino-acids were oxidized to yield ammonia. Apart from the evidence of Van Slyke that ammonia and nitrous acid decompose relatively slowly under his conditions, Barritt [1931] has also considered this possibility and concludes that this reaction does not take place at a measurable rate at ordinary temperatures. We therefore assume that this reaction is of negligible importance in our experiments.

SUMMARY

1. Under certain conditions and when inorganic sources of nitrogen are supplied, plant tissues may lose considerable amounts of nitrogen.
2. These losses are greatest in older tissues where proteolysis is in progress.
3. A mechanism from which nitrogen loss may result is suggested.

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