CLXX. THE ASSIMILATION OF INORGANIC NITROGENOUS SALTS, INCLUDING SODIUM NITRITE, BY THE GRASS PLANT.

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THE primary object of this investigation was to obtain information regarding the effect of applying sodium nitrite to grassland and to determine whether the nitrite accumulated in sufficient quantity in the herbage to cause metabolic disturbance. For comparative purposes, however, three other treatments were included: ammonium sulphate, sodium nitrate and potassium sulphate. By sampling and analysing the herbage at intervals after the application of the salts to the soil, it was hoped to follow to some extent the course of the elaboration of protein-N from the various forms of inorganic nitrogen assimilated by the plant. For this purpose the amide-N, pepsin-HCl-soluble N, true protein-N and total-N were determined as well as ammonia-N, nitrate-N and nitrite-N. It had been hoped to obtain a measure of the total amount of dry matter at each sampling, so that the nitrogen partition could be considered in terms of absolute weight per unit area, but unfortunately other considerations prevented this being done on a sufficiently accurate basis. Consequently the amounts of nitrogen present in various forms in the herbage are of necessity expressed as parts per 100 of dry matter, and this must be borne in mind when making comparisons. The course of the nitrogen transformations in the soil was followed to some extent by analyses of samples of soil taken from the various plots at time of grass sampling, the ammonia-N, nitrate-N and nitrite-N being determined in the soil extract.

EXPERIMENTAL.

The experimental plot, a five by five latin square, was situated in Drownboy Field, at this Station. The various salts were applied in solution from a small watering can fitted with a fine rose, in amounts corresponding to an application of ammonium sulphate at the rate of 3 cwt. per acre; the potash was applied at the rate of 2 cwt. per acre. The foliage was well watered afterwards to ensure that no salts adhered to the grass, the control plots receiving the same total quantity of water, 0·25 in., as the treated plots.

The soil was sampled to a depth of 3 inches with a soil sampling tube of 1 inch diameter, and the grass by plucking the required quantity from random positions over the five replicates.

So far as the analyses of the soil nitrogen are concerned, the methods used were substantially the same as those already described by the author [1934].

The "true protein"-N in the dried and finely ground grass was determined by Stützer's method, which involves precipitation of the proteins by a suspension of cupric hydroxide in glycerol and water and subsequent determination of the nitrogen content of the washed precipitate.

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The amide-N was determined by the difference in ammonia-N content of a known weight of dried and ground grass before and after boiling with HCl (5 %) to convert amides into ammonium chloride.

The pepsin-HCl-soluble N, which may be taken as a good comparative measure of the digestibility of the herbage, was determined by comparing the total nitrogen content of the original dried and ground grass with that of the residue left after digestion with pepsin-HCl. Since the figure obtained includes inorganic nitrogen if present, the necessary corrections were subsequently applied.

The ammonia-N content of the dried and ground grass was determined by distillation with magnesia.

Estimation of nitrite-N in grass. Immediately after sampling, 10 g. of the wet grass, the dry matter content of which was subsequently determined, were macerated in a stone mortar with 50 g. of sand previously extracted with HCl and ignited. The grass was effectively disintegrated only when the very minimum of water was added. After maceration, the remainder of 100 ml. water was added, and the whole filtered on a Büchner funnel. An aliquot (50 ml.) of this filtrate was immediately clarified by the addition, in order, of 2 ml. of N $CuSO_4$, 0·5 g. $MgCO_3$ and 0·5–1·0 g. $Ca(OH)_2$. After filtration, the nitrite was determined colorimetrically on an aliquot by the Griess-Ilosva method, the whole operation not taking more than half an hour.

Estimation of nitrate-N in grass (due to Mr F. L. Ashton[1]). The method consisted of boiling the finely ground grass with water and determining the nitrate content of an aliquot part of the clarified extract by the phenoldisulphonic acid method. Since the accuracy of the method depends entirely on the efficiency of the clarification, both as regards removal of organic material and the non-removal of nitrate, a brief description of the method used to clarify the water extract is necessary. The bulk of the organic matter present was precipitated by the addition of a solution of basic lead acetate, and, after filtering and washing, the cold filtrate was shaken with a small quantity of "norite". The excess lead and any chlorides present were then precipitated by the addition of $(NH_4)_2SO_4$ and Ag_2SO_4 and the combined precipitates, including the norite, filtered off. The nitrate determination was then conducted colorimetrically on an aliquot of the filtrate after taking to dryness. In control experiments satisfactory recoveries of known amounts of added nitrate were obtained.

Nitrogen partition in the soil.

In Table I are set out the amounts of ammonia-N, nitrate-N and nitrite-N found in the soil on the various sampling dates.

The nitrogen was added at the rate of 69·5 lb. per acre which, since the weight of 1 acre of dry soil from Drownboy Field to a depth of 3 in. is $0·9 \times 10^6$ lb., corresponds to 77 p.p.m. of dry soil. It will be seen that the figures for ammonium sulphate, sodium nitrate and sodium nitrite, given in Table I, fall short of this amount by 42, 49 and 54 p.p.m. respectively. Whilst, as is shown by Table III, some of this deficiency is undoubtedly due to assimilation by the grass during the interval between applying the salts and sampling the soil, by far the greater part must have arisen from inefficient separation of soil and root by the sieving process, particularly as regards the surface layer, where the added salts were mainly located. It is consistent with this that the amounts recovered were materially higher on the following day after a night's rain, when presumably the salts were washed down into layers where the smaller amount of organic débris

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[1] A detailed account of this method, including several modifications subsequently introduced, is now in the press.

Table I. *Inorganic nitrogen partition in the soil following the addition of various salts.*

p.p.m. dry soil.

Days commencing 1. v. 33 ...	1	2	3	9	24
Ammonia-N:					
Control	6·7	8·2	7·6	11·1	6·8
(NH₄)₂SO₄	5·6	34·9	43·4	14·8	10·0
NaNO₃	6·6	9·9	8·7	12·1	10·1
NaNO₂	7·0	8·7	13·7	14·9	9·4
K₂SO₄	6·3	6·5	13·3	5·9	9·8
Nitrate-N:					
Control	(12·7)	4·1	2·2	1·4	0·3
(NH₄)₂SO₄	2·8	1·7	0·5	2·5	1·0
NaNO₃	3·1	27·7	(37·2)	7·6	0·6
NaNO₂	2·1	0·1	1·4	2·7	0·6
K₂SO₄	3·5	1·0	12·7	2·1	0·6

(Some doubt attaches to the figures in brackets.)

	1	2	3	9	24
Nitrite-N:					
Control	<0·1	0·04	0·06	0·07	Traces
(NH₄)₂SO₄	<0·1	0·07	0·17	0·09	Traces
NaNO₃	<0·1	0·22	0·08	0·09	Traces
NaNO₂	<0·1	22·8	27·0	0·32	Traces
K₂SO₄	<0·1	0·06	0·25	0·06	Traces

permitted a more efficient separation of the soil. The deficiencies on this occasion were less; 34, 40 and 50 p.p.m. respectively. It is apparent from this that if the assimilation of nitrogenous salts were to be followed in any detail from the soil side, adequate provision would have to be made to ensure a much more effective separation of soil from root than that provided by the sieving method now employed. It might even be advisable to consider soil and root together by drying the soil sample *in vacuo*, sieving and re-incorporating the organic matter thus separated after grinding in a suitable mill.

However, in spite of the complication mentioned above, several points are abundantly clear. Firstly, only a very small fraction of the nitrogen originally added remained in the soil in the form of ammonia, nitrate or nitrite 8 days after addition. So far as can be judged from the above figures, ammonium sulphate has disappeared least quickly and sodium nitrite most quickly, with sodium nitrate intermediate. So much is in accord with accepted opinion. The disappearance of the nitrite, however, does not appear to have been accompanied by an increase in nitrate-N in the soil. Moreover, there was no corresponding accumulation of nitrite-N or nitrate-N in the herbage. For example, on the 24th day there were still 580 p.p.m. of nitrate-N in the grass which had been treated with sodium nitrate, but the grass which had been treated with sodium nitrite had by this time no more nitrate-N than it had at the beginning of the experiment, *viz.* 10 p.p.m. This rather gives the impression that the nitrite has been dealt with either in the soil or in the plant much more quickly than the nitrate and, moreover, in a way which presumably has not entailed its conversion into nitrate, in which case it should have been detected in this form either in the grass or in the soil. This might be explained by assuming an enhanced rate of synthesis of protein, but the fact that the amount of pepsin-HCl-soluble N, which fraction includes amino-acids, was lowered in the nitrite-treated grass (see Tables V and VII), lends colour to the view that a proportion of the nitrite had reacted with amino-compounds present in the grass with the resulting formation of free nitrogen.

Another feature of Table I is that the amount of ammonia-N in the soil was increased following the application not only of ammonium sulphate (which is, of course, expected), but also of sodium nitrate and, to a greater extent, of sodium nitrite. This increase in ammonia-N, which has been observed in the case of sodium nitrate on other occasions, indicates that some of the added nitrate and nitrite had undergone a process of reduction.

Finally, it may be noted that a slight increase in nitrite-N took place immediately following the application of sodium nitrate, although the added sodium nitrate was free from nitrite.

Nitrogen partition in the herbage.

All the nitrogenous salts had the effect of increasing the proportion of total nitrogen in the dry matter of the herbage, the greatest increase occurring with

Table II. *Increases in the percentage of total nitrogen in the dry matter of spring grass treated with various salts.*

Fertilisers added 2 hours before sampling on 2nd day.

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·27	0·59	0·98	0·33
$NaNO_3$	—	0·27	0·52	1·15	0·46
$NaNO_2$	—	0·27	0·32	0·32	−0·07
K_2SO_4	—	0·24	0·69	0·37	0·19

(Mean initial percentage N in control = 3·93 ± 0·12.*)

* The estimate of error has been determined in all cases from the six values obtained from the five parallel observations on the first day together with that for the control on the second day.

Table III. *Inorganic nitrogen partition in the dry matter of spring grass before and after application of various salts.*

Application made 2 hours before sampling on 2nd day. Results in p.p.m.

Days commencing 1. v. 33 ...	1	2	3	9	24
Ammonia-N:					
Control	430	380	450	500	390
$(NH_4)_2SO_4$	390	920	640	830	320
$NaNO_3$	380	600	530	630	390
$NaNO_2$	420	490	420	420	250
K_2SO_4	380	500	480	480	320
		(S.E. = ±23.)			
Nitrate-N:					
Control	<10	<10	<10	<10	<10
$(NH_4)_2SO_4$	<10	<10	<10	510	290
$NaNO_3$	<10	880	560	1250	580
$NaNO_2$	<10	60	20	310	<10
K_2SO_4	<10	<10	<10	<10	<10
		(Accuracy approx. 10 p.p.m.)			
Nitrite-N:					
Control	0·25	0·7	f. tr.	—	—
$(NH_4)_2SO_4$	0·25	1·1	f. tr.	f. tr.	—
$NaNO_3$	0·25	4·3	f. tr.	f. tr.	—
$NaNO_2$	0·25	44·0	tr.	—	—
K_2SO_4	0·25	0·7	f. tr.	f. tr.	—
		(S.E. < ±0·1 p.p.m.)			
9 a.m. soil temperature (°F.) at 4 in. (10 cm.)	51·0	51·0	50·0	—	—
Sunshine (hours)	4·3	0·0	2·4	—	—

sodium nitrate and the least with sodium nitrite. Potassium sulphate also increased the total nitrogen content. The results are shown in Table II as increases relative to the control.

Ammonium sulphate, sodium nitrate, potassium sulphate and sodium nitrite all increased the proportion of ammonia-N in the dry matter, in order of descending magnitude, the maximum increase occurring shortly after application to the soil (see Table III). The increase in ammonia-N content of the herbage following application of potassium sulphate may be explained in terms of base exchange, the potash liberating ammonia from the soil colloid complex.

The proportion of nitrate-N was very markedly increased soon after addition of the sodium nitrate. Ammonium sulphate and sodium nitrite in the order mentioned also increased the nitrate content, but the increases were not immediately apparent. No change in nitrate content followed the application of the potassium sulphate.

The proportion of nitrite-N showed immediate increases in all cases where a nitrogenous salt was added, the increase being greatest for sodium nitrite and least for ammonium sulphate. Potash was without effect. Only faint traces of nitrite-N were detected in any treatment 24 hours after application, and none was detected subsequently. So far as the eye could judge, the grass suffered no harm from the application of sodium nitrite at the rate of 3 cwt. per acre.

Table IV. *Differences in percentage of amide-N in the dry matter of spring grass treated with various salts.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	−0·030	+0·033	+0·075	−0·039
$NaNO_3$	—	−0·041	+0·029	+0·052	−0·033
$NaNO_2$	—	−0·012	+0·044	+0·035	−0·043
K_2SO_4	—	−0·017	+0·034	−0·003	−0·012

(Mean value for control on 1st day = 0·109 % ±0·013.)

The proportion of amide-N was increased after an initial drop, due to the application of other forms of nitrogen and reached a maximum about 8 days after application. At the end of the experiment the proportion of amide-N on all the treated plots was substantially lower than on the control.

Table V. *Increases in $\%$ pepsin-HCl-soluble N (less inorganic N) in the dry matter of spring grass, following treatment with various fertilisers.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·17	0·50	0·63	0·24
$NaNO_3$	—	0·12	0·45	0·88	0·41
$NaNO_2$	—	0·16	0·23	0·25	−0·03
K_2SO_4	—	0·17	0·71	0·37	0·13

(Mean value for control on 1st day = 2·81 % ±0·08.)

The proportion of pepsin-HCl-soluble N was also increased, sodium nitrate showing the greatest and most permanent increase, followed in order by ammonium sulphate and potassium sulphate. The pepsin-HCl-soluble N was only slightly increased by the sodium nitrite treatment and was actually slightly less than that of the control at the end of the experiment.

A rise in the proportion of "true protein"-N occurred in all cases on the day after application; this increase was maintained with ammonium sulphate and sodium nitrate until the end of the experiment, but with sodium nitrite the proportion was substantially the same as in the control by the 9th day and was

Table VI. *Increases in $\%$ "true protein"-N in the dry matter of spring grass following treatment with various fertilisers.*

Days commencing 1. v. 33 ...	1	2	3	9	24
$(NH_4)_2SO_4$	—	0·07	0·33	0·22	0·26
$NaNO_3$	—	0·13	0·36	0·32	0·26
$NaNO_2$	—	0·19	0·30	0·06	−0·25
K_2SO_4	—	0·02	0·44	−0·16	0·02

(Mean value for control on 1st day = 3·23 % ±0·08.)

markedly lower on the 24th day. Potassium sulphate increased the proportion of "true protein"-N on the day after application, but the increase did not persist.

It is of interest to consider the sum of the following forms of nitrogen, which according to the methods of analysis employed are mutually exclusive, in relation to the total amount of nitrogen found to be present by separate Kjeldahl determination.

Table VII. *Amount of nitrogen in spring grass in the form of "true protein", amide, NH_3, NO_3 and NO_2, expressed as $\%$ of the total N present.*

Days commencing 1. v. 33 ...	1	2	3	9	24
Control	88·6	89·3	88·8	98·5	101·6
$(NH_4)_2SO_4$	88·9	86·0	85·1	85·2	102·4
$NaNO_3$	88·1	87·9	88·3	84·4	98·3
$NaNO_2$	88·2	87·8	90·0	92·8	96·0
K_2SO_4	92·9	88·4	91·1	88·6	104·7

At the commencement of the experiment all four samples agree in showing that between 7 and 12 % of the nitrogen known to be present remains undetermined. On the 24th day of the experiment this undetermined fraction, which, from the nature of the analysis, consists of fairly simple amino-acids, peptides, and other simple soluble organic nitrogen compounds other than amides, not precipitable with Stützer's reagent, has decreased to negligible proportions in all cases; indeed, on the control grass this state of affairs has been reached by the 9th day. It would appear, therefore, that by the beginning of May the rate of protein synthesis is such that the margin of partially elaborated nitrogen compounds is extremely small, and that this condition may not be reached until some weeks later when inorganic nitrogenous salts are added to the soil.

The presence of nitrite-N in the expressed juice of spring grass.

Schimper [1888] showed that nitrites are always present in the living leaf when the plant is in the dark, and that they disappear when the leaf is exposed to light. With regard to its occurrence in the plant under illuminated conditions, Rimington and Quin [1933] have found traces in the fresh juice of certain members of the genus *Tribulus*. It has also been detected in the stem of grape vines, in the leaves of carrot and in the leaves of soya bean in the early stages of growth, but so far as can be ascertained, its occurrence normally in spring grass has not been recorded. The amounts found by the author were always small (< 1 p.p.m. of dry matter).

In the experiment described above, the amount of nitrite in the expressed juice was increased within 2 hours of application to the soil, not only on the plots treated with sodium nitrite and sodium nitrate, but also on those treated with ammonium sulphate, a fact which seems to indicate that some at least of the ammonium sulphate was oxidised in the leaf. (There was no increase of

nitrate in the leaf until a few days later.) Attention has been already drawn to the rapid assimilation of nitrogenous salts by Eckerson [1924], who observed nitrate in the leaves of tomato 24 hours after applying calcium nitrate to the roots—she also observed nitrite 36 hours after treatment.

Mevius and Dikussar [1930] showed that in neutral or alkaline solution *Zea Mays* could utilise nitrites without metabolic disturbances. They found that the optimum nitrite concentration was about 50 p.p.m. in a neutral solution, and that at greater concentrations protein synthesis was retarded with simultaneous accumulation of amides and ammonia but not nitrite. In the experiment recorded here no evidence was obtained of accumulation of the forms of nitrogen mentioned. During the first 9 days after application the nitrite materially increased the percentage of true protein-N and pepsin-HCl-soluble N, as did the other fertilisers tested but to a smaller extent, but by the 24th day after application, the percentages of total-N and pepsin-HCl-soluble N, true protein-N, amide-N and ammonia-N were found to be depressed below the corresponding values in the untreated grass, although the applied nitrite had already disappeared from the soil and the herbage by the 9th day. The highest concentration of soil nitrite-N recorded in this experiment was 27 p.p.m., corresponding to 190 p.p.m. of soil solution, and the p_H of the soil was 6·8.

The temporary accumulation of nitrite in unmanured grass is undoubtedly conditioned by rapid assimilation of nutrients and by low intensity of solar radiation, a state of affairs which may be presumed likely to occur only in early spring or late autumn (or in summer under heavy shade). It appears likely, therefore, that it is a contributory factor in the so-called "grass tetany" of cattle which is sometimes observed at these times and is usually associated with high inorganic nitrogen content of the herbage.

In this connection it is of interest to record an observation made at Jealotts Hill. On May 9th, 1933 it was noticed that stock had broken out of a field (A) which had been treated with nitrochalk a few days previously. They were found grazing contentedly in the adjoining field (B) which had not been so treated. To the eye, no difference in the herbage of the two fields was apparent, but in view of the possibility that the stock had detected some disagreeable quality in the herbage, samples were taken from both fields and examined for nitrite by the above method, with the following results.

Table VIII.

| | Nitrite-N | | Potassium nitrite |
	p.p.m. wet grass	p.p.m. dry matter	g./cow/day*
Field A	1·2	7·0	0·6
Field B	0·4	2·0	0·19

* Assuming a daily consumption of 30 lb. dry matter per cow per day.

Thus it will be seen that there was at least three times as much nitrite present in the grass which the stock found unpleasant as in the grass which they found palatable.

Unfortunately nitrite was the only nitrogenous constituent looked for on this occasion, so that there still remains the probability that nitrate was also present in the treated grass, although this is known to be considerably less toxic than nitrite. Nevertheless, apart from the certain amount of circumstantial evidence concerning the part played by nitrite in "grass tetany", the figures demonstrate that nitrite occurs naturally in grass in the spring, and that the amount may increase after treatment with a nitrogenous manure.

The significance of nitrite-N in protein synthesis.

Berthelot and Gaudechon [1911] demonstrated nearly twenty-five years ago that nitrite was formed when solutions of ammonium salts were exposed to ultra-violet radiation, and more recently Dhar *et al.* [1933] and Corbet [1934] have confirmed that nitrite formation can be effected by ultraviolet light in the absence of micro-organisms. It would appear likely that the same reaction occurs in the grass leaves under the action of sunlight. The appearance of nitrite after the application of sulphate of ammonia certainly supports this view and raises the question whether the ammonia taken up as such all goes through the nitrite stage or whether part is directly synthesised into amino-compounds and only a fraction is converted into nitrite. In any case, the simultaneous occurrence of amino-compounds and nitrites may constitute a fruitful source of loss of nitrogen in the elementary form, the reaction forming what is probably a natural safeguard against undue accumulation of nitrite.

It might be mentioned in passing that the much vexed question whether ammonia-N or nitrate-N is more readily utilised by the grass plant has still to be answered, and although there is a mass of circumstantial evidence based on the observed ready uptake of applied ammonia by the plant under certain conditions, no conclusive evidence has yet been brought forward to show that nitrogen is freely taken up by grass in the ammoniacal form *in natura*. It is true that plants grown *in vitro* and supplied with ammonium salts as a sole source of nitrogen definitely do assimilate ammonium salts as such, but the conditions are far from natural. Even the entirely practical conditions of manuring in the field with ammonium sulphate, when again ammonia can be shown to be taken up, as in the above experiment, can hardly be described as normal to the plant. Nevertheless, the point to be stressed is that even when the plant is faced with the necessity of dealing with its nitrogen in the ammoniacal form, a mechanism certainly exists whereby a part, if not all, can be oxidised to nitrite, thereafter to be dealt with possibly as suggested by Baly *et al.* [1922].

Work has been in progress in this laboratory on the photosynthesis of amino-acids from solutions of nitrite and sugars, and definite evidence of a reaction has been obtained, and recently also Dhar and Mukherjee [1934] have been successful in synthesising amino-acids by exposing solutions of glycol or glucose and nitrates to sunlight in the presence of titania as a catalyst. The reaction obviously proceeds through the nitrite stage. There would thus appear to be substantial grounds for believing that the photochemical formation of nitrite from nitrate and ammonia is of primary and fundamental importance in the synthesis of protein in plant tissue.

SUMMARY.

An attempt has been made to follow the transformation of various forms of inorganic nitrogen applied to the soil into elaborated organic nitrogen in the grass plant.

Under the conditions of the experiment, applied inorganic nitrogen was detected in substantial amounts in the herbage only 2 hours after application to the soil.

In early spring, synthetic processes in the leaf are slow enough to permit the accumulation of simple inorganic and organic compounds. This margin of partially elaborated nitrogen rapidly disappears, but at a diminished rate, when inorganic nitrogenous salts are applied to the soil.

Nitrite occurs naturally in spring grass, and the amount is increased when the grass is treated with ammonium sulphate or sodium nitrate at this time. It

is suggested that nitrites play a significant part in the so-called "grass tetany" of cattle, which is usually associated with herbage of high inorganic nitrogen content.

Grass treated with sodium nitrite at the rate of 3 cwt. per acre showed no visible signs of distress, although chemical analysis of the herbage gave evidence of metabolic disturbance.

The possible significance of nitrite in the synthesis of protein is briefly discussed.

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