Effect of Ammonium and Nitrate Nutrition on Protein Level and Exudate Composition [1, 2]

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Research efforts of recent years on the protein metabolism of plants have been primarily directed toward clarification of the cellular mechanism of protein synthesis. Less attention has been devoted to the analysis of those factors that determine protein concentration levels in organs of whole plants. A review on this subject is included in the monograph of Webster (18). Past efforts of the present investigator in this area have been concerned with the influence of the ammonium: nitrate ratio of the culture solution on protein level in shoots of young wheat seedlings grown in darkness (19, 20). The shoot protein level of plants provided with a simultaneous supply of ammonium and nitrate was found to be greater than that of plants grown with either ammonium or nitrate alone. The present study concerns itself with the effect of ammoninum and nitrate on protein synthesis in young leaves of sunflower grown in the light. Preliminary experiments indicated that protein levels were again at a maximum under ammonium plus nitrate conditions. Among the factors that may play a role in the determination of leaf protein level, one is the nature of the nitrogenous substances transferred to the leaves from the root system. In an attempt to explain the decline in protein experienced by detached leaves, Chibnall (5) suggested that roots provide some factor or factors necessary for leaves to maintain their protein levels. This hypothesis is relevant to the problem of protein level in attached leaves under investigation in this laboratory. It is possible that the simultaneous presence of ammonium and nitrate in the culture solution may lead to the synthesis in the roots of a complex of nitrogenous compounds which, when transported to the leaves, permits the establishment of a high protein level. Accordingly, a study was made of the amino acids, amides, and inorganic nitrogen ions in the exudate of decapitated sunflower plants that had been grown under ammonium, ammonium plus nitrate, and nitrate conditions.

Materials and Methods

Seeds of *Helianthus annuus* L. var. Mammoth Russian were soaked in distilled water for 1 hour and individually planted in paraffined cups containing washed sand. The cups were supported in 400 ml beakers which served as collectors of culture solution applied to the seedlings. Excess solution dripped through glass wool covered perforations in the bottom of the cups. Distilled water was added to the seedlings during the first 6 days of growth and this was followed by the application of a previously described culture solution lacking nitrogen (20) for a period of 4 days. On the morning of the eleventh day the plants were divided into 3 classes of 12 plants each and, after the removal of the cotyledons, 25 ml of complete culture solution containing nitrogen as either 0.01 M NH_4Cl, 0.005 M NH_4Cl plus 0.005 M KNO_3, or 0.01 M KNO_3 were supplied to the plants of the respective classes. A similar addition of complete culture solution was made the morning of the twelfth day and the following morning, after receiving their third allotment of complete culture solution, the plants were sliced in the hypocotyl region about 1 cm above sand level. Exudates were collected over a 1-hour period with fine-tipped 10 μl graduated pipettes and were applied directly to chromatography paper for amino acid and amide analysis. The complete procedure was repeated 4 times and each time the 2 sets of opposite leaves that had developed were collected, separated into lots of older and younger leaves, and dried in a forced draft oven at 75°. The dry tissue of each trial was weighed and ground, pooled with the tissue collected in other trials, and saved for analysis of total and protein nitrogen by Kjeldahl methods previously described (19). In a fifth repetition of this experiment the exudate was subjected to analysis of its ammonia and nitrate content. Ammonia was determined by the vacuum distillation method of Archibald (1) and nitrate was determined colorimetrically with phenoldisulfonic acid (9).

In a second experiment, 2 groups of plants were grown in order to determine the effect of a single application of nitrogen on the nitrogenous composition of the exudate. Plants were grown in the usual manner for a period of 10 days, and the next morning, after removal of the cotyledons, each group of plants was divided into 4 classes. The first class received culture solution lacking nitrogen, and the other 3 received solutions containing ammonium, ammonium plus nitrate, or nitrate. The plants were decapitated and exudate was collected for 1 hour. Exudates from 1 group were used in the determination of amino acids

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and amides; the second group provided exudate for ammonia and nitrate analysis.

A third experiment was performed in which plants were grown as in the first experiment for 12 days. On the morning of the thirteenth day, NH_4 plants, $NH_4 + NO_3$ plants, and NO_3 plants were each divided into 4 subclasses. The first subclass of each nitrogen class was decapitated and exudate was collected for chromatographic analysis. The remaining 3 subclasses received their normal allotment of culture solution containing nitrogen and were decapitated after 15, 120, and 480 minutes. Exudates were collected for 1 hour.

Amino acids and amides were determined by 2-dimensional chromatography of the exudates applied to Whatman No. 1 filter paper. Butanylalcohol: acetic acid: water (4: 1: 4) was used in the first direction and water-saturated phenol in the second. The substances in the ninhydrin positive spots were separately eluted with 50 % ethanol in phosphate buffer. The color density was determined in a Klett-Summerson photoelectric colorimeter and compared with amino acid standard curves prepared, for each separate exudate analysis, from chromatograms of known amino acids.

During all of the period of growth, the plants were exposed to continuous illumination provided by a bank of fluorescent lamps in a controlled temperature room.

Results

Growth and Nitrogen Content of Leaves. Experiment 1. Table I refers to the dry weight per plant of the 2 sets of opposite leaves that had developed by the thirteenth day after planting. Of the two, leaf set 1 is the older and appeared 5 days after planting. It will be noted that leaf growth with ammonium plus nitrate was superior to that with either ammonium or nitrate alone. These leaves received nitrogen from the cotyledons during the first 5 days of their growth and from the culture solution for the final 3 days. An even greater measure of superior growth under ammonium plus nitrate conditions occurred in leaf set 2. These leaves appeared 10 days after planting and it was at this time that the cotyledons were removed and nitrogen was supplied from the culture solution.

Table I also reveals that on both a concentration and set-of-leaves basis protein nitrogen accumulation in leaf sets 1 and 2 of $NH_4 + NO_3$ plants was superior to that of plants receiving either ammonium or nitrate alone. Particularly striking are the results for leaf set 2 in which protein nitrogen on a concentration basis for $NH_4 + NO_3$ plants was 24 % and 18 % greater than that of NH_4 and NO_3 plants, respectively; on a set-of-leaves basis protein nitrogen for $NH_4 + NO_3$ plants was 100 % greater than that in NH_4 or NO_3 plants. Also to be noted is the high concentration of total nitrogen in the younger leaves of NO_3 plants.

Exudate Composition. Experiment 1. Within the collection period of 1 hour after decapitation, the yield of exudate from each plant ranged from 7 to 10 μl. No constant difference in the rate of exudation from plants treated with the 3 forms of nitrogen could be detected and in this respect the results differ from reports of a superior rate of exudation in tomato with ammonium (10). Table II records the results of analyses performed on exudates collected from plants that had been treated with 3 applications of the appropriate nitrogen-containing solution. Total nitrogen per ml of exudate was at a maximum with NO_3 plants and primarily responsible was a high level of nitrate nitrogen. This was present in the exudate at a concentration 3.6 times that of the culture solution. Ammonia, too, was at a maximum concentration in the exudate of NO_3 plants with the result that inorganic nitrogen constituted 79 % of the total nitrogen. The exudate of $NH_4 + NO_3$ plants contained 54 % of the total nitrogen in the inorganic form while only ammonia, constituting 25 % of the total nitrogen, was found in NH_4 plant exudate.

Table I. *Dry Weight, Total Nitrogen, and Protein Nitrogen of Sunflower Leaves as Influenced by Three Applications of Nitrogen*

Treatment	Dry wt	Total nitrogen		Protein nitrogen	
	mg/ set	mg/ g	mg/ set	mg/ g	mg/ set
		Leaf set 1			
NH_4	15.2	59.5	0.90	29.9	0.45
$NH_4 + NO_3$	16.9	59.0	1.00	33.1	0.56
NO_3	13.2	57.4	0.76	29.1	0.38
		Leaf set 2			
NH_4	0.9	86.8	0.08	37.1	0.03
$NH_4 + NO_3$	1.3	93.8	0.12	46.1	0.06
NO_3	0.7	149	0.10	39.0	0.03

The amides composed a significant portion of the total nitrogen in all exudates, contributing 71, 43, and 19 % of that present in NH_4, $NH_4 + NO_3$, and NO_3 plants, respectively. Compared to the large quantities of inorganic and amide nitrogen, amino acids constituted only a small portion of the total nitrogen. The concentration of this form of nitrogen was slightly higher in NH_4 plants than in $NH_4 + NO_3$ and NO_3 plants. As indicated in table II, the individual amino acid content in the exudate of $NH_4 + NO_3$ plants was intermediate between that of NH_4 plants and NO_3 plants. Alanine, arginine, leucine, serine, and valine were favored by ammonium treatment while γ-aminobutyric acid, aspartic acid, glutamic acid, and lysine were greater with nitrate. The differences between NH_4 and NO_3 plants were significant to the less than 4 % level for every amino acid and amide except alanine.

Exudate Composition. Experiment 2. . . No difference in the rate of exudation could be detected among the 4 classes of 11-day plants, whether treated with a single application of culture solution lacking in nitrogen or a single application of solution containing nitrogen. Table II indicates that the effects of nitro-

Table II. *Soluble Nitrogen Composition of Sunflower Exudate as Influenced by Three Applications and One Application of Nitrogen*

	Three applications					One application		
	NH_4*	NH_4 + NO_3**	NO_3*	C***	Initial	NH_4	NH_4 + NO_3	NO_3
		μg N per ml				μg N per ml		
Ammonia	169	232	261		9.9	29.6	19.8	12.3
Nitrate	...	256	505		...	...	15.1	20.1
Asparagine	119	81	29	0.4	24	40	29	25
Glutamine	350	312	155	0.1	48	170	149	130
Alanine	1.1	0.9	0.8	4.2	1.9	0.9	0.6	0.6
γ-Aminobutyric acid	0.3	0.5	1.4	14.6	1.6	0.7	0.7	0.6
Arginine	3.2	1.9	1.0	10.6	1.9	0.6	0.9	1.5
Aspartic acid	0.8	1.1	1.8	2.7	1.5	1.4	1.5	1.7
Glutamic acid	0.3	0.7	1.8	5.4	1.7	1.1	1.6	1.7
Leucine	3.8	3.3	2.0	1.4	1.6	1.9	2.1	1.9
Lysine	3.5	4.4	5.7	1.1	2.5	1.3	3.5	4.7
Serine	5.1	3.7	2.7	0.5	3.2	4.7	3.9	3.2
Valine	5.1	3.2	1.9	1.3	2.8	3.3	2.7	2.6
Total inorganic	169	488	766		9.9	29.6	34.9	32.4
Total amide	469	393	184		72	210	178	155
Total amino acid	23	20	19		19	16	18	19
Total nitrogen	661	900	969		101	256	231	206

* Amide and amino acid data represent the mean of 4 replicate trials; ammonia and nitrate data derived from 1 trial.
** Amide and amino acid data represent the mean of 3 replicate trials; ammonia and nitrate data derived from 1 trial.
*** C = coefficient of variation as per cent of mean value, all treatments; 3 application experiment.

gen treatment differ from those observed after the third application of nitrogen. Total nitrogen after 1 addition was at a maximum in NH_4 plants and inorganic nitrogen was approximately equal under the 3 conditions of nitrogen supply. Ammonia, at its maximum in NO_3 plant exudates after 3 applications, was at its lowest level in NO_3 exudates after a single application. A relatively low content of nitrate accompanied the low concentration of ammonia in NO_3 plants after a single addition. The exudate of these plants contained only 4 % of the nitrate found in the exudate of plants after the third addition of nitrate.

As was the case in the 3-application experiment, amide was highest in the exudate of NH_4 plants. However, whereas 1 application of ammonium brought the level of amide nitrogen to about one-half that in the exudate after 3 applications, the concentration of amide nitrogen in NO_3 plants after a single addition was already approximately equal to that in the exudate after 3 additions. Total amino acid nitrogen in the exudate of NH_4 plants was somewhat lower than that in NO_3 plants but, as was the case for plants treated with 3 applications, both the total of amino acid nitrogen and most of the individual amino acids in $NH_4 + NO_3$ exudate were intermediate in quantity between that in NH_4 and NO_3 exudates.

Exudate Composition. Experiment 3. No difference in rate of exudation could be detected among the plants whether they were decapitated before the third addition of nitrogen or at various times after the addition. Table III indicates that, as expected, the third application of each form of nitrogen led to an initial increase of amide in the exudates. For each treatment, however, the quantity of this fraction dropped to the original level within 8 hours. Table III further reveals that when collection of exudate was initiated 15 and 120 minutes after the third addition of nitrogen, the total amino acid content was highest in NH_4 plants, intermediate with $NH_4 + NO_3$, and lowest in NO_3 plants. However, exudates collected prior to and 8 hours after the third addition displayed maximum total amino acid under $NH_4 + NO_3$ conditions.

The changes in individual amino acid content of the exudates with time are also shown in table III. Exudates of plants treated with ammonium displayed a sharp increase, followed by a decrease to approximately the original level, of alanine, arginine, leucine, serine, and valine. No trace of γ-aminobutyric acid was detected at any time in the exudates of these plants. Upon the addition of nitrogen to NO_3 plants, aspartic acid and glutamic acid increased markedly and then fell to their original levels. The consistently low level of arginine in NO_3 plants is also to be noted. The high total amino acid level in the exudate of $NH_4 + NO_3$ plants prior to and 8 hours after the third addition of nitrogen has been referred to and table III reveals that this is due to the presence of all the amino acids in relatively high amounts.

Discussion

Since the early observations of Vickery et al. (17) on tobacco, a level of protein production with ammonium plus nitrate superior to that achieved with either ammonium or nitrate alone has been noted by Pucher

Table III. *Amide and Amino Acid Composition of Sunflower Exudate Collected before and after the Third Application of Nitrogen*

	Treatment											
	NH_4				$NH_4 + NO_3$ μg per ml				NO_3			
	*A	B	C	D	A	B	C	D	A	B	C	D
Asparagine	429	543	690	472	225	338	357	244	140	133	137	143
Glutamine	1330	1930	2068	1530	802	1508	1482	918	536	744	796	550
Alanine	4	10	12	5	7	8	6	7	5	4	5	4
γ-Aminobutyric acid	...	...	...	...	6	4	4	6	7	9	12	9
Arginine	7	15	14	10	7	8	8	7	...	4	4	...
Aspartic acid	8	8	8	6	8	12	12	12	8	19	19	11
Glutamic acid	3	5	3	5	12	12	15	16	12	19	24	14
Leucine	22	32	34	25	20	28	30	24	15	16	18	16
Lysine	14	12	12	16	20	19	21	23	22	28	28	23
Serine	24	37	40	28	22	30	24	24	15	21	16	16
Valine	20	44	48	24	16	23	28	20	16	14	12	16
Total amide	1759	2473	2758	2002	1027	1846	1839	1162	676	877	933	693
Total amino acid	102	163	171	119	118	144	148	139	100	134	138	109

* A, before; B, after 15 minutes; C, after 2 hours; D, after 8 hours.

et al. (13) with *Bryophyllum*, Weissman (19) with wheat seedlings, and Gilmore (7), again with tobacco. The data of Delwiche (6) reveal that although the protein content of detached tobacco leaves declined during culture with either ammonium or nitrate provided singly, an increase in protein occurred in leaves receiving a simultaneous supply of the 2 types of nitrogen. Steeves et al. (15) report that the growth of excised sunflower leaves provided with ammonium nitrate was superior to the growth with sodium nitrate even though the total nitrogen supply to the leaves was the same. In the present study it is clearly indicated that the growth and protein level of leaves of young sunflower plants are enhanced when ammonium plus nitrate, rather than ammonium or nitrate, is made available to the plants for the last 3 days of the experimental growth period. The most striking enhancement occurred in the younger set of leaves which was under the influence of the ammonium plus nitrate solution for all of its 3-day growth period.

That the leaves of $NH_4 + NO_3$ plants are characterized not only by a high protein nitrogen content, but also by the degree to which their available nitrogen is converted into protein is indicated by the data in table I. It may be calculated that of the total nitrogen in leaf set 1, 50.2, 56.1, and 50.7 % is in the form of protein for NH_4, $NH_4 + NO_3$, and NO_3 plants, respectively, while for leaf set 2, the same series of treatments resulted in leaves in which protein constituted 42.8, 49.1 and 26.2 % of the total nitrogen. Similar observations, indicative of a greater utilization of available nitrogen in the synthesis of protein by plants supplied with ammonium plus nitrate, have been reported (13, 19, 20). It is clear that factors other than, or in addition to, the high concentration of nitrogen in the leaves of $NH_4 + NO_3$ plants are responsible for the establishment of the high protein level; among these factors may be the nature of the nitrogenous compounds transferred from root to shoot.

Bollard (2, 3) has made an extensive examination of the nitrogenous substances found in the tracheal sap of a variety of plants. In most species examined, nitrogen was translocated principally in the form of glutamine or asparagine, with smaller quantities of glutamate, aspartate, alanine, serine, γ-aminobutyric acid, arginine, methionine, leucine, and valine usually present. The results of the present study are generally in keeping with these findings except for the absence of methionine. In this regard it may be noted that Tolbert and Wiebe (16) found sulfur to be present in only the sulfate form in the xylem exudate of barley, willow, and tomato.

The high concentration of nitrate found in the exudate of NO_3 plants in the present study suggests that much of the nonprotein nitrogen detected in the younger leaves of these plants was probably in the form of unreduced nitrate. High nitrate in the exudate is in keeping with exudate analyses on corn (12), squash (8), and tobacco (11), but is at variance with the data of Bollard who found that nitrate was present in less than half the saps tested and was always in trace amounts. It may be that the application of nitrate in luxury amounts, as exemplified in the present study by the 3-application experiment, led to an accumulation of nitrate assimilation products and so prevented further nitrate reduction. Instead, nitrate was taken up by the vascular tissue and appeared in the exudate. This view is supported by the high concentration of amide in the exudate after a single application of nitrate and by the high concentration of ammonia after 3 applications.

Of the various nitrogen fractions found in the exudate, only the amino acids, in this study, appear to be of determinative significance in establishing the level of leaf protein. That such a determinative role cannot be ascribed to the total nitrogen of the exudate is indicated by the observation that although protein was at a maximum in $NH_4 + NO_3$ leaves, total exudate nitrogen was greatest in NO_3 plants after 1 treat-

ment and in NH_4 plants after 3 treatments. Similarly, the quantity of inorganic nitrogen entering the shoot cannot be responsible for the higher protein content of $NH_4 + NO_3$ leaves because, as revealed in experiment 1, both ammonia and nitrate were greater in NO_3 plant exudate than in $NH_4 + NO_3$ exudate. Despite the quantitative importance of the amides in the exudates, this fraction, too, does not appear to be of primary significance in the final determination of protein level. Support for this view is obtained from the consistent observation that both asparagine and glutamine were present in greater amounts in the exudates of NH_4 plants than in $NH_4 + NO_3$ plants. The relatively low quantity of total amino acid in $NH_4 + NO_3$ exudates of experiments 1 and 2 would suggest that this fraction could not be responsible for the high protein level of $NH_4 + NO_3$ leaves. Experiment 3 reveals, however, that low total amino acid in $NH_4 + NO_3$ exudate obtains only immediately after the application of nitrogen and that for most of the 24-hour period between nitrogen applications the total amino acid content is maximum in the exudate of these plants. Because no significant difference could be detected in the volume of exudate produced under the 3 conditions of nitrogen supply, it may be concluded that for most of the period of time between nitrogen applications the quantity of amino acids received by the shoots of $NH_4 + NO_3$ plants was greater than that received by either NH_4 or NO_3 plants. In the shoots the amino acids may contribute to the establishment of a high protein level by a mass-action effect that prevents protein breakdown or they may act by providing a nonlimiting supply of protein precursors (18). No attempt has been made in the present study to explain the presence of a higher concentration of amino acids in $NH_4 + NO_3$ exudate. It has been previously suggested (20) that the simultaneous presence of ammonium and nitrate could stimulate the formation of glutamic acid, an amino acid known to play a central role in the synthesis of other amino acids.

Also involved in the formation of a higher level of protein in $NH_4 + NO_3$ leaves may be the concentration of individual amino acids that enter the shoot. In experiment 3, arginine could be detected in the exudate of NO_3 plants for only a short period after the third addition of nitrogen while γ-aminobutyric acid, not believed to be a constituent of plant protein, was not detected in NH_4 exudates during any of the collection periods. Many investigators (18) have established the generally accepted principle that protein synthesis proceeds at maximal rates only in the presence of all of the amino acids. This situation appears to have obtained in the present study only when ammonium and nitrate were simultaneously available to the plants. The inability of the root systems of NH_4 and NO_3 plants to supply the shoots with adequate amounts of all the amino acids may have contributed to the lower level of protein in the leaves of these plants.

Racusen and Aronoff (14) have observed that detached leaves are either completely unable to produce certain amino acids or synthesize others at very low rates. This suggests the necessity of supplementation from some other source in order to maintain the normal steady state of protein synthesis. The present study indicates that the root system may act as such a source of supplementary amino acids and that the proportions of these compounds translocated to the shoot may be influenced by the available supply of ammonium and nitrate. Furthermore, in keeping with the Chibnall hypothesis, the protein level attained in the leaves appears to be dependent upon the amino acid constituents received from the roots.

Summary

After removal of the cotyledons from 10-day old sunflower plants, nitrogen was provided for 3 days in the form of ammonium chloride, ammonium chloride plus potassium nitrate, or potassium nitrate. Dry weight, total protein, protein concentration, and protein as a percentage of total nitrogen were all greater in leaves of $NH_4 + NO_3$ plants. Neither the total nitrogen, inorganic nitrogen, nor amide nitrogen of the exudates, collected after the final addition of nitrogen, appeared to be of determinative significance in the establishment of protein level. For most of the period between nitrogen applications total amino acid nitrogen was greatest in the exudate of $NH_4 + NO_3$ plants, suggesting that this fraction may contribute to the high protein level. Also indicative of a role played by translocated amino acids in the control of leaf protein level was the low concentration of arginine in the exudate of NO_3 plants and the absence of γ-aminobutyric acid in NH_4 plant exudate.

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Dependence upon Wavelength of Stomatal Movement in Epidermal Tissue of Senecio odoris [1]

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The effect of wavelength on stomatal opening has previously been studied at rather broad spectral intervals. In general, it has been found that blue light and red light are more effective than green (4). Most authors agree that the spectral dependence of stomatal opening resembles the action spectrum of photosynthesis of leaves and the absorption spectrum of chlorophyll.

Karvé (3) used interference filters. He observed a strong stimulation of opening in blue light (439 mμ) and a smaller one in red light (680 mμ). The response in green and yellow was much smaller.

Zelitch (8) reported some preliminary observations on the stomatal opening movement in tobacco leaf discs, with use of a high power spectrograph. This instrument allows a much more detailed examination than do interference filters. He observed opening in red light and blue light, while the stomata remained closed in green.

Mouravieff (6) measured the opening movement of isolated stomata at 3 different wavelengths (464, 526, and 660 mμ). The advantage of this technique is that the stomatal movement can be studied without the effect of CO_2 depletion by mesophyll cell chloroplasts. He found an optimal opening at 464 mμ.

Hitherto no detailed spectral dependence curve of stomatal movement has been made. The aim of the present study is the detailed measurement of stomatal movement at as many wavelengths as possible with use of epidermal tissue devoid of mesophyll cells so that the effect of light on guard cells alone can be evaluated. In addition, the effect of 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) on stomatal movement was studied.

Materials and Methods

The high power spectrograph of the Plant Physiology Pioneering Laboratory, Agricultural Research Service at Beltsville was used. The energy output of this instrument could be varied by changing the width of the slit, by the use of a screen before the slit, and by the use of reflecting glass plates and converging mirrors at the focal position. Light intensity was measured with a thermopile that was calibrated against a standard lamp. The optical resolution of the instrument varied with the slit width, the number of prisms used and the wavelength. It is shown ap-

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