Commenced work 4/6/1940 Should we index? No, but file reprint when received

STUDIES ON THE NITROGEN METABOLISM OF THE BARLEY PLANT (*HORDEUM SATIVUM*)

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[*Manuscript received July* 7, 1950]

Summary

In detached barley leaves held at constant temperature in the dark, there is an increase in "soluble" and a corresponding decrease in "solid" nitrogen. In mature leaves, glutamine accumulates in the early stages of starvation and later decreases concurrently with an increase in asparagine. In detached seedling leaves, the glutamine content remains low throughout, the sequence of events otherwise resembling that found with mature leaves. Changes in the nitrogenous fractions during autolysis, in sterile conditions, of barley leaf tissue differ greatly from those found with intact detached leaves. "Soluble" nitrogen increases, but neither amide nor ammonia accumulates.

In germinating seedlings, there is a considerable accumulation of asparagine. This accumulation is more marked in the dark than in the light, and in plants supplied with nitrogen as an ammonium salt than in those supplied with nitrate. Small amounts only of glutamine were found in seedlings. The relation of the results to our knowledge of nitrogen metabolism in plants is discussed.

I. Introduction

There is strong evidence (Yemm 1937) that much of the carbon dioxide produced in the respiration of detached barley leaves arises, especially in the later stages of starvation, from nitrogenous compounds. The present paper is mainly concerned with transformation of nitrogenous compounds in detached leaves and developing seedlings of barley. It is thought that a study of changes in nitrogenous substances, known from previous work to be of metabolic importance, may throw some light on the processes whereby products of protein catabolism provide substrates for respiration.

II. Methods

All material used came from plants of *Hordeum sativum* (Plumage Archer variety) grown in conditions of known nitrogen supply. Mature leaves were taken from plants grown in glazed earthenware pots on the laboratory roof. Seedlings were grown in sand in glazed earthenware dishes, kept either in a warmed greenhouse, in a dark incubator at 21°C., or in a dark room at ordinary temperatures. All germinating seed was kept for the first 24 hours in an incubator at 25°C. The composition of the culture solution is given in Table 1; the three levels of nitrogen supply used are designated "2N," "N," and "2N/5."

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In the treatments with reduced supplies of nitrate, the supply of potassium was maintained by adding potassium chloride. In some cultures, the potassium nitrate was replaced by enough ammonium sulphate to supply an equal amount of nitrogen.

TABLE 1

COMPOSITION OF CULTURE SOLUTION (G./L.)

Calcium sulphate	0.374
Acid calcium phosphate	0.500
Magnesium sulphate	0.500
Sodium chloride	0.160
Ferric chloride	Trace
Potassium nitrate	2.72 for treatment "2N"
	1.36 for treatment "N"
	0.54 for treatment "2N/5"

III. EXPERIMENTAL CONDITIONS

Samples of detached leaves were held at 21°C. in darkened glass chambers immersed in a water-bath. In experiments where the respiratory output of carbon dioxide or uptake of oxygen was measured, these chambers remained unopened throughout; where samples were withdrawn for chemical analysis, the chambers were opened at each time of sampling. Air from outside the laboratory was drawn through the chambers after passing through cotton wool filters to remove solid particles and through baryta bubblers and soda-lime towers to remove atmospheric carbon dioxide. Finally it passed through a water bubbler, taking up enough moisture to prevent desiccation of the material. The carbon dioxide produced was absorbed by baryta in Pettenkofer tubes changed every three or six hours. In some experiments, the gas stream was switched from one to the next of a series of tubes by a clockwork device and in others the tubes were titrated and replaced at the end of each period. Mixed samples of leaves from the third, fourth, and fifth positions on the stem were used in all experiments. At the end of the experiments, the leaves were brown and sodden, but visible infection was rare, though no elaborate precautions were taken to ensure sterility.

IV. ANALYTICAL METHODS

(i) *Carbon Dioxide.*—The Pettenkofer tubes contained baryta of known strength (approximately N/10). The residual baryta was washed out and titrated with standard hydrochloric acid. Where respiratory quotients were determined, carbon dioxide and oxygen were both measured in the Haldane gas analysis apparatus.

(ii) *Extraction of Soluble Nitrogenous Compounds.*—Chibnall's ether-water method, as modified by Yemm (1937) was used. Material (5-10 g.) was cut finely, allowed to stand 15 minutes with 2 ml. ether and 15 ml. water, and pressed (2 tons/sq. in.) between tinned plates in a hydraulic press. Two

further lots of 15 ml. water were added and the sample pressed again 15 minutes after each addition of water. The juice from the three pressings was mixed, boiled for two minutes, filtered hot, cooled, and made to a known volume, aliquots being taken for the subsequent determinations. The residue on the filter paper was added to that from the press in some experiments and their total nitrogen ("solid nitrogen") determined.

(iii) *Estimation of "Solid Nitrogen."*—The residues from pressing and filtration were digested with concentrated sulphuric acid, potassium sulphate, and a little cupric sulphate, digestion being continued for six hours after the digest cleared, and the nitrogen content determined, using the Parnas-Wagner micro-Kjeldahl apparatus.

(iv) *Estimation of "Soluble Nitrogen."*—An aliquot of the filtrate was treated in the same way to determine the "soluble nitrogen."

(v) *Estimation of Ammonia.*—An aliquot of the filtrate was distilled in the micro-Kjeldahl apparatus at 45°C. under reduced pressure with a 10 per cent. aqueous suspension of magnesium oxide and the ammonia given off estimated as in the "solid nitrogen" determination.

(vi) *Estimation of Glutamine.*—The procedure of Chibnall and Westall (1932) was followed. An aliquot of the filtrate was hydrolysed for three hours in a boiling water-bath with an acetate buffer (pH 4) and the ammonia produced estimated as before. The extra ammonia, compared with that produced on distillation of the unhydrolysed extract with magnesium oxide, was taken as arising by hydrolysis of glutamine. Certain modifications of the method of Chibnall and Westall (1932) were recommended by Vickery *et al.* (1935); in particular, that the pH of the hydrolysing buffer should be raised from 4 to 6.5, as urea and allantoin can interfere at the lower pH. At pH 6.5 hydrolysis of urea is stated to be incomplete after 20 hours on a boiling water-bath. Eight extracts from leaves starved for periods up to 166 hours were hydrolysed (*a*) for 2 hours with a phthalate buffer at pH 6.3, (*b*) for 3 hours at pH 6.3, (*c*) for 3 hours at pH 4. Differences between the three sets of figures were negligible and it was therefore assumed that interfering substances were not present in significant amounts in the experimental material, though urea is known to occur in barley leaves in very small amounts (Fosse 1913; Klein and Tauböck 1931).

(vii) *Estimation of Asparagine.*—It was found, in accordance with the results of Yemm (1937), that 4 hours heating in a boiling water-bath of an asparagine solution made 2N with respect to sulphuric acid gave 98 per cent. hydrolysis and that further heating gave no increase on this figure. These conditions were therefore adopted. The buffer (pH 4) used for the glutamine hydrolysis gave 3.4 per cent. hydrolysis with asparagine; this causes no serious error in the glutamine estimations, as is shown by the very small amounts of glutamine found on some occasions in the presence of large amounts of asparagine.

(viii) *Estimation of Nitrate.*—The residue from the glutamine estimations (after distillation of the ammonia) was made more strongly alkaline by addition of 3 ml. of 30 per cent. sodium hydroxide to 20 ml. of solution, and Devarda's alloy (Sessions and Shive 1928) added to reduce nitrate to ammonia, which was estimated by distillation as usual, though serious difficulty was met through excessive frothing under reduced pressure. For this reason, nitrate determinations were made only when the results seemed likely to be of particular interest. The method gave excellent recoveries on pure nitrate solutions and it was also established that asparagine was not hydrolysed in the alkaline conditions used.

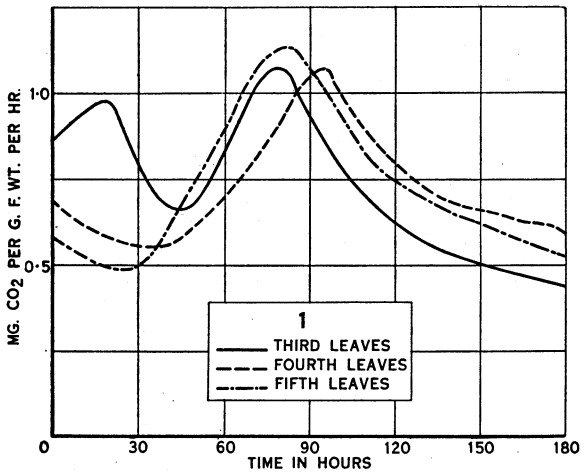


Fig. 1

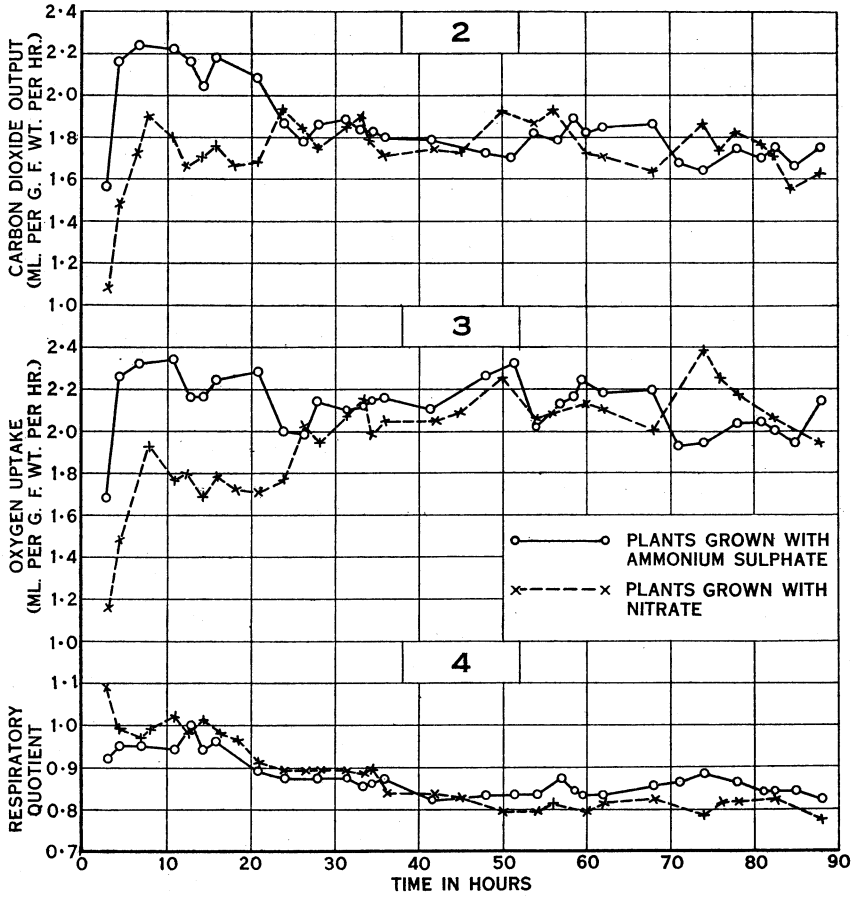
IV. Expression of Results

A single sample was used throughout an experiment for measurement of carbon dioxide output, but the chemical determinations require a new sub-sample for each occasion of sampling. The regularity of the changes in the various constituents found in most experiments indicates that the sub-samples used have been reasonably comparable with one another. For organs that have ceased to develop, such as detached leaves, the amount of each metabolite per unit fresh weight is recorded. This, however, may obscure metabolic changes in such rapidly developing material as seedlings, and for these the results are expressed in terms of both individuals and fresh weight. In the respiration experiments, the fresh weight used is that at picking and in the analytical work that at the time of sampling. Changes in weight during the experimental period are thus disregarded, but it is believed they are not great enough to distort seriously the general metabolic picture.

V. Results

(*a*) *Carbon Dioxide Output*

Plotted against time, the carbon dioxide output of isolated darkened barley leaves, held at a constant temperature, gives a characteristic two-humped curve. Figure 1 shows a set of such curves for leaves from each of three positions on the stem. Similar data were collected for leaves from plants grown with other



Figs. 2-4

levels of nitrogen supply, but no consistent effect of nitrogen supply on the form or position of the respiration curve could be demonstrated. The respiratory behaviour of leaves from seedlings grown with nitrogen supplied as ammonium and nitrate was compared. Over the first 20 hours the former showed a higher carbon dioxide output and oxygen uptake, but later the curves came together. The "ammonium" leaves had a lower respiratory quotient for the first 35 hours, after which the curves crossed and the "ammonium" leaves had the higher quotient till the end of the experiment (Figs. 2, 3, and 4).

(*b*) *Changes in Nitrogenous Constituents*

(i) *Detached Mature Leaves.*—Six experiments were run with this type of material, three in one season and three in the next. The results all conform to the general pattern found for field-grown material by Yemm (1937). The qualitative course of nitrogen metabolism is similar in detached leaves from plants grown at the three levels of nitrogen supply, though, as might be expected, leaves from plants grown with the higher supplies of nitrogen form,

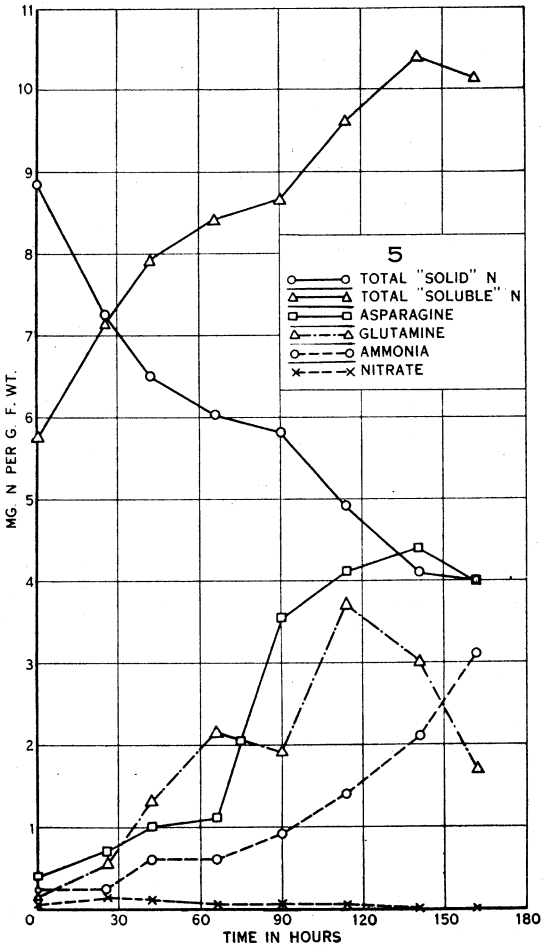


Fig. 5

during starvation, larger amounts of ammonia and the amides. Glutamine reaches a high concentration comparatively soon after the leaves are detached; it then decreases concurrently with an increase in the asparagine concentration. The latter also falls towards the end of the experiment, sometimes much more steeply than in the case illustrated. The ammonia concentration rises slowly in the earlier part of the experiment and becomes large towards its end. The

final decrease in asparagine content occurs after about 140 hours, the precise time varying from sample to sample, and coincides with the final collapse of the starved material (Fig. 5).

At this stage, the leaves are changing from yellow to brown and the content of soluble carbohydrate is probably very low (Yemm 1935). The figures for ammonia, high as they are towards the end of the experiments, do not represent all that is formed in the tissues of the detached leaves, for some escapes in gaseous form. In one respiration experiment with detached leaves from etiolated seedlings the gas stream from the respiration chamber was passed through a bubbler containing a known amount of dilute acid. In this, ammonia was identified using Nessler's reagent and estimated by back titration of the

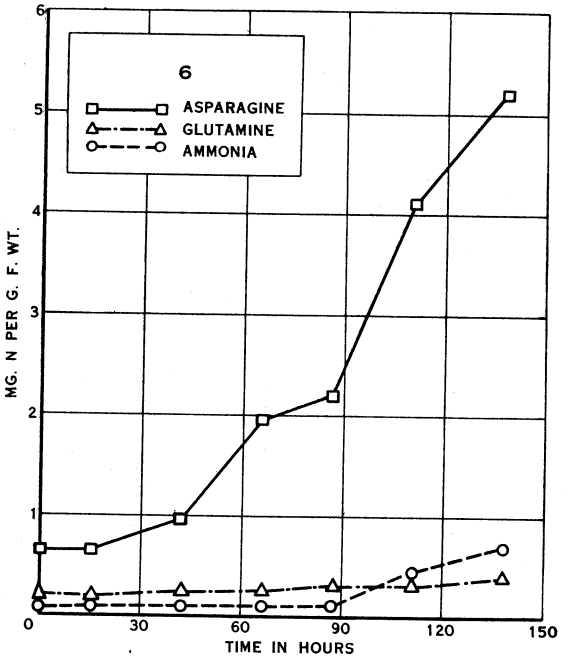


Fig. 6

acid to be equivalent to 0.35 mg. of ammonia per g. of original fresh weight, given off in 150 hours. Very small amounts of ammonia have been shown (Klein and Steiner 1928; Steiner and Löffler 1931) to be lost from leaves of many plants under normal conditions, but the losses in starvation are much greater. The results shown in Figure 5 may be taken as typical of the results with mature detached leaves. The increase in "soluble" and decrease in "solid" (presumably mainly protein) nitrogen are rapid and continuous from the beginning of the experiment; there is no indication of an initial lag in protein hydrolysis or of a sparing of protein by carbohydrate, such as were found with detached tobacco leaves by Vickery *et al.* (1933), with vine leaves by Deleano (1912), and with Kikuyu grass leaves by Wood, Cruickshank, and Kuchel

(1943). The amounts of nitrate found are small throughout, but there is an increase during the first 24 hours after the isolation of the leaves. This is a surprising finding, but similar results have been reported for tobacco (Vickery *et al.* 1933), tomato, and Swiss chard (*Beta vulgaris* var. *cicla*) leaves (McKee and Lobb 1938).

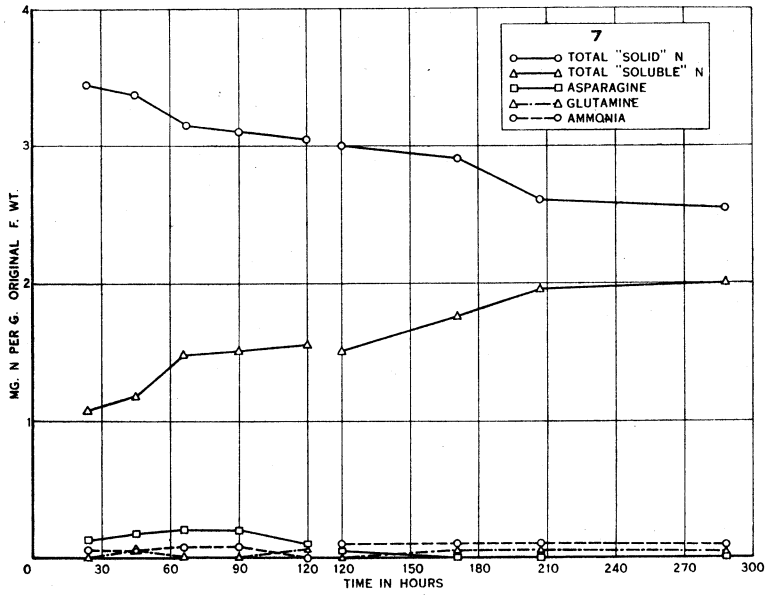


Fig. 7

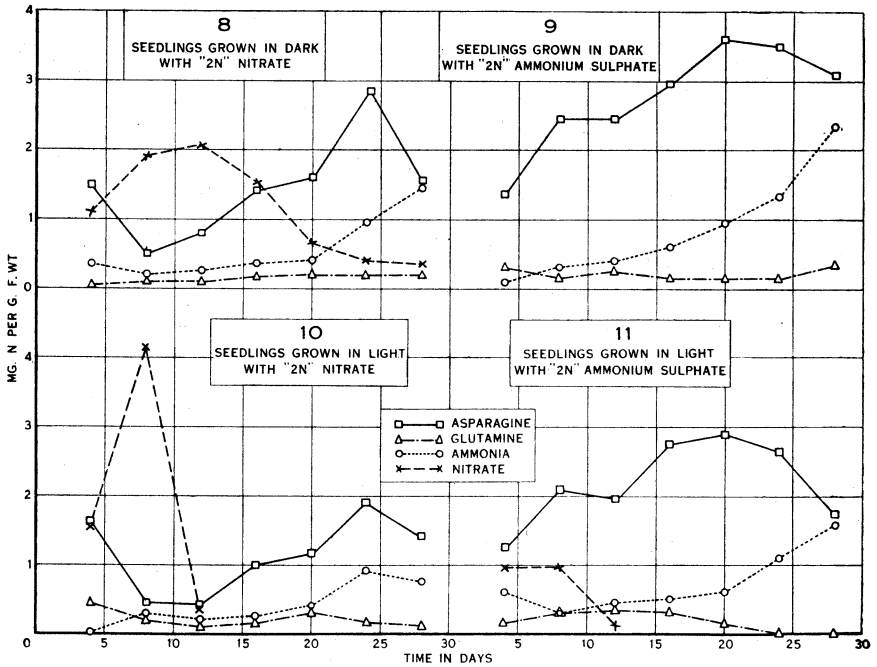
(ii) *Results with Detached Seedling Leaves.*—Five experiments were run with detached seedling leaves. The material included leaves from plants grown in the light and in the dark, and with nitrogen supplied as nitrate and as ammonium. The leaves from the plants supplied with ammonium had more asparagine initially than those given nitrate; their subsequent rate of asparagine accumulation and maximum asparagine concentration were also higher than in the "nitrate" leaves. Glutamine in both sets was present in low concentrations, but there was rather more in the "ammonium" leaves.

Leaves detached from seedlings accumulate much less glutamine than those from mature plants. The glutamine content also fails to show the early maximum found in starvation experiments with mature leaves. Typical results are shown in Figure 6.

(iii) *Autolysis Experiments.*—Two experiments were run, both using four-weeks-old seedling material grown in the light with "2N" nitrate supply. The material was cut finely and ground in a mortar. Samples (5 g.) were incubated at 21°C. with 50 ml. water and a little toluol in flasks fitted with cotton wool stoppers. In one experiment five analyses were made at intervals from 24 to 120 hours, and in the other four analyses from 120 to 287 hours. The results, shown in Figure 7, differ markedly from those with detached intact leaves.

There is a steady decrease in "solid" and a corresponding increase in "soluble" nitrogen, which does not appear to any important extent as ammonia or amide, but in some other form. After 287 hours, the ammonia content is still low and amides have almost disappeared.

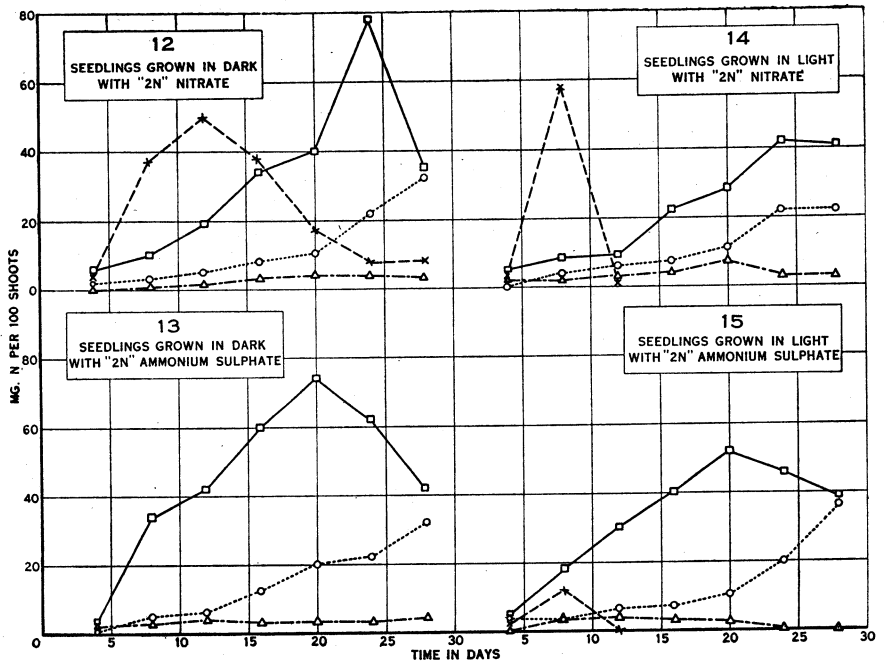
(iv) *Changes in Nitrogenous Constituents of Developing Seedlings.*—Four experiments were run, using seedlings grown with nitrate and ammonium (both "2N") in light and darkness. The results are presented both in terms of mg. N/g. fresh weight (Figs. 8, 9, 10, and 11) and of mg. N/100 shoots for the various constituents (Figs. 12, 13, 14, and 15). Over the first 20 days there is



Figs. 8-11

in all sets a great increase in the amount of asparagine per shoot. After 20 days, the asparagine content falls considerably in all sets except that grown with nitrate in light. In the other three sets, a decrease in the asparagine content is associated with a rise in that of ammonia, which rises steadily from the beginning in all sets except that grown with nitrate in light. Glutamine in all sets is present always in very small amounts compared with asparagine; it tends, in most cases, to decrease towards the end of the experiment. Both sets of etiolated seedlings reach a maximum weight between 16 and 20 days and subsequently lose weight. Both in the light and dark the "nitrate" seedlings are heavier than the "ammonium" seedlings (see Fig. 16). The nitrate content of seedlings supplied with nitrate and grown in the dark increases up to 12 days and then declines till at 28 days only comparatively small amounts are present. In the light, nitrate concentration is very high after eight days, but falls to a low value within three days.

A marked decrease in the concentrations of ammonia and asparagine occurs in the "nitrate" seedlings, both in the light and dark, between the fourth and eighth days. This is masked, when the results are calculated per shoot, by the rapid increase in bulk of the seedlings at this period. In the "ammonium" sets, asparagine and ammonia increase from the beginning even when calculated on the fresh weight basis. The finding of Vickery, Pucher, and Clarke (1936) that glutamine accumulates mainly in the root of sugar beet suggests that in any repetition of these experiments, it would be desirable to analyse the roots as well as the shoots of the seedlings, especially as Yemm (1949) has shown that barley roots assimilating nitrate or ammonium ions form glutamine rapidly.



Figs. 12-15

VI. Discussion

It has long been held that the formation of amides in the plant is a response to the presence in the cell of dangerously high concentrations of ammonia. This view can be traced back to Boussingault (1864), and has more recently been strongly supported by Pryanishnikov (1924). In the experiments reported here, formation of ammonia in the tissues or its supply from outside led to the formation of large amounts of asparagine, both in intact seedlings and in leaves detached from seedlings and mature plants. Glutamine, however, shows, in the conditions of these experiments, no tendency to accumulate with rising ammonia concentration, and is never found in large amounts in seedling material. The relation between asparagine and glutamine may be expressed by the regression equation:

$$y = a + bx,$$

where y is the concentration of glutamine in mg. N per 100 g. fresh weight and x that of asparagine. Values of a and b for different types of material are shown in Table 2.

TABLE 2
VALUES OF a AND b IN BARLEY LEAVES

Material	No. of Observations	a	b	Standard Error of b	Significance of Difference of b from Zero
Detached mature leaves	38	0.73	0.43	0.06	Highly significant
Detached seedling leaves	17	0.19	0.05	0.02	Not significant
Intact seedlings	28	0.24	0.04	0.05	Not significant

There is, therefore, in mature but not in seedling material, a statistically demonstrable increase of glutamine content as that of asparagine rises.

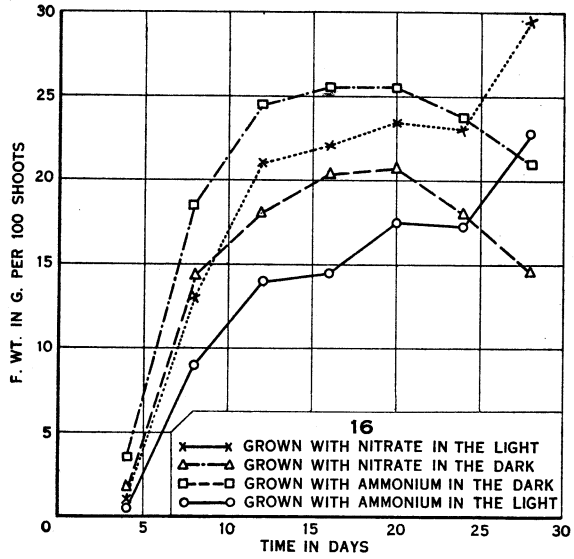


Fig. 16

In all experiments with young material, whether intact seedlings or young leaves, grown in the light or the dark, on nitrate or ammonium salts, the glutamine content is always much lower than that of asparagine. It is thus clear that the formation of ammonia in barley tissues or its introduction from outside the plant leads to accumulation of asparagine rather than of glutamine, and we must seek some other explanation for the accumulation of glutamine in the early stages of the starvation experiments with detached leaves. It may arise in part by hydrolysis of reserve protein. The leaf proteins of barley yield large amounts of glutamic acid and ammonia on acid hydrolysis (Yemm 1950) and it seems possible that part at least of this may represent glutaminyl units in the

protein molecule, as demonstrated for gliadin by Damodaran, Jaaback, and Chibnall (1932). The key to the behaviour of glutamine may also involve the intermediate products of carbohydrate breakdown. Glutamine is closely related to glutamic acid and so to α-ketoglutaric acid, a component of the tricarboxylic acid cycle which plays a major part in the catabolism of carbohydrate (via pyruvic acid) and fat (via a two-carbon-atom compound whose exact nature is uncertain). The relations between asparagine, aspartate, and oxalacetate and between glutamine, glutamate, and α-ketoglutarate show the close connection of the cycle with some of the most important compounds in the intermediate nitrogenous metabolism of plants. The consequent interrelations are discussed elsewhere in some detail (McKee 1937, 1949), and have also been considered recently by Yemm (1949).

VII. Acknowledgments

The experimental work described in this paper was carried out in the Botany Department, University of Oxford, during 1935 and 1936. The author wishes to thank Dr. W. O. James of that Department for his interest in the problem and Dr. E. W. Yemm, now of the University of Bristol, for advice on experimental techniques and for discussion of the interpretation of the results. He is indebted also to Professor J. G. Wood of the University of Adelaide and to Dr. R. N. Robertson of the Division of Food Preservation and Transport, C.S.I.R.O., for criticism of the manuscript, and to Mr. G. E. Ferris, Section of Mathematical Statistics, C.S.I.R.O., for calculating regression equations to illustrate the relations between asparagine and glutamine in different types of material.

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