ON THE UTILIZATION OF γ-AMINOBUTYRIC ACID BY WHEAT SEEDLINGS

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Summary

Feeding experiments and enzyme tests were carried out to investigate the utilization of γ-aminobutyric acid (ABA) by wheat seedlings.

In the feeding experiments embryos were excised from the grain 20–24 hr after soaking, explanted on a basal medium to which different nitrogen sources were added, and incubated at 25°C.

ABA nitrogen was utilized for protein synthesis. The optimal concentration was 6·0 mM. Different amino acids tested at equivalent nitrogen concentration produced the following sequence in insoluble nitrogen content per seedling: control = L-α,γ-diaminobutyric acid < DL-α-aminobutyric acid = ABA < L-proline < L-alanine = L-glutamic acid < L-asparagine = L-glutamine = nitrate.

Growth in length of the first leaf was on all occasions stimulated by ABA, but root growth was not.

Extracts of roots of 2- and 3-day-old intact seedlings had about 2·5 times the glutamic acid decarboxylase activity (per seedling) as that of shoot extracts; on a protein basis the ratio became more than 6.

No ABA–glutamic acid transaminase could be shown in root and shoot extracts, even after attempts to induce the enzyme, but alanine–glutamic acid transaminase activity was high in similar extracts. Neither was any evidence found for the presence of ABA oxidase in the seedlings.

I. INTRODUCTION

γ-Aminobutyric acid (ABA) has become recognized as a common component of the soluble nitrogen fraction of plants, but it is not found as a constituent of proteins.

ABA is of interest because of its relationship with the key metabolite, glutamic acid, from which it may be formed by decarboxylation. A puzzling aspect of the role of this amino acid is that the decarboxylases described do not favour carboxylation (Meister 1957), and the question has arisen whether this amino acid is a catabolic side product. In leaves of barley ABA content increases under anaerobic conditions (Naylor and Tolbert 1956). Kretovich and Yakovleva (1959) found a notable increase in ABA content after infiltrating potassium or ammonium α-ketoglutarate into leaves of wheat seedlings, but they question the conversion of ABA to glutamic acid. In a number of lower plant forms ABA has been shown to be an active intermediate, but its role in higher plants is still not clear.

Re-utilization of ABA nitrogen can be envisaged by means of transamination. Miettinen and Virtanen (1953) showed that ABA may act as a donor substrate in a transamination reaction with α-ketoglutarate in pea root extract. Carboxylation

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is implied in the conclusion by Steward, Bidwell, and Yemm (1956) that this amino acid is "en route to glutamic acid and glutamine". Amongst other evidence for this view Steward, Bidwell, and Yemm (1958) showed by feeding ^{14}C-labelled ABA to explants of carrot root phloem that the carbon of the glutamine and glutamic acid being formed in the tissue was partly derived from the ABA. Warburg, Klotsch, and Krippahl (1957) deduced from observations on *Chlorella* that an equilibrium between ABA and glutamic acid is maintained by the metabolic state of the cells. Fowden (1959) has suggested oxidative pathways as an alternative for the re-utilization of ABA.

As a contribution towards assessing the role of this amino acid, some results are reported here of a study, similar to the approach by Brown (1906), on the effect of externally applied ABA on nitrogen assimilation and growth of isolated germinating wheat embryos. The possibility was investigated that shoot–root ratios in the activity of some enzymes relevant to ABA metabolism could throw light on the utilization of ABA.

II. Materials and Methods

(a) *Feeding Experiments*

Grains of *Triticum vulgare* L., cv. Nabawa, produced locally, were selected for uniform weight.

The grains were surface-sterilized, soaked for 2 hr, and laid out in sterile petri dishes on moistened filter paper for 20–24 hr at 25°C. The embryos with the primary root piercing the grain coats were excised with the scutellum attached and transferred to 1-in. diameter tubes containing 16 ml slanted agar medium. The embryos were orientated on the surface of the agar with the root pointing downwards. Each treatment consisted of 10 replicates. As the manipulation of 80 or more embryos lasted some hours, batches of explants containing all treatments were incubated at time intervals.

The basal medium was prepared by adding 8 g agar (Bacto-Difco) and 18 g glucose to 1 l. solution consisting of:

	Concn. (mM)		Concn. (μM)
KCl	0·2	$MnCl_2$	2·0
$CaCl_2$	0·4	H_3BO_3	1·0
$MgSO_4$	0·2	$ZnSO_4$	1·0
KH_2PO_4	0·2	Na_2MoO_4	0·2
K_2HPO_4	0·07	$CuSO_4$	0·1
Fe (chelated with EDTA)	5·0 mg/l		

and of:

	Concn. (mg/l)		Concn. (mg/l)
Pyridoxal	1·0	Thiamine	0·1
Nicotinamide	0·1	Pantothenic acid	0·1
Folic acid	0·1	Riboflavin	0·1
p-Aminobenzoic acid	0·1	Biotin	0·01

TABLE 1

EFFECT OF γ-AMINOBUTYRIC ACID ON TOTAL AND INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS

Mean values per seedling after 120 and 240 hr of incubation at 25°C in the dark. The grains from which the embryos were taken weighed 55·0–59·9 mg. The designation "control" applies to embryos cultured on the basal medium, and "γ-aminobutyric acid" to embryos cultured on the same medium to which γ-aminobutyric acid (6·0 mM) was added

Embryo Treatment	Period of Incubation (hr)	Total Nitrogen per Seedling (μg)		Insoluble Nitrogen per Seedling (μg)						Coleoptile Growth (mm)	First Leaf Growth (mm)	Root Growth	
		Shoots + Roots		Shoots + Roots		Shoots		Roots				Number	Total Length (mm)
		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.				
Control	0	117·1	3·4	98·1	1·8								
Control	120	120·3	1·3	104·1	3·3	68·6	2·1	35·6	1·8	46·8	50·5	4·0	147·3
γ-Aminobutyric acid	120	181·4	2·2	144·4*	3·1	95·3*	2·1	49·2*	1·9	48·7	64·0*	4·8	162·7
Control	240	121·0		106·7	2·3	69·3	2·6	38·0	0·7	50·8	98·4	4·4	158·4
γ-Aminobutyric acid	240	222·5*		167·5*	5·3	118·2	4·1	49·7	5·0	48·7	163·3*	5·1	170·0

* This value differs significantly at least at the 1 per cent. level from the value recorded for control plants harvested at the same time.

Nitrogen sources (except glutamine) were added to the basal medium prior to heat sterilization at 110 lb/sq. in. for 10 min. ABA appeared stable under these conditions. Glutamine was added separately as a filter-sterilized solution to the heat-sterilized medium. The pH was *c.* 5·8.

In all experiments incubation of embryos was at 25°C in the dark, except for occasional inspection in orange light.

At harvest time the lengths of plant parts were measured and the plants stored in 70 per cent. alcohol. After several days the plants were boiled in fresh 70 per cent. alcohol and after a few days the alcohol was again renewed. The plants were washed twice with distilled water prior to determination of insoluble nitrogen content by the microKjeldahl method and Nesslerization of distillates. Where the insoluble nitrogen content of the shoot and the roots was estimated separately, the shoot included the scutellum.

(*b*) *Tests for Enzyme Activity*

(i) *Glutamic Acid Decarboxylase.—Preparation of extracts:* Wheat grains were soaked and germinated on moistened filter paper for 2 or 3 days at 25°C in darkness. The seedlings were separated from the endosperm and divided into root and shoot parts. These parts were ground separately in a chilled mortar in the presence of 0·2M phosphate buffer, pH 5·3. The extracts were strained through muslin, made up to volume to the equivalent of 20 seedlings per ml with additional buffer, and then centrifuged for 10 min at 500 *g* and 1°C. The supernatant was tested for enzyme activity. *Manometry:* Enzyme activity was measured in duplicate by conventional Warburg technique at 30°C. The vessels contained 2·0 ml extract, 0·3 ml 0·1M glutamic acid (pH 5·3), 0·5 ml 1M sodium fluoride, 0·1 ml pyridoxal phosphate (approx. 50 μg), and 0·1 ml water. Water replaced glutamic acid in the controls.

(ii) *Transaminase.—Principle:* Extracts were incubated with amino acid and α-ketoglutarate, and the glutamic acid produced was assayed with bacterial glutamic acid decarboxylase (Cohen 1955). *Preparation of extracts:* Extracts of roots and shoots were prepared as for glutamic acid decarboxylase assay except that an 0·2M phosphate buffer, pH 7·5, was used. The extracts were dialysed overnight against 0·001M phosphate buffer, pH 7·5. In the interval the buffer was renewed once. On other occasions the extract was prepared with 0·15M pyrophosphate buffer, pH 8·5, and an ammonium sulphate precipitation (70 per cent. saturation) was carried out. The precipitate was redissolved in 0·015M pyrophosphate buffer, pH 8·5, to the original volume. *Procedure:* Into the main compartment of a Warburg vessel was pipetted 1·7 ml extract, 0·1 ml pyridoxal phosphate (approx. 50 μg), 0·2 ml 0·5M amino acid dissolved in 0·01M buffer (either pH 7·5 or 8·5), and in the side-arm 0·2 ml 0·5M α-ketoglutarate adjusted to pH. After incubation for 90 min at 30°C the reaction was stopped and the glutamic acid formed was assayed with bacterial glutamic acid decarboxylase (Worthington Biochem. Corp., U.S.A.).

(iii) *Amino Acid Oxidase.*—Oxygen consumption of extracts, either dialysed or redissolved from ammonium sulphate precipitates, was measured by the conventional

TABLE 2

EFFECT OF NITRATE AND DIFFERENT CONCENTRATIONS OF γ-AMINOBUTYRIC ACID ON INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS

Mean values per seedling after 120 hr of incubation at 25°C in the dark. The grains from which the embryos were excised weighed 55·0–59·9 mg. The mean value of the insoluble nitrogen content of the embryos at the time of excision was 98·3 μg per embryo and the standard error 2·6 μg

Substance Added	Concn. (mM)	Insoluble Nitrogen Content per Seedling (μg)		Coleoptile Growth (mm)	First Leaf Growth (mm)	Root Growth	
		Mean	S.E.			Number	Total Length (mm)
Nil		95·2	2·6	53·8	49·0	4·4	137·8
γ-Aminobutyric acid	3·0	112·1*	3·7	53·9	61·8*	5·3	138·7
γ-Aminobutyric acid	6·0	133·1*	4·1	58·3	69·5*	5·3	132·3
γ-Aminobutyric acid	12·0	137·0*	5·4	52·8	72·0*	5·7*	108·2*
Potassium nitrate	3·0	221·0*	8·2	56·7	99·6*	4·2	300·3*

* This value differs significantly at least at the 1 per cent. level from the value recorded for the treatment in which no substance was added to the basal medium.

Warburg technique. The central cup contained KOH, the main compartment 2 ml of extract, and the side-vessel 0·4 ml 0·05M amino acid.

III. Results

(a) *Feeding Experiments*

Table 1 summarizes the results of an experiment in which excised embryos were fed with ABA at 6·0 mM concentration and cultured for 120 and 240 hr. The insoluble nitrogen content of the ABA-treated seedlings is significantly higher than the total nitrogen content of the control embryos or of the zero-time embryos. This demonstrates that ABA nitrogen was utilized for protein synthesis. The growth in length of the first leaves was significantly stimulated by ABA, but the growth in length of the roots was not. This phenomenon was also observed in a series of experiments mentioned below. However, the insoluble nitrogen content of the roots of the ABA-treated seedlings was nearly 40 per cent. higher than that of the roots of the control seedlings. The roots virtually stopped growing after 120 hr of incubation, and their insoluble nitrogen content remained constant. In the shoots the insoluble nitrogen content continued to increase in the ABA-treated seedlings but at a reduced rate.

In another experiment in which embryos were cultured for 144 hr the roots of the control treatment attained an insoluble nitrogen content of 32·5 S.E. 2·3 μg and a total length of 156·3 mm, and the corresponding values for the ABA treatment (3 mM) were 43·2 S.E. 1·4 μg and 157·0 mm. The difference in insoluble nitrogen content was again significant but the difference in total root length was not.

Table 2 gives the results of an experiment in which different concentrations of ABA and potassium nitrate at 3·0 mM were applied to the embryos under conditions similar to those for the experiment of Table 1. All concentrations of ABA used effected a significant increase of insoluble nitrogen content. This increase approached its maximum value at 6·0 mM concentration. It is noteworthy that the seedlings appeared tolerant to a high concentration (12·0 mM) of the amino acid. The only adverse effect of this concentration was in total root length. At the other concentrations of ABA used, total root length was not increased over the control. Potassium nitrate surpassed the effects of ABA considerably in insoluble nitrogen content and in the lengths of the first leaf and of the roots.

In Table 3 the effects of ABA can be compared with those of the control, DL-α-aminobutyric acid, L-α,γ-diaminobutyric acid, monosodium-L-glutamate, L-proline, L-alanine, L-asparagine, and L-glutamine. The amino acids were applied on a basis of nitrogen equivalence.

Only the control and the L-α,γ-diaminobutyric acid treatments resulted in significantly less insoluble nitrogen content than the ABA treatment. The diamino acid proved very inhibitory to growth. It is noteworthy that α-aminobutyric acid, although applied as a racemate, afforded as much nitrogen assimilation as ABA; except for a remarkable increase in root number, it had an inhibiting effect on growth. Glutamic acid, proline, alanine, asparagine, and glutamine gave significant increases over ABA in insoluble nitrogen content and growth. Root growth was stimulated

TABLE 3

EFFECT OF γ-AMINOBUTYRIC ACID AND SOME OTHER AMINO ACIDS ON INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS

Mean values per seedling after 120 hr of incubation at 25°C in the dark. The grains from which the embryos were excised weighed 60·0–64·9 mg. The mean value of the insoluble nitrogen content of the embryos at the time of excision was 109·5 μg per embryo and the standard error 2·6 μg

Substance Added	Concn. (mM)	Insoluble Nitrogen Content per Seedling (μg)		Coleoptile Growth (mm)	First Leaf Growth (mm)	Root Growth	
		Mean	S.E.			Number	Total Length (mm)
Nil		105·4	4·4	61·1	50·1	4·7	168·4
DL-α-Aminobutyric acid	3·0	123·1*	1·8	45·7*	33·7*	7·9*	60·1*
L-α,γ-Diaminobutyric acid	1·5	103·2	3·1	4·9*	4·8*	3·6	16·2*
γ-Aminobutyric acid	3·0	124·9*	2·8	61·9	57·2*	5·2	156·7
Monosodium L-glutamate	3·0	174·9*	0·7	61·8	73·0*	5·0	229·3*
L-Proline	3·0	158·0*	4·9	68·2	81·5*	5·2	146·4
L-Alanine	3·0	181·8*	9·4	64·4	80·1*	5·0	192·7*
L-Asparagine	1·5	207·8*	7·8	69·8	89·6*	4·9	244·0*
L-Glutamine	1·5	235·6*	10·4	68·9	94·7*	4·8	250·6*

* This value differs significantly at least at the 1 per cent. level from the value recorded for the treatment in which no substance was added to the basal medium.

by glutamate but not by ABA and not by proline. There are no significant differences between the effects of asparagine and glutamine. Although Tables 2 and 3 are not strictly comparable, it is of interest to note that results with nitrate (Table 2) and with asparagine or glutamine (Table 3) are more or less equal.

(*b*) *Enzyme Tests*

(i) *Glutamic Acid Decarboxylase.*—Carbon dioxide evolution of root and shoot extracts of 2- and 3-day-old seedlings from whole grains incubated with glutamic acid was nearly linear for 80 min. The values observed for an incubation period of 60 min expressed per 40 seedlings and per mg insoluble nitrogen are recorded in Table 4. It is evident from these data that the roots show considerably more

TABLE 4

GLUTAMIC ACID DECARBOXYLASE IN SHOOTS AND ROOTS OF WHEAT SEEDLINGS

Carbon dioxide production by extracts from shoots and roots of plants germinated for 48 and 68 hr on water and on γ-aminobutyric acid (6·0 mM). The values represent differences found between manometric estimations in the presence and absence of glutamic acid at 30°C

Source of Extract	Carbon Dioxide (μl) Produced in 60 Min at 30°C			
	Per 40 Plants		Per Mg Insoluble Nitrogen of Extract	
	Shoot	Root	Shoot	Root
48-hr seedlings germinated in water	43	105	23	128
68-hr seedlings germinated in water	71	185	20	137
68-hr seedlings germinated in γ-aminobutyric acid	77	204	27	162

enzyme activity than the shoots; on a protein basis, the activity is more than six times greater in the roots than in the shoots. Treatment of grains with ABA (6·0 mM) during soaking and germination had no effect on observed activities.

(ii) *Transaminase.*—Attempts to demonstrate the presence of ABA–glutamic acid transaminase in dialysed extracts of roots and shoots of 2- and 3-day-old seedlings from both whole grains and excised embryos were unsuccessful, although alanine–glutamic acid transaminase activity could readily be shown. Further, although Fowden (1959) reported ABA–glutamic acid transaminase to be inducible in yeast, treatment of grains or excised embryos with 6 mM ABA failed to yield seedlings with detectable activity of this enzyme. The enzyme would have been detected in such seedlings if it had had 2–3 per cent. of the activity of the alanine–glutamic acid transaminase. When extracts of ABA-treated seedlings were incubated at pH 8·5 with alanine and α-ketoglutarate, glutamate formed, expressed as μl CO_2 evolved, was 356 per equivalent of 34 seedlings and 306 per mg insoluble nitrogen in extracts for shoot preparations, and 464 and 574 respectively for root preparations.

(iii) *Amino Acid Oxidase.*—Addition of 20 μmoles ABA to dialysed extracts from whole seedlings or to redissolved ammonium sulphate precipitates of crude extracts tested at pH 6·0, 7·5, and 8·5 did not stimulate oxygen consumption, the endogenous rate of which did not exceed 10 μl per hour. Ascorbic acid was readily oxidized by such extracts tested at pH 6·0. The failure to find any activity is consistent with the work by Kenten and Mann (1952) who stated that extracts of barley seedlings did not possess any amine oxidase activity.

IV. Discussion

In this period of rapidly increasing information on plant nitrogen metabolism it has become evident that it is difficult to generalize on the relative role of the different amino acids therein. ABA, once thought to form a side product, has been ranked as a central amino acid in *Endomycopsis* (Steiner 1959). Steward and co-workers (1956, 1958) have drawn attention to its possible role in higher plants. Barnes and Naylor (1959) made the remarkable observation that ABA stimulated the growth of excised pine roots much better than a series of amino acids including glutamic acid. Nitrate stimulated root growth better than ABA which indicates that in the other cases nitrogen was deficient. Apparently ABA is utilized readily as a source of nitrogen by pine roots.

The results recorded above for wheat demonstrate that ABA nitrogen is utilized in protein synthesis by this cereal. However, the utilization is slow when compared with nitrate or glutamine and other amino acids such as alanine, proline, and glutamic acid. The relative efficiency of various nitrogen compounds probably reflects the readiness with which a system takes up a substance and incorporates its nitrogen into glutamic acid. The central assignment of glutamic acid and glutamine has now been proved for yeast in physiological experiments by Folkes (1959) who showed that at least 87 per cent. of the nitrogen of ammonium sulphate passes primarily through this amino acid. The limiting factor in feeding glutamic acid itself is most likely to be found in its uptake; the fact that glutamic acid has strongly polar groups lends support to this interpretation. On this basis alanine and ABA might be expected to be taken up more rapidly than glutamic acid. The fairly good utilization of alanine is then further explained by the presence of highly active alanine–glutamic acid transaminase. In contrast, judging from its utilization, ABA nitrogen cannot readily be passed on to glutamic acid. The enzyme tests appear to support this conclusion.

Since ABA–glutamic acid transaminase was not detectable, the direct transfer of ABA nitrogen via such a transaminase reaction is probably not a significant pathway. Judging from the utilization of alanine and of ABA, ABA–glutamic acid transaminase should have had approximately 25 per cent. of the activity of the alanine–glutamic acid transaminase, but no trace of activity was found when extracts were tested at pH 7·5 or 8·5.

The shoot–root distribution of glutamic acid decarboxylase does not fit in with the relative increases in length and insoluble nitrogen content of these parts when fed with ABA. After 120 hr of incubation with ABA (Table 1) increase in

insoluble nitrogen content in roots stopped but continued in the shoots. It therefore seems unlikely that direct conversion of ABA to glutamic acid by a reversal of this enzyme reaction is a possible mechanism.

It seems probable from the results that ABA nitrogen is not passed on to glutamic acid in a single-step reaction in wheat. Furthermore, the participation of an ABA oxidase in a more indirect pathway is unlikely because of the negative results of the tests for this enzyme.

Another problem raised by this study concerns root growth because, although growth in length of roots is stimulated by glutamic acid, in general, it is not stimulated by ABA. Therefore, whilst stimulating protein synthesis in the root, ABA must effect a deviation from normal root development. Perhaps there is a connection between this fact and the observation by Barnes and Naylor (1959) that the ABA-treated pine roots contained a high number of dichotomous laterals.

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