REVISTA ESPAÑOLA DE FISIOLOGIA, 35, 37-40. 1979

Enzymatic Disappearance of Hydroxylamine in the Presence of GABA

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(Received on April 17, 1978)

M. P. GONZALEZ, S. CAÑADAS and A. SANTOS-RUIZ. *Enzymatic Disappearance of Hydroxylamine in the Presence of GABA.* Rev. esp. Fisiol., 35, 37-40. 1979.

This paper presents data on the elimination of hydroxylamine from *Lupinus albus* seeds when they were germinated in the presence of GABA and hydroxylamine. The possibility of an enzymatic reaction, ATP dependent, between GABA and hydroxylamine is discussed. Some kinetic properties from this reaction are studied.

In a previous work (1) it was shown that hydroxylamine at low concentrations (1-10 mM) increased germination from *Lupinus albus* seeds; although it inhibited GABA-2-oxoglutarate aminotransferase and succinic semialdehyde dehydrogenase. Higher hydroxylamine concentrations (20 mM) produced a strong inhibition on both, germination and enzyme activities.

This effect could be due to its effect on oxygen consumption because it is known that hydroxylamine is a strong chelating agent which reacts to form oximes with aldehydes and ketones and probably can form oximes with the formyl group of cytochrome «a» limiting in this way the electron flow to oxygen (9).

GABA protected the germination and the enzyme activities against hydroxylamine action. The mechanism of that protection seems to be due to the fact that *in vivo* and, in the presence of GABA the free hydroxylamine disappeared from the seeds. This reaction could arise at least, in two ways: 1) by an enzymatic reaction with the intervention of ATP or 2) by a condensation between GABA and hydroxylamine. Both possibilities are discarded in this paper.

Materials and Methods

Plant Material. Lupinus albus seeds from Sevilla (Spain) were recollected on August and were germinated on vermiculite moistened with distilled water or different concentrations of hydroxylamine used as hydroxylamine-HCl, at pH 7 with sodium hydroxyde, or GABA, as indicated in each case. Germination was carried out at 25° C under constant illumination. In order to keep the humidity degree constant, the seeds were sprinkled each day with 10 ml of the suitable solution.

Emergence of the radicle was the adopted criterion for germination. Hydroxylamine was prepared directly before using and then kept in cold at 4° C during germination time (about 10 days).

Preparation of extract. Embryos or cotyledons were ground in a mortar with 10 volumes of 0.05 M Tris-ClH buffer pH 7.9 at 2-4° C. The extracts were stirred during 1 minute, then were filtered through cheesecloth and centrifuged at 25,000 × *g* for 30 minutes. Supernatants were used as enzymatic solution.

Determination of hydroxylamine. Free hydroxylamine was assayed essentially according to CSAKY (2).

Determination of hydroxamates and ADP. Hydroxamates were checked by IQBAL and OTTAWAY method (3). ADP formation was followed by NADH disappearance in the presence of pyruvate kinase and phosphoenol-piruvate.

GABA-hydroxylamine-ATP reaction. It was checked as follows: 500 µl of enzymatic extract were incubated with 40 µmoles of hydroxylamine; 40 µmoles of GABA; 10 µmoles of ATP; 60 µmoles of $MgCl_2$; 50 µmoles of Tris-HCl buffer pH 7.9; in a total volume of 1.5 ml; incubation was carried out at 37° C for 30 minutes. The reaction was stopped by addition of 1 ml of $HClO_4$ (1 M) when ADP was measured, or with 0.5 ml of 20 % (w/v) trichloroacetic acid when hydroxylamine was assayed. When hydroxamates were checked the IQBAL *et al.* method was used (3).

Determination of proteins. Proteins were measured by LOWRY *et al.* method (7).

Results and Discussion

The relative activities of various substrates of glutamine synthetase with hydroxylamine vary in a wide extent (4-6, 10) beig the higher activity always found with L-glutamate as substrate. According to our results hydroxylamine *in vivo* presents higher activity with GABA than with glutamate, in both, embryos and cotyledons; this indicates that GABA is more effective in hydroxylamine elimination.

GABA reacts with hydroxylamine and this reaction seems to be attended by hydroxamate formation (table I), because hydroxamate concentrations increase in the presence of GABA. It would mean that the reaction between GABA and NH_2OH could be a condensing enzyme or a synthetase, probably, a GABA-hydroxylamine synthetase or a glutamine synthetase which used GABA besides glutamate.

Table I. *Contents of free hydroxylamine in* L. albus *seeds germinated in the presence of hydroxylamine or hydroxylamine plus GABA.*
Seeds were germinated in the presence of 20 mM of hydroxylamine or hydroxylamine plus the indicated effector. After germination (1.5 cm radicle) free hydroxylamine was checked in extracts prepared as indicated in Methods.

Conditions	nmoles free NH_2OH		Hydroxamates (ΔOD/ml)	
	Embryos	Cotyl.	Embryos	Cotyl.
Control	0.0	0.0	0.0	0.0
+ NH_2OH	238 ± 10.3	321 ± 10.3	0.004	0.004
+ NH_2OH + 20 mM GABA	80 ± 10.3	129 ± 6.4	0.040 ± 0.005	0.125 ± 0.010
+ NH_2OH + 30 mM GABA	85 ± 8.6	130 ± 10.2	0.083 ± 0.010	0.155 ± 0.016
+ NH_2OH + 40 mM GABA	82 ± 8.4	132 ± 11.5	0.132 ± 0.012	0.187 ± 0.020

Table II. *Mechanism of hydroxylamine elimination by* L. albus *seeds.*
Extract from seeds germinated with distilled water and 1.5 cm radicle were used. A part of this extract was boiled at 100° C during 30 minutes. Incubations were carried out as indicated in Methods with the exception that the total volume was 2.6 ml.

Conditions	nmoles of free hydroxylamine/mg protein Embryos	Cotyledons
Control	0.0	0.0
+NH_2OH	153	143
+NH_2OH+GABA	128	117
+NH_2OH+GABA+ATP	15	76
Boiled extract		
Control	0.0	0.0
+NH_2OH	159	152
+NH_2OH+GABA	155	150
+NH_2OH+GABA+ATP	155	153

Table III. *Appearance of ADP when GABA and hydroxylamine react in the presence of ATP.*
Incubations were carried out as indicated in Methods. ADP was measured in 0.1 ml sample and 0.5 μmoles of NADH; 10 μmoles of phosphoenol pyruvate; 5 μmoles EDTA-Na; 100 μmoles of potassium phosphate buffer pH 7.9. Total volume 1 ml.

Conditions	μmoles ADP/mg protein Embryos	Cotyledons
Extract	1.7	1.4
Extract+ATP	1.9	1.7
Extract+GABA+NH_2OH+ATP	9.5	3.8
Boiled extract		
Extract	1.3	1.4
Extract+GABA+NH_2OH+ATP	1.9	2.0

Table IV. *Aparent Km values for hydroxylamine and GABA.*

Substrate	Km × 10^{-2} M Embryos	Cotyledons
NH_2OH	3.1	3.2
GABA	3.2	3.1

The *in vivo* results are not enough to confirm this hypothesis, although the studies presented in table II and III show that *in vitro* there is an enzymatic reaction between hydroxylamine and GABA. This reaction was ATP dependent and it catalized a coupling between: *a*) the cleavage of ATP to ADP and inorganic phospate (table III), and *b*) the formation of GABA hydroxamates from hydroxylamine and GABA (table II). That is:

$$GABA + NH_2OH + ATP \longrightarrow GABA\text{-}NHOH + ADP + Pi$$

The possibility that this reaction to be an enzimatic reaction is favoured by several results: 1) The hydroxylamine and GABA reaction did not take place when the extract was boiled (table II and III). 2) The reaction increased with the extract concentration (fig. 1). 3) With incubation time (fig. 2). 4) The reaction

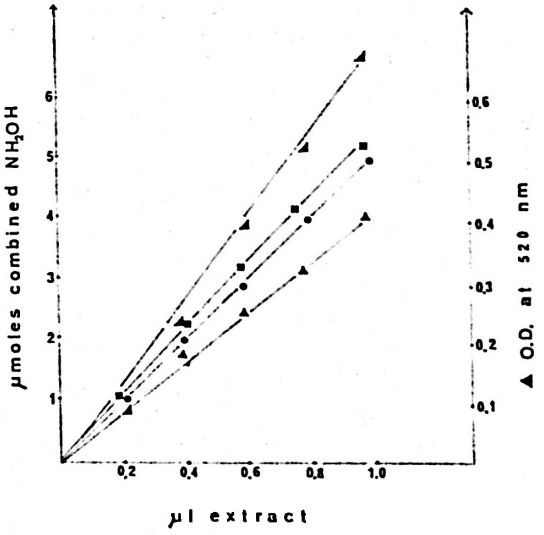

Fig. 1. *GABA-hydroxylamine reaction: Effect of extract concentration.*
The reaction was followed by hydroxylamine combination and by hydroxamates formation. Combined hydroxylamine: embryos (●); cotyledons (■). Hydroxamates formation: embryos (◣); cotyledons (▲).

was pH dependent (fig. 3). 5) The reaction was ATP dependent (table II and III).

The optimum pH was 7.3-7.5 for both enzymes, embryos and cotyledons (fig. 3). The apparent K_m for hydroxylamine and GABA were similar (table IV), these apparent K_m for hydroxylamine and GABA were higher than those found for sheep-brain glutamine synthetase (8).

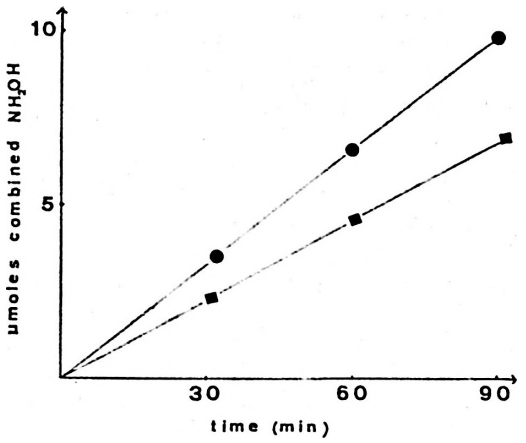

Fig. 2. *Effect of incubation time.* Hydroxilamine combined: embryos (●); cotyledons (■).

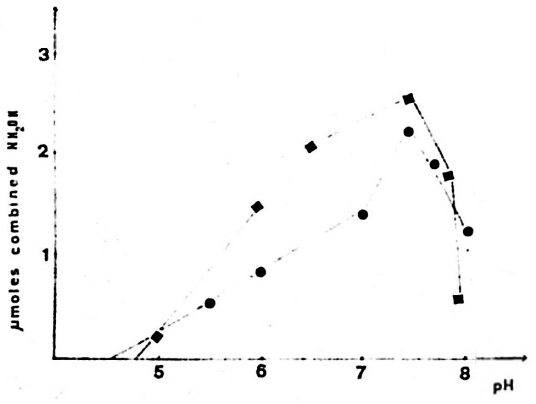

Fig. 3. *Effect of pH on GABA hydroxylamine reaction.* Embryos (●); cotyledons (■).

Resumen

Se estudia la desaparición de hidroxilamina en semillas de *Lupinus albus* que han germinado en presencia de ésta más 4-aminobutirato (GABA). Se discute sobre la posibilidad de que la eliminación de hidroxilamina se efectúe mediante el concurso de una reacción enzimática, ATP dependiente, entre el 4-aminobutirato y la hidroxilamina. Se estudian algunas propiedades cinéticas de esta reacción.

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