Effects of Magnesium Chloride and Manganous Nitrate upon the Content of Ascorbic Acid in Soybean during Germination, with Observations on the Activity of Ascorbic Acid Oxidase

By T. SHEN, K. M. HSIEH AND T. M. CHEN, *The Physiological Laboratory, Tsinghua University, Kunming, China*

(Received 9 November 1944)

The effect of chemical substances upon the ascorbic acid content of seeds during germination has been investigated. In wheat seedlings, Gal (1938) reported that various kinds of organic acids in a concentration of 5:1000 caused an increase of 40–50% in the amount of ascorbic acid present, and some inhibition of germination. By germinating Bengal Gram (*Cicer arietinum*), Rudra (1938) found that minute quantities of manganese added to distilled water for germination favoured ascorbic acid synthesis. In this connexion it is interesting to note that manganese in the form of manganous sulphate, when added to the soil, increased the ascorbic acid content in tomato pulp (Hester, 1941).

It might be thought that the ascorbic acid formed in the seed during germination is dependent upon reserve food material, though the specific nature of the precursor is not known (King, 1939); that enzymes, probably including ascorbic acid oxidase, play a rôle in the synthesis; and that effects of some chemical substances on the ascorbic acid content of germinated seed are through their actions on enzymes. With these possibilities in mind, we made an investigation of effects of $MgCl_2$ and $Mn(NO_3)_2$ upon the content of ascorbic acid in soybean during germination, with special observations on the activity of ascorbic acid oxidase.

METHODS

Germination of soybean. The dry beans were obtained from Kunming market. Only the mature seeds nearly of the same size were used for germination. Three hundred beans were washed with distilled water and loaded in each bag made of cotton towel. They were soaked in distilled water for 24 hr., then hung up for 24 hr., and were soaked in water once every 24 hr. Germination took place in the dark at a room temperature which varied from 17 to 24°. To learn whether germination will take place satisfactorily in this manner and how fast the sprout will grow under these conditions, observations were made on eight bags of germinated beans, each of which contained 300 seeds. The beans in the eight bags were germinated in the same manner as we have just described and at the same time. The results are tabulated in Table 1.

Table 1. *Growth of the sprouts of soybean under experimental conditions*

Days of soaking	Length of the sprout (epicotyl, hypocotyl and radicle)		
	Mean (cm.)	Mode (cm.)	Range (cm.)
2	0.72	0-1 (78)*	0- 1
3	2.22	2-3 (65)	0- 5
4	4.03	4-5 (39)	0- 8
5	5.15	6-7 (20)	0-12
6	7.27	6-7 (16)	0-15
7	9.18	6-7 (12)	0-23
8	11.67	6-7 (8)	0-25
9	12.42	8-9 (11)	0-29

* Percentage of beans in the class of the mode is given in parenthesis.

Stage of germination. The length of the sprout as well as the number of days after the beginning of the soaking process were chosen arbitrarily.

Stage of germination	Days of soaking	Length of sprout (cm.)
I	2	0- 1
II	3	2- 3
III	4	4- 5
IV	5	6- 7
V	6	8- 9
VI	7	10-11
VII	8	12-13

Method of estimating ascorbic acid. In the present study the content of l-ascorbic acid was estimated. No effort was made to estimate the reversibly oxidized ascorbic acid in the germinated bean. Harris & Olliver's working directions for estimating ascorbic acid by titration against 2:6-dichlorophenolindophenol (Harris & Olliver, 1942) were followed with some changes due to lack of certain materials. The changes were the omission of metaphosphoric acid, the use of copper-free redistilled water only in preparing reagents and the use of boiling 8% acetic acid in extraction. It was found that ordinary distilled water used only in diluting the extract would not lower the titration value to a significant extent, if the reagents were prepared with copper-free water. It was also found that boiling 8% acetic acid, when enough was used to cover the beans before grinding, would arrest almost completely the activity of ascorbic acid oxidase.

The detailed procedure used was as follows. The 300 germinated soybeans in a bag were sampled in a chamber into which only red light could pass. Cotyledons (5 g.) or sprouts (2 g.) were covered by 25 ml. of boiling 8% acetic acid and ground in a glass mortar. The material was afterwards transferred into a flask and diluted to 100 ml. with ordinary-distilled water. The titration was then done immediately with a microburette. Blank tests were also made on all the reagents. The standard solution of ascorbic acid was prepared just before being used. The 2:6-dichlorophenolindophenol solution was prepared without metaphosphoric acid. It was not kept for longer than 5 days.

Method of estimation of ascorbic acid oxidase. The activity of ascorbic acid oxidase was estimated by titrating a known solution of ascorbic acid incubated with some ground soybean in distilled water at room temperature for 10 min. The amount of ascorbic acid destroyed could thus be estimated. Only redistilled water free from copper and microorganisms was used to prepare the reagents and to dilute the ascorbic acid solution before titration.

The detailed procedure is as follows. Grind 0.5 g. of cotyledon or 0.2 g. of sprout with 3 ml. of water in a glass mortar. Transfer with 2 ml. of H₂O into a 150 ml. flask. Add 1 ml. of ascorbic acid solution (about 2 mg./ml.). Let the mixture stand for 10 min. at room temperature. Dilute to 50 ml. with H₂O and acidify with 2 drops of glacial acetic acid. Titrate against 2:6-dichlorophenolindophenol solution, 1 ml. of which is equivalent to approximately 0.4 mg. of ascorbic acid.

The activity of ascorbic acid oxidase was expressed by the percentage of ascorbic acid destroyed, the calculation of which is shown below:

The percentage of ascorbic acid destroyed

$$= \left(1 - \frac{\text{ascorbic acid left}}{\text{ascorbic acid added}} \right) \times 100$$

$$= \left(1 - \frac{A - B_1}{C - B_2} \right) \times 100.$$

In the above equation,

A = The titration value of 1 ml. ascorbic acid incubated with a ground paste of 0.5 g. cotyledon or of 0.2 g. sprout.

B_1 = The titration value of 0.5 g. cotyledon or 0.2 g. sprout.

B_2 = The titration value of 50 ml. acidified H₂O.

C = The titration value of 1 ml. ascorbic acid solution.

RESULTS

The change of ascorbic acid content in the cotyledons and in the sprout of an average soybean during germination. In the course of mastering our technique of estimating the ascorbic acid content in the soybean sprout, we measured 720 germinated beans for fresh weights of cotyledons and of sprouts at different stages of germination. Triple estimations of ascorbic acid content in a 5 g. portion of cotyledons and in a 2 g. portion of sprouts were made, and the number of milligrams of ascorbic acid per 100 g. fresh cotyledons or sprouts was calculated. Based upon these data, the content of ascorbic acid in an average soybean during germination could be found. The results are tabulated in Table 2.

Table 2. *The ascorbic acid content in an average soybean during germination*

Stage of germination	Fresh weight (g.)		Ascorbic acid (μ g.) content of	
	Cotyledons	Sprout	Cotyledons	Sprout
I	0.377	—	17.2	—
II	0.343	0.049	44.4	11.6
III	0.336	0.094	55.2	17.7
IV	0.333	0.147	61.5	23.2
V	0.346	0.220	64.7	27.3

Fig. 1. The change of the activity of ascorbic acid oxidase in an average soybean during germination.

The activity of ascorbic acid oxidase in soybean during germination

The activities of the oxidase in cotyledons and in sprouts were tested at different stages in duplicates and were expressed as percentages of ascorbic acid destroyed. If we assumed that the oxidase

activity increased in a direct proportion to the amount of cotyledons or sprouts used in the test, we could find out the change of the oxidase activity in an average soybean during germination. The results are shown in Fig. 1.

Effects of $MgCl_2$ and $Mn(NO_3)_2$ upon the content of ascorbic acid in soybean during germination

The effect of $Mn(NO_3)_2$ was first studied with a set of beans germinated in distilled water as controls. The effect of $MgCl_2$ was studied later with another set of controls. The stock solutions of $MgCl_2$ and $Mn(NO_3)_2$ were prepared beforehand. They were diluted with distilled water before use. The final concentration of the metallic ions in the salt solutions was approximately 10 p.p.m., when the degree of ionization of these salts was assumed to be unity. Solutions for soaking the beans in the experimental groups as well as the distilled water in the control group were renewed every other day.

The results are given in Tables 3 and 4. In these tables, the averages for every five estimates are presented.

Table 3. *Ascorbic acid contents of soybean germinated in water and in $Mn(NO_3)_2$ solution*

Stage of germination	Ascorbic acid (mg./100 g. fresh material)			
	In cotyledons		In sprouts	
	H ₂ O group	Mn group	H ₂ O group	Mn group
	(0.5 g.)	(0.2 g.)	(0.5 g.)	(0.2 g.)
I	5	65	10	100
II	16	51	21	92
III	19	80	28	82
IV	25	80	31	84
V	25	64	26	66
Average	11.90	12.65	9.76	10.54
Difference		0.75*		0.78
		(6.3%)		(8.0%)

* If the second stage of germination were not taken into consideration the difference would be 1.14 or 9.6%.

Table 4. *Ascorbic acid contents of soybean germinated in water and in $MgCl_2$ solution*

Stage of germination	Ascorbic acid (mg./100 g. fresh material)			
	In cotyledons		In sprouts	
	H ₂ O group	Mg group	H ₂ O group	Mg group
	(0.5 g.)	(0.2 g.)	(0.5 g.)	(0.2 g.)
I	10.0	11.8	—	—
II	10.9	14.2	17.4	20.2
III	12.0	12.5	11.6	13.2
IV	14.6	16.9	10.2	9.7
V	15.7	16.6	6.2	7.3
VI	15.4	17.9	4.3	4.8
Average	13.10	14.98	9.94	11.04
Difference		1.88		1.10
		(14.4%)		(11.1%)

As observed under these experimental conditions, the effect of the salts upon the ascorbic acid content of soybeans throughout the whole process of germination may be considered. The differences between the experimental group and the control group tested by means of Fisher's *t* value for small samples (Love, 1936) showed that $MgCl_2$ treatment caused the significant increase of the ascorbic acid content in cotyledons by 14.4% with the odds more than 99:1, while other differences might be considered insignificant with the odds less than 19:1.

Effects of $MgCl_2$ and $Mn(NO_3)_2$ upon the activity of ascorbic acid oxidase in soybean during germination

The effect of $MgCl_2$ and $Mn(NO_3)_2$ upon the activity of ascorbic acid oxidase was investigated with another set of beans. The concentration of the salt solutions was the same as that used in the previous experiment. The results are given in Table 5.

Table 5. *The activity of ascorbic acid oxidase in soybeans germinated in H₂O, $MgCl_2$ solution and $Mn(NO_3)_2$ solution*

Stage of germination	Relative activity of oxidase					
	H ₂ O group		Mn group		Mg group	
	Cotyledon	Sprout	Cotyledon	Sprout	Cotyledon	Sprout
	(0.5 g.)	(0.2 g.)	(0.5 g.)	(0.2 g.)	(0.5 g.)	(0.2 g.)
I	5	65	10	100	6	96
II	16	51	21	92	20	72
III	19	80	28	82	28	81
IV	25	80	31	84	33	92
V	25	64	26	66	26	80

A study of the results tabulated in Table 5 reveals that $MgCl_2$ treatment and $Mn(NO_3)_2$ treatment cause the activity of ascorbic acid oxidase to increase in both cotyledons and sprouts.

DISCUSSION

Lee & Read (1936) reported that soybeans germinated in the dark form increasing amounts of dehydroascorbic acid. Large amounts of this form arise from ascorbic acid by oxidation during the extraction of fruits and vegetables, especially when ascorbic acid oxidase is present (Harris & Olliver, 1942). Lee & Read seem to be unaware of the presence of this enzyme in germinated soybean and their claim needs confirmation. In the present work care was taken to avoid the effects of oxidation and of the oxidase.

The activity of ascorbic acid oxidase in cotyledons and in sprouts was found to be enhanced by the treatment of $MgCl_2$ and $Mn(NO_3)_2$. It is, however, still uncertain whether the enhancement of the activity did occur in the intact germinated soybeans

or whether it happened when the sprouts and the cotyledons were ground and measured according to the procedure adopted.

The relationship between the activity of ascorbic acid oxidase and the content of ascorbic acid in the soybean during germination is yet to be investigated. In the present study it was shown that both the ascorbic acid content and the oxidase activity increased, but at different rates as the germination of soybean proceeded. It is interesting to note that the activity of ascorbic acid oxidase in the sprout of an average soybean during germination is much greater than that in the two cotyledons, and the reverse is true for the content of ascorbic acid (Fig. 1 and Table 2).

Magnesium chloride in a concentration of about 10 p.p.m. of magnesium ion increases significantly the ascorbic acid content by 14.4% in cotyledons of soybean during germination. Manganous nitrate in the same concentration would also increase significantly the ascorbic acid content by 9.6% in cotyledons, if the earliest stage of germination were not taken into consideration (see Table 3). The *t* value for the mean difference, 1.14, is 7.00. Degrees of freedom are 4; odds are more than 99 to 1. This might be done on account of the slow imbibition of the beans in the Mn group at the early stages of germination. In both the Mn group and the Mg group the salt solution caused different growth rates of the sprouts as compared with that in the water group (Table 6).

Table 6. *Mean lengths in cm. of the sprouts of soybean germinated in water and salt solutions*

Days of soaking ...	2½	3½	4½	5½	6½
H ₂ O group	0.72	2.22	4.03	5.15	7.27
Mg group	0.65	2.10	4.07	6.24	8.58
Mn group	0.64	2.23	4.12	6.20	8.06

The delayed germination in the Mg group and the Mn group might be explained by slow imbibition,

but the faster growth (elongation) in the later stages of germination needs clarification. It is yet to be investigated how the effect on growth is related to the effect on ascorbic acid content of the soybeans germinated in the solutions of MgCl₂ and Mn(NO₃)₂.

The actions of MgCl₂ and Mn(NO₃)₂ are not necessarily alike. The role of Mg as a carrier of phosphorus should lead us to inquire into the effect of MgCl₂ upon the hydrolytic changes in soybean cotyledons during germination, the findings of which will probably help to explain its effect on ascorbic acid. Meanwhile the nature of ascorbic acid oxidase demands further investigation, particularly in the direction of elucidating the mechanism of the formation of ascorbic acid in soybean during germination.

SUMMARY

1. The gradual increase in the content of ascorbic acid and the activity of ascorbic acid oxidase in soybean during germination is demonstrated.

2. MgCl₂ and Mn(NO₃)₂ in a concentration of approximately 10 p.p.m. of the metallic ions cause some increase in the ascorbic acid content of the soybean germinated in the salt solutions in the dark. The 14.4% increase in the cotyledons caused by MgCl₂ treatment was considered to be significant.

3. MgCl₂ and Mn(NO₃)₂ also enhance the activity of ascorbic acid oxidase in the soybean during germination.

We wish to express our sincere gratitude to friends at the Cornell Nutrition Laboratory for their gift of ascorbic acid and also to Dr Leslie J. Harris of Cambridge University for the supply of indophenol dye used in these experiments. We also wish to acknowledge our indebtedness to the British Council Cultural Scientific Office which has been a source of help and encouragement. Our thanks are also due to Drs P. S. Tang, H. C. Yin and C. H. Lou of this laboratory for helpful criticism, and to Prof. C. Y. Chang and Prof. T. T. Li of South-West Associated University for continued interest and encouragement in this work.

REFERENCES

- Gal, E. (1938). *Nature, Lond.*, **142**, 1119.
 Harris, L. J. & Olliver, M. (1942). *Biochem. J.* **36**, 155.
 Hester, J. B. (1941). *Science*, **93**, 401.
 King, C. G. (1939). *Ann. Rev. Biochem.* **8**, 397.
 Lee, W. Y. & Read, B. E. (1936). *J. Chin. chem. Soc.* **4**, 208.
 Love, H. H. (1936). *Application of Statistical Methods of Agricultural Research*. Shanghai: The Commercial Press.
 Rudra, M. N. (1938). *Nature, Lond.*, **141**, 203.