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Selective Effect of Hormones on Nucleic Acid Metabolism during Germination of Pear Embryos

By A. A. KHAN and C. E. HEIT
New York State Agricultural Experiment Station, Cornell University, Geneva, N.Y. 14456, *U.S.A.*

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1. The effect of hormones on ^{32}P incorporation into various RNA fractions in germinating pear embryos was studied by fractionation on methylated albumin–kieselguhr columns. Abscisic acid inhibited labelling of soluble RNA, DNA–RNA hybrid and light-ribosomal RNA fractions with ^{32}P and this effect was reversed by both kinetin and gibberellic acid. 2. Kinetin reversed the inhibition by abscisic acid of ^{32}P incorporation into total ribosomal RNA and appeared to promote labelling of heavy-ribosomal RNA. Gibberellic acid was more active than kinetin in reversing the inhibition by abscisic acid of labelling of the DNA–RNA hybrid fraction with ^{32}P, but in contrast with kinetin appeared to increase further the inhibition by abscisic acid of labelling of total ribosomal RNA. 3. The percentage of radioactivity in various RNA fractions showed marked variation in response to hormones. 4. The pattern of labelling of RNA in pear embryos during reversal of inhibition by abscisic acid with a combination of kinetin and gibberellic acid was similar to that after cold-treatment of dormant pear embryos. This is suggestive of hormonal interplay in dormancy release by cold-treatment in pear embryos.

Seed germination is among the physiological processes profoundly influenced by hormones. Plant hormones, both inhibitors and promoters, at low concentrations control germination. It is therefore widely accepted that natural control of germination may involve an interplay of hormones.

A decrease in concentration of inhibitor, or an increase in concentration of promoter, or both, may occur during dormancy release by cold-treatment. Sondheimer, Tzou & Galson (1968) showed a decrease by 68% in the concentration of ABA* by cold-treatment of dormant *Fraxinus americana* embryos. Other workers showed very little change in the inhibitor content of seeds requiring cold-treatment for germination (Luckwill, 1952; Villiers & Wareing, 1960; Frankland & Wareing, 1962). Increased amounts of gibberellin and gibberellin-like substances were found during dormancy release by cold-treatment in hazel (Frankland & Wareing, 1962). Wareing (1964) concluded that dormancy may be due to the presence of inhibitors and the removal of dormancy by prechilling may depend on a build-up of promoting substances that overcome the effects of the inhibitors. Our work (Khan & Tolbert, 1965; Khan, 1966, 1967*a*, 1968, 1969; Khan & Downing, 1968) showed a widespread antagonism in germination and dormancy between cytokinins and naturally occurring germination inhibitors at low concentrations.

Cold-treatment ('stratification') is known to break the dormancy in pear embryos. No part of the embryo emerges from the seed during cold-treatment for as long as 3 months. A preliminary report showed that the capacity of the excised embryos to synthesize nucleic acids increased with increasing length of cold-treatment of the pear seed (Khan, Heit & Lippold, 1968). The present paper describes the effect of three hormones, ABA, GA_3 and kinetin, on ^{32}P incorporation into various RNA fractions and growth in pear embryos.

* Abbreviations: ABA, abscisic acid; GA_3, gibberellic acid; MAK, methylated albumin on kieselguhr; s-RNA, soluble RNA; lr-RNA, light-ribosomal RNA; hr-RNA, heavy-ribosomal RNA; m-RNA, messenger RNA; tb-RNA, tenaciously bound RNA.

MATERIALS AND METHODS

Plant material. Pear seeds, *Pyrus communis* var. Bartlett (common pear), were prechilled at 5° on moistened blotters for various times. Seeds were soaked for 15min. in 1% (w/v) sodium hypochlorite solution and rinsed thoroughly with water. Embryos were then excised by the method described by Heit (1955) and kept on ice until ready for use.

Growth experiments. When the effect of cold was studied, embryos were grown on 20ml. of 0·75% agar (Bactoagar) in 9cm. Petri plates (20embryos/plate) at 25° in continuous light (cool fluorescent, 10ft.-candles). When the effects of hormones were studied embryos were grown on 8ml. of

0·75% agar containing the hormones in 5·5cm. Petri plates (ten embryos/plate). Boiled agar solution was added to the aqueous solution of the hormones to give the appropriate concentrations. This method gave extremely uniform growth responses, free of contamination. After 4 days the embryos were weighed.

Preparation of nucleic acid. In each experiment 80 embryos were preincubated for 1hr. in the aqueous solution of the hormone or hormones, washed thoroughly with sterile water and then transferred to the heat-sterilized medium containing 0·03M-sucrose, 0·01M-tris–HCl buffer, pH6·5, 0·01M-$MgCl_2$, 0·06M-KCl and carrier-free $H^{32}PO_4$ in 5ml. of solution. Incubation was carried out for 3–4hr. at 25° on a Warner–Chilcott metabolic shaker. Extraction and purification of total nucleic acid from embryos were essentially as described by Cherry & Chroboczek (1966). Embryos were homogenized on ice in a mixture containing 15ml. of 0·01M-tris–HCl buffer, pH7·6, 0·06M-KCl, 0·01M-$MgCl_2$, 1ml. (40mg.) of bentonite, 3ml. of 11% (w/v) sodium dodecyl sulphate and 15ml. of phenol (washed with tris–HCl buffer). The aqueous layer was drawn off after centrifugation at 20000*g* for 10min. and re-extracted twice with an equal volume of cold phenol in the presence of bentonite. Nucleic acids were precipitated with 2vol. of cold ethanol in the presence of 0·4g. of potassium acetate, then dissolved in 0·05M-sodium phosphate buffer, pH6·7, and dialysed for 2 days against the same buffer at 12°.

Fractionation on MAK columns. Purified total nucleic acid was fractioned on an MAK column (Mandell & Hershey, 1960) with a linear gradient of NaCl (0·35–1·1M) in 0·05M-phosphate buffer, pH6·7. Fractions (5ml.) were collected and assayed for E_{260} and for radioactivity.

Nucleotide-composition analysis. The eluate comprising each RNA fraction from the MAK column was dialysed for 2 days at 12° before hydrolysis in 0·5M-KOH for 16hr. at 37°. Hydrolysates were neutralized in the cold with 2·5M-$HClO_4$ and centrifuged. The mononucleotides in the supernatant were separated by stepwise elution with formic acid from columns (21cm.×1·2cm.) of Dowex 1 (formate form) essentially by the procedure of Ewing & Cherry (1967). Base compositions were estimated from the relative amounts of ^{32}P in each of the four nucleotide peaks.

RESULTS

Nucleic acid fractionation. Fractionation on MAK columns of purified nucleic acids from pear embryos gave nucleic acid profiles essentially similar to that shown for other plant tissues. The designation of

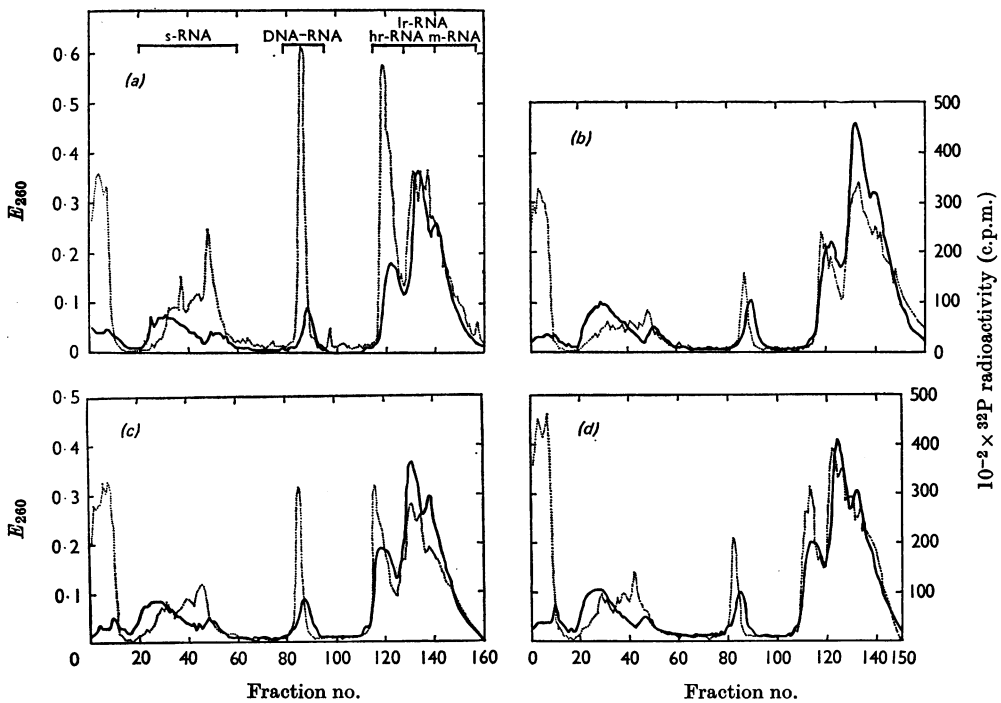

Fig. 1. Fractionation on MAK columns of nucleic acids of excised pear embryos grown in the presence of plant hormones. Each sample contained 80 embryos. Embryos were excised from seeds prechilled for 36 days at 5°. Embryos were preincubated for 1hr. in hormone solution before being incubated for 4hr. in the presence of 32μc of carrier-free $H^{32}PO_4$ in the incubation medium. See the Materials and Methods section for details. (*a*), water; (*b*), 0·02mM-ABA; (*c*), 0·02mM-ABA+0·02mM-GA_3; (*d*), 0·02mM-ABA+0·02mM-kinetin. ——, E_{260}; ····, ^{32}P radioactivity.

Table 1. *Nucleotide composition of various* ^{32}P-*labelled RNA species from excised pear embryos from experiment reported in Table* 2 (*'water' column*) *and Fig.* 1(*a*)

Fraction	Percentage of total radioactivity				Nucleotide ratios
	CMP	AMP	GMP	UMP	(A+U/C+G)
s-RNA	28·4	19·4	31·9	19·9	0·65
DNA–RNA	27·4	20·5	30·4	21·5	0·73
lr-RNA	22·5	25·5	29·6	22·2	0·91
hr-RNA	21·2	25·7	30·3	22·5	0·93
m-RNA	22·3	27·0	28·7	21·8	0·95

various nucleic acids as s-RNA, DNA–RNA hybrid, lr-RNA, hr-RNA, m-RNA and tb-RNA [Fig. 1(*a*)] is based on characterization of these fractions in soya-bean hypocotyls (Ingle, Key & Holm, 1965), peanut cotyledons (Cherry & Chroboczek, 1966) and pea roots (Ewing & Cherry, 1967).

The base composition of all nucleic acid fractions with the exception of tb-RNA is shown in Table 1. It has been shown that in plant tissues the fraction eluted (in MAK-column fractionation) after hr-RNA has the highest AMP content (Key & Ingle, 1964; Chroboczek & Cherry, 1966). This appears to be the case in the present instance also (Table 1).

The results further show that the base composition of the fraction represented as m-RNA resembles most closely that of r-RNA. However, base-composition analyses of high-molecular-weight fractions separated on MAK columns cannot be considered reliable because of the lack of resolution of such fractions by this technique and the possibility of formation of ribosomal-RNA aggregates (Ingle & Key, 1968).

Effect of hormones on ^{32}P *incorporation in pre-chilled embryos.* The effect of hormones on ^{32}P incorporation in excised pear embryos from seeds kept in cold for 36 days is shown in Fig. 1 and Table 2. It is evident that the radioactivity profile does not precisely follow the E_{260} peaks of the various RNA species. Nevertheless, radioactivity and specific-radioactivity (c.p.m./E_{260}) results show parallel changes in response to hormone treatments (Table 2).

ABA inhibited ^{32}P incorporation into s-RNA, DNA–RNA and lr-RNA fractions, but had little or no effect on hr-RNA and m-RNA. The inhibitory effect of ABA on ^{32}P incorporation into s-RNA, DNA–RNA and lr-RNA was reversed by both kinetin and GA_3. Effects of kinetin and GA_3 on labelling with ^{32}P of r-RNA and DNA–RNA in the presence of ABA, however, differed markedly. GA_3 was more active than kinetin in reversing inhibition by ABA of labelling of the DNA–RNA fraction and appeared to increase the inhibition of

Table 2. *Effect of hormones on* ^{32}P *incorporation into various RNA fractions*

RNA fractions were obtained as shown in Fig. 1. See the Materials and Methods section for details. Numbers in parentheses are the results of a separate experiment with embryos prechilled for 36 days and 26 μC of carrier-free $H^{32}PO_4$. The specific radioactivity represents c.p.m./E_{260}. Concentrations: ABA, 0·02 mM; kinetin, 0·02 mM; GA_3, 0·02 mM.

RNA fractions	Water		ABA		ABA+GA_3		ABA+kinetin	
	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity
s-RNA	388237	178859	198529	80025	292088	105825	305169	102465
	(263273)		(132683)		(180389)		(239347)	
DNA–RNA	257932	406350	71694	92338	138187	169180	103421	122741
	(156388)		(54101)		(81594)		(63533)	
lr-RNA	385024	200884	224578	93333	279880	115414	288961	116236
	(258547)		(142789)		(207205)		(215248)	
hr-RNA	449106	118699	409961	77511	325596	83775	464777	109567
	(268336)		(277998)		(43203)		(364004)	
m-RNA	304164	108943	311974	86440	262607	74818	382195	100761
	(178328)		(262927)		(194442)		(210428)	
Total r-RNA	1138294		946513		868083		1135933	
	(705211)		(683714)		(619850)		(789680)	
Total RNA	1785000		1217225		1298750		1543600	
	(1125100)		(871197)		(882100)		(1095410)	

^{32}P incorporation into total r-RNA observed in the presence of ABA. As ABA did not affect ^{32}P incorporation into hr-RNA and m-RNA, a decrease in labelling in these fractions owing to the addition of GA_3 may reflect changes brought about by GA_3. Kinetin, in contrast with GA_3, reversed the inhibition by ABA of ^{32}P incorporation into r-RNA and appeared to promote labelling of hr-RNA. Although these results point to the selective nature of the action of kinetin and GA_3, it is extremely difficult from the results presented to suggest a mode of action of these hormones. The complex nature of the ribosomal fractions obtained by this technique makes the task all the more difficult (Ingle & Key, 1968).

Sabota, Leaver & Key (1968) and other workers showed that ^{32}P incorporation in the fraction designated DNA–RNA from MAK columns may reflect bacterial contamination. Work in our laboratory showed, however, that embryos treated with 1% sodium hypochlorite grow free from contamination. The rate of ^{32}P incorporation into DNA–RNA (as well as other nucleic acids) in embryos all incubated under identical conditions depends on the length of cold-treatment, with little or no incorporation in embryos with no prechilling treatment (Khan *et al.* 1968). In the experiments reported here the bacterial counts in the incubation medium, before and after incubation of embryos, never exceeded 400 colonies, a value considered insignificant.

The results presented in Fig. 1 were further analysed in terms of percentage of radioactivity incorporated into different fractions and are shown in Table 3. The differences in ^{32}P incorporation due to hormonal treatments were highly significant as determined on a probability considerably less than 0·05 ($n_1 = 3$, $n_2 = 4$, $F = 6·95$). Error variance was used to compare the mean percentage of radioactivity in each treatment. The decrease by ABA of the percentage of radioactivity incorporated into the DNA–RNA fraction and its reversal by GA_3 was highly significant (at the 1% level). Kinetin did not appear to have any significant effect on the rate of ^{32}P incorporation into DNA–RNA. The decrease by ABA of the percentage of ^{32}P incorporated into s-RNA and lr-RNA and its reversal by both kinetin and GA_3 were significant (at the 5% level). ABA appeared to increase significantly (at 5% level) the percentage of ^{32}P incorporated into hr-RNA and m-RNA. The increase in radioactivity in m-RNA caused by ABA was not counteracted by either kinetin or GA_3. However, unlike kinetin, GA_3 was able to reverse significantly (at 5% level) the increase by ABA of the percentage of ^{32}P incorporated into hr-RNA.

Effect of hormones on ^{32}P incorporation in embryos from seeds prechilled for 45 days. The effect of ABA, in the presence and absence of a combination of kinetin and GA_3, on ^{32}P incorporation in nucleic acid fractions of excised pear embryos from seeds prechilled for 45 days is shown in Table 4. The presence of kinetin and GA_3 increased labelling with

Table 3. *Effect of hormones on percentage of radioactivity in each RNA fraction*

RNA fractions were obtained as shown in Fig. 1. Concentrations: ABA, 0·02 mM; kinetin, 0·02 mM; GA_3, 0·02 mM. Numbers in parentheses correspond to those in parentheses in Table 2.

	Percentage of radioactivity in fraction				
Treatment	s-RNA	DNA–RNA	lr-RNA	hr-RNA	m-RNA
ABA	16·31	5·89	18·45	33·68	25·63
	(15·23)	(6·21)	(16·39)	(31·91)	(30·18)
ABA+kinetin	19·77	6·70	18·72	30·11	24·76
	(21·85)	(5·80)	(19·65)	(33·23)	(19·21)
ABA+GA_3	22·49	10·64	21·55	25·07	20·22
	(20·45)	(9·25)	(23·49)	(24·17)	(22·61)
Water	21·75	14·45	21·57	25·16	17·04
	(23·40)	(13·90)	(22·98)	(23·85)	(15·85)

Table 4. *Effect of hormones on ^{32}P incorporation into various RNA fractions*

RNA fractions were obtained as shown in Fig. 1. In each treatment embryos were preincubated for 1 hr. in hormone solution before incubation for 3 hr. in the presence of 22·7 μC of carrier-free $H^{32}PO_4$ added to the incubation medium. See the Materials and Methods section for details. The specific radioactivity represents c.p.m./E_{260}. Concentrations: ABA, 0·02 mM; kinetin, 0·02 mM; GA_3, 0·02 mM.

	ABA		ABA+kinetin+GA_3	
RNA fraction	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity	Radioactivity (c.p.m./80 embryos)	Sp. radioactivity
s-RNA	36949	17764	63573	25128
DNA–RNA	3149	5431	36699	42674
lr-RNA	29373	12771	65374	27701
hr-RNA+m-RNA	40492	10944	39349	15370

Table 5. *Effect of different times of cold-treatment on growth of pear embryos*

Embryos were excised after cold-treatment of pear seeds for different times. After growth for 4 days on agar plates, 80 embryos were weighed in each case.

Time of cold-treatment (days)	Wt. of embryos (g.)
0*	2·85
5	2·87
10	3·56
20	4·55
30	7·72
40	7·91
50	7·94

* Seeds were presoaked for a few hours in water before the embryos were dissected.

Table 6. *Effect of hormones on dormant (unchilled) and non-dormant (prechilled for 36 days) excised pear embryos grown on agar plates for 4 days*

Ten embryos were weighed for each treatment; the values given are means of ten embryos. Concentrations: ABA, 0·06 mM; kinetin, 0·02 mM; GA_3, 0·02 mM.

Treatment	Wt. of one embryo (mg.)	
	Dormant*	Non-dormant
Water	40·1	85·4
Kinetin	62·3	83·7
GA_3	57·1	87·4
Kinetin + GA_3	74·8	89·4
ABA	36·9	38·7
ABA + kinetin	44·5	48·7
ABA + GA_3	45·4	54·9
ABA + GA_3 + kinetin	48·1	60·1

* Embryos were dissected after being soaked for 12 hr. in water.

^{32}P in all RNA fractions except that designated 'hr-RNA + m-RNA'. The results completely mask the inhibitory effect of GA_3 in the presence of ABA on ^{32}P incorporation into hr-RNA and m-RNA (Table 2). There was a marked increase in the labelling of r-RNA in presence of the hormones. These results are consistent with the individual and selective effects of GA_3 and kinetin shown in Table 2.

Effect of hormones and cold-treatment on growth. The effect of cold-treatment of pear seeds for different times on the subsequent growth of excised pear embryos is shown in Table 5. Cold-treatment for approx. 20–30 days appears to be sufficient for a complete release of dormancy of pear embryos.

The effect of hormones on the growth of dormant and non-dormant embryos is shown in Table 6. Either kinetin or GA_3 released the dormancy. However, maximum release of dormancy was obtained by a combination of kinetin and GA_3. Kinetin and GA_3 even in the presence of ABA were able to release dormancy to some extent. The growth of non-dormant (prechilled) embryos was inhibited by ABA and this inhibition was reversed by either kinetin or GA_3. A combination of kinetin and GA_3 caused maximum reversal of inhibition by ABA.

DISCUSSION

Pear embryos provide an excellent material for the study of dormancy and germination. The embryo fails to grow during prechilling treatment of up to 3 months. However, its capacity to incorporate ^{32}P into nucleic acids changes during this period (Khan *et al.* 1968). The results presented here clearly show that GA_3, kinetin and ABA profoundly influence the nucleic acid metabolism during germination of excised pear embryos. The results show that GA_3 is more active than kinetin in reversing the inhibition by ABA of ^{32}P incorporation in the fraction designated as 'DNA–RNA' (Table 2). This selective action of GA_3 on the DNA–RNA fraction is also shown by the results on percentage of radioactivity incorporated into each fraction (Table 3). Kinetin, on the other hand, was more active than GA_3 in reversing inhibition by ABA of ^{32}P incorporation into r-RNA (Table 2). Specific-radioactivity results also revealed similar selective effects of the two hormones on inhibition by ABA (Table 2). The increase in labelling of hr-RNA and m-RNA, as shown by results on percentage distribution of radioactivity (Table 3), but the little or no effect on total labelling of these fractions (Table 2) in the presence of ABA, suggests that inhibition by ABA of germination may be due to its having preferential effects on sites controlling the synthesis of other RNA species.

Jarvis, Frankland & Cherry (1968*a*,*b*) reported that, before dormancy is released in hazel seeds, GA_3 increases the DNA template and RNA polymerase activity necessary for RNA transcription. In the results presented in the present paper GA_3 by itself does not seem to be as effective in releasing dormancy or reversing inhibition by ABA of ^{32}P incorporation or growth as when kinetin is also included in the germination medium. However, GA_3 acts by removing significantly the inhibition by ABA of ^{32}P incorporation into DNA–RNA in preference to other RNA species. Natural DNA–RNA complexes have been described by several workers (Yankofsky & Spiegelman, 1962; Schulman & Bonner, 1962). According to Hayashi & Spiegelman (1961), the DNA–RNA complex is an intermediate in the transcription of the genetic information of the DNA. Cherry (1964) showed that DNA–RNA in peanut cotyledon consists of 25% of RNA, 25% of rapidly metabolized DNA

and 50% of non-metabolic DNA. It is, therefore, tempting to speculate that GA_3 may induce formation of template RNA through such a route of DNA–RNA hybridization.

In peanut cotyledon (Carpenter & Cherry, 1966) and radish leaves (Burdett & Wareing, 1968) cytokinins were shown to enhance RNA synthesis. Osborne (1962) suggested that cytokinins may regulate the synthesis of a particular RNA fraction. The effect of kinetin in our present experiments appears to be similar to that reported by Srivastava (1967, 1968) in that a parallel increase in amount of ribosomal components and ribosomal RNA occurs in response to kinetin.

Participation of more than one hormone in biological systems has been repeatedly demonstrated (Tata, 1966). ABA and other natural growth inhibitors are known to induce in many seeds a dormancy that can be broken by cytokinins, GA_3 or by a combination of the two (Khan & Tolbert, 1965; Khan, 1966, 1967*a*,*b*, 1968, 1969; Sondheimer & Galson, 1966; Sondheimer *et al.* 1968; Bradbeer, 1968; Khan & Downing, 1968; Jarvis *et al.* 1968*a*,*b*).

In barley and lettuce seeds, exogenously applied GA_3 (and perhaps the endogenous gibberellins) appears to be the primary stimulus for germination (Khan, 1968, 1969). Cytokinins have little effect on germination of these seeds. However, they are extremely effective in counteracting the inhibition of germination imposed by natural growth inhibitors (Khan, 1967*a*,*b*, 1968). In rosaceous or ash embryos requiring cold-treatment for germination both cytokinins and gibberellic acid are effective in releasing the dormancy (Sondheimer & Galson, 1966; Khan & Heit, 1967; Khan *et al.* 1968). Post-harvest dormancy in grain seeds can be overcome, among other means, by treatment with GA_3, but not by cytokinins (Pollock, 1959). In other instances, e.g. *Xanthium* seed, dormancy can be released by kinetin, but not by GA_3 (Khan, 1966). All these cases are consistent with our working hypothesis (Khan, 1968, 1969) that gibberellin or related compounds are perhaps the primary stimulus for germination. The endogenous inhibitors block the gibberellin-mediated germination response. The cytokinins antagonize the action of the endogenous inhibitors and thereby allow gibberellin to function.

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