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Lettuce Seed Germination

MODULATION OF PREGERMINATION PROTEIN SYNTHESIS BY GIBBERELLIC ACID, ABSCISIC ACID, AND CYTOKININ[1]

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ABSTRACT

Protein synthesis in gibberellin-treated lettuce (*Lactuca sativa*) seeds has been studied during the lag phase between the beginning of imbibition and the first signs of radicle protrusion. When compared to the water-imbibed controls, both polyribosome populations and radioactive leucine incorporation into protein increase in the embryos of GA_3-induced seeds early in the imbibition period. Since these results are contradictory to previously published studies, the reasons for the differences are outlined and various alternative possibilities eliminated. The protocol for protein extraction, particularly the speed at which the supernatant from the seed homogenate is cleared, is important for demonstrating the GA_3-mediated changes. Embryos maintained in the dormant state by abscisic acid still conduct considerable amounts of protein synthesis, and this is enhanced by concentrations of 6-benzylaminopurine which also promote germination. Therefore, the actions of GA_3, abscisic acid, and cytokinin on lettuce seed germination are mediated, directly or indirectly, via protein synthesis.

In the absence of a suitable germination stimulus, hydrated Grand Rapids lettuce seeds maintained in darkness at 25 C fail to germinate. Such dormant seeds exhibit a capacity for protein synthesis however (4, 6, 11), accompanied by the establishment of an extractable polyribosome population (11). Thus, lettuce seeds resemble those of other species, in that ribosome activation is characteristic of the early stages of germination (1, 14, 20, 21, 23). Promotion to germination of dark-imbibed lettuce seeds can be achieved by the introduction of low concentrations of gibberellic acid (5, 16). Since GA_3 has been reported to enhance germination and protein synthesis in some seeds, *e.g. Avena fatua* (9), wheat (8), and hazel (15), we decided to reexamine the contention that GA_3 does not enhance protein synthesis in germinating lettuce seeds (4).

Also of interest to us is the complex response of lettuce seeds to GA_3, ABA, and CK[3] (5, 17). The promotive effect of GA_3 can be reversed by ABA, and this inhibition is overcome by CK, but not by increasing GA_3 concentrations. Isolated embryos imbibed in darkness do not require GA_3 to stimulate their germination. Abscisic acid alone inhibits radicle elongation and this inhibition can be overcome by CK, but not by high levels of GA_3 (5, 7). We interpret these results to mean that ABA and CK have a common site of action, which is distinct from that where GA_3 acts. We also examined the relationship of these promoters and inhibitor to the protein activity of the lettuce seed embryo.

MATERIALS AND METHODS

Seeds of *Lactuca sativa* cv. Grand Rapids (Ferry Morse Seed Company: batch 18639) stored at 4 C were used for all experiments. Seeds from the 1971 harvest were used for all experiments except those reported in Figure 2, for which the 1970 harvest was used (as it was for our previous polyribosome extraction experiments; 11). Both seed harvests germinated little (5–15%) when imbibed at 25 C in darkness on water.

Procedures for embryo preparation, surface sterilization, imbibition, and polyribosome extraction have been described previously (11). When seeds or embryos were imbibed in GA_3, a concentration of 100 μg/ml (0.29 mM) was used. Pricked seeds were obtained by first surface-sterilizing intact dry seeds, transferring them on moist filter paper to the stage of a binocular dissecting microscope, and then pricking them through one side with a No. 1 entomological pin, midway along their length. This treatment was effected under a dim green safelight and it neither increased the low level of dark germination of these seeds nor lowered their high level of GA_3-induced germination. Concentrations of GA_3, ABA, and CK used to obtain the required embryo response were based on those reported previously (5) and are given in the appropriate figures and tables.

Proteins were extracted from seeds or embryos previously incubated on L-leucine 4,5-T (New England Nuclear, 42.7 Ci/mmol) (except for results of Fig. 8, see legend) as previously described (4, 6), except that the extraction buffer was modified and based upon that of Jarvis and Hunter (15): 250 mM sucrose, 50 mM tris-Cl(pH 8.5), 20 mM KCl, 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 1% (w/v) DOC (Fisher Scientific). The homogenate supernatant to which trichloroacetic acid was added to precipitate protein was collected by centrifugation at 20,000*g* (4, 6). Uptake of labeled leucine into the seed was estimated by extracting the seeds in ice-cold 80% ethanol and sampling the 20,000*g* supernatant for radioactivity.

The extent of bacterial contamination on our seeds was determined using a surface viable colony count of serial dilutions incubated at 30 C for 36 hr on 23 g/l Bacto Nutrient Agar (Difco, Detroit).

For analysis of the free amino acid pool, 250 mg of surface-sterilized seeds were incubated on filter paper in a 9-cm Petri dish for 9 hr. They were then ground in 10 ml of 0.1 M phosphate buffer (pH 7.6), centrifuged at 20,000*g* for 15 min, and the protein precipitated with trichloroacetic acid. The collected supernatant was evaporated under vacuum to near dryness and

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[3] Abbreviations: DOC: sodium deoxycholate; CK: cytokinin; 6-BA: 6-benzylaminopurine.

quantitatively analyzed on a Beckman model 121 automatic amino acid analyzer equipped with a curve-integrating attachment.

RESULTS AND DISCUSSION

Time Course of GA_3-promoted Germination. We counted a seed as having germinated when the radicle protruded visibly through the enclosing seed layers. Germination of isolated embryos was scored at the first indication of geotropic curvature of the radicle. Intact seeds imbibed in darkness on filter paper in the presence of GA_3 commenced germination after 10 hr (Fig. 1a), whereas those imbibed in water failed to germinate until after 14 hr of imbibition, and then only to a low percentage (Fig. 1d). Experiments using radiolabeled leucine were conducted in Conway units following surface sterilization of the seeds. Both of these treatments resulted in delay of GA_3-induced germination (Fig. 1, b and c) and reduced dark germination (Fig. 1e). Since our studies are aimed at determining the effects of GA_3 on germination, we confined our experiments to times before radicle protrusion, since only those events occurring between the start of imbibition and the commencement of visible germination can be important in establishing the promotive action of GA_3. Our experiments on the effects of GA_3 on polyribosome formation were performed within the lag phase of Figure 1a on seeds imbibed on filter paper, and our leucine incorporation experiments within the lag phase of Figure 1c, on surface-sterilized seeds imbibed in Conway units.

The time course of germination of isolated embryos imbibed on water was faster than GA_3-induced intact seeds (Fig. 2, a and b). Embryos imbibed on 20 μM ABA in darkness failed to germinate even after 7 days. If 10 μM 6-BA was added along with the ABA, germination was achieved after a 24-hr lag period (Fig. 2c). The germination kinetics shown here for seeds and embryos incubated on filter paper in Petri dishes was retarded approximately 2 hr if the incubation was carried out in the outer rim of a Conway unit, and so experiments involving radioactive incorporation into embryos were conducted within the time limits of this expanded lag period.

Ribosomal Fraction. Ribosomal fractions were separated by sucrose density gradient centrifugation and analyzed as described previously (11). The polyribosome component in Figure 3 is expressed as a percentage of the total extractable ribosomal fraction (11). At times prior to germination, GA_3-treated seeds yielded more polyribosomes than their equivalent dark-imbibed

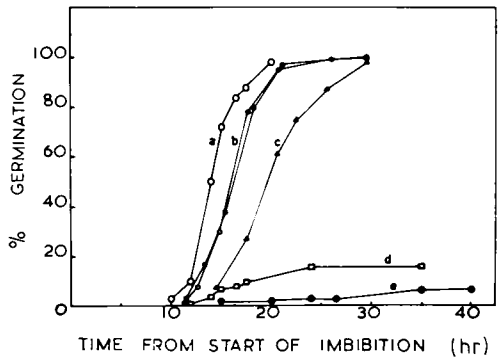

FIG. 1. Effect of incubation conditions on the time course of seed germination. Seeds were incubated at 25 C in darkness with the following modifications: a: in 9-cm Petri dishes containing 1 layer of Whatman No. 1 filter paper and 5 ml GA_3 (100 μg/ml); b: incubated in the outer well of a Conway unit containing 2 ml GA_3 (100 μg/ml). ○: 1970 harvest; ●: 1971 harvest; c: surface-sterilized seeds incubated in Conway units as in (b); d: imbibed on water as in (a); e: surface-sterilized seeds imbibed as in (b) and (c), but on water. All seeds from the 1970 harvest except where indicated otherwise.

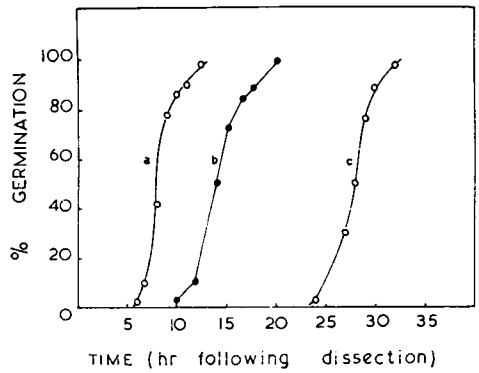

FIG. 2. Time course of embryo germination response. a: embryos in water; b: seed germination in GA_3 (reference time course as in Fig. 1b); the abscissa for this curve is "time from start of imbibition."; c: embryos in ABA (0.02 mM) + 6-BA (0.01 mM).

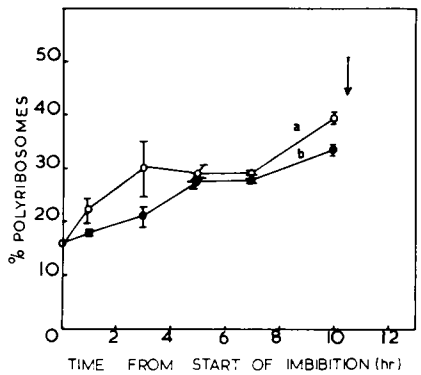

FIG. 3. Effect of GA_3 on the polyribosome content of lettuce seeds. a: Seeds incubated in GA_3 (100 μg/ml); b: seeds incubated in water; each point is the mean (±SE) of a minimum of three replicate extractions of 200 mg of seeds; the arrow indicates the time of initiation of germination by seeds in (a).

control. Similar results were obtained when embryos alone were extracted after they had been dissected out of intact seeds, imbibed over the same time period in the presence or absence of GA_3 (results not presented). This means that the observed changes in polyribosomes occur within the embryo itself and not in the surrounding structures, *i.e.* endosperm. While naked embryos do not require GA_3 to stimulate radicle elongation (the germination response), they still respond to this promoter in the same way as the embryos from intact seeds. It is widely accepted that the site of action of germination promoters is in the embryos of intact lettuce seeds. The advantages of using isolated embryos over whole seeds are outlined later.

***In Vivo* Protein Synthesis.** Seeds were imbibed in darkness on GA_3 or water and the incorporation of [^{3}H]leucine into protein determined (Fig. 4). Protein extracted from GA_3-treated seeds was of approximately twice the specific radioactivity of the water controls. In the latter, both the rate and magnitude of incorporation were less. The results in Figure 4 are expressed as a function of total extractable protein (determined by the Lowry method, ref. 19), which did not vary during the experimental time period in either treatment, nor was there any difference between the treatments (data not presented). The observed increase in GA_3-induced incorporation is probably a reflection of a relatively small change in *in vivo* activity, rather than massive synthesis of one or more proteins.

The results presented here do not agree with the earlier contention that GA_3 does not induce any detectable increases in protein synthesis (4). We thus decided to study the possible reasons for this discrepancy and to eliminate alternative explanations for the GA_3 effect.

Uptake of [^{3}H]Leucine into the Embryo. A variety of organic (2, 25) and inorganic (13) compounds are restricted in their entry into the embryo by the thin sac-like enclosing endosperm. The entry of leucine may be severely retarded by this tissue (18), so we have determined if the increased incorporation observed in the presence of GA_3 is merely a consequence of enhanced uptake of labeled precursor through the endosperm into the embryo. Figure 5 shows that leucine uptake into the seed was essentially completed after the 2nd hr of imbibition, and that the minor differences in uptake between the GA_3-treated and water-imbibed seeds thereafter were not significant and could not account for the large differences in its incorporation into protein.

Localization of GA_3-Associated Changes in Protein Synthesis Capacity. Black and Richardson (6) have shown that the endosperm of dormant seeds of Grand Rapids lettuce incorporated radioactive amino acid into protein to at least 5 times the level of the axis tissue. To show that the GA_3-promoted increase in protein synthesis was associated in some way with embryo (*i.e.* with that part of the seed which is induced by GA_3 to germinate), this tissue was dissected out of intact seeds (pricked or unpricked) following a 4-hr incubation period in labeled leucine. Table I shows that the embryos dissected from intact and pricked GA_3-treated seeds showed over a 50% increase in protein synthesis, and that this increase was unrelated to the change in uptake of leucine into the embryos. While incorporation of label into the proteins of the endosperm might occur, considerably enhanced protein synthesis stimulated by GA_3 takes place in the embryo.

Changes in Amino Acid Pool. An analysis was made of the amino acid pool of dry seeds and of seeds incubated in the presence or absence of GA_3 for 9 hr. These tests were performed to check that the GA_3-induced stimulation of [^{3}H]leucine incorporation was not due to differential dilution of the radiotracer by the endogenous amino acid pool. A reduced endogenous pool size resulting from the action of GA_3 would be expected to result in a more concentrated labeled pool, and hence, a greater availability for incorporation (4).

From Table II it is obvious that considerable quantities of most amino acids were present in dry seeds (aspartic acid, cystine, and proline were not detected). Large increases were observed in the pools of the aromatic amino acids phenylalanine and tyrosine during imbibition, with glycine, threonine, alanine, and arginine showing decreases. In almost all cases (and in the particular case of leucine), the pool size changes exhibited over the 9-hr imbibition period were of approximately the same magnitude irrespective of the presence or absence of GA_3; furthermore, the total μmol of the endogenous pools were more or less equivalent. Thus, GA_3-induced incorporation of leucine cannot be explained through changes in the pool size. We do not know if there are any GA-induced modifications of the active and inactive pool sizes, but there is no reason to suspect that this should occur.

It has been suggested that phytochrome-induced lettuce seed germination occurs because the ability of the embryo to push itself out through the surrounding layers is enhanced. One way in which this could happen is by the lowering of the osmotic potential of the radicle cells because of an increase in small mol wt catabolites of lipid and protein storage materials (22). Our analysis of the free amino acid pool after a 9-hr imbibition on GA_3 (a time just prior to the start of visible germination) shows that this does not increase sufficiently to account for any changes in osmotic potential at this time. No GA-induced increase in proteases has been found either (3, 4).

Bacterial Contamination and Protein Synthesis. Many seeds possess a surface flora of microorganisms which can compete for radioactive precursors. If no precautions are taken to reduce this

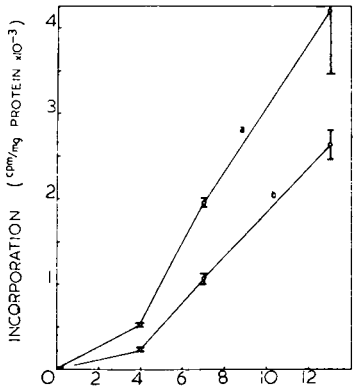

FIG. 4. *In vivo* incorporation of [^{3}H]leucine into protein by lettuce seeds. a: Seeds incubated in 100 μg/ml GA_3; b: seeds incubated in water. Two replicates of 75 seeds were incubated in 100 μCi L-leucine 4,5-T and extracted as described in "Materials and Methods:" seed homogenate cleared at 20,000*g*. Standard error of the mean is presented for each point.

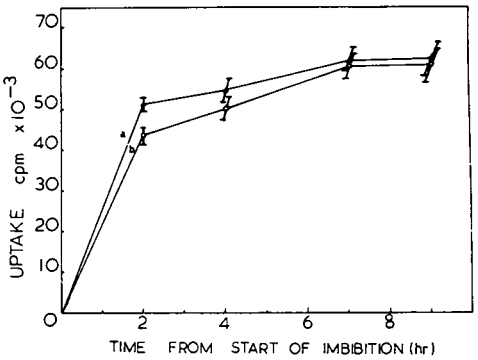

FIG. 5. Uptake of [^{3}H]leucine by lettuce seeds. a: Seeds incubated in water; b: seeds incubated in 100 μg/ml GA_3. Two replicates of 75 seeds were incubated in 10 μCi L-leucine 4,5-T. Uptake was estimated from an 80% ethanol-soluble extract. Standard error of the mean is presented for each point.

Table I. *Uptake of [^{3}H]Leucine and Protein Synthesis in Embryo Tissue*

Duplicate treatments of 30 intact or pricked seeds were incubated for 4 hr in 5 μCi L-leucine 4,5-T. 25 embryos were dissected following the incubation period and then protein was extracted.

Seed Treatment	Uptake (cpm/replicate)[1]	Change %	Incorporation (cpm/mg protein)	Change %
intact H_2O	6790.0	-	118.1	-
intact GA_3	4950.0	-27.2	180.7	+52.9
pricked H_2O	8810.0	-	266.4	-
pricked GA_3	9460.0	+7.4	420.7	+57.9

[1] Duplicate treatments were extracted in 80% ethanol for estimation of uptake. Uptake is expressed in cpm/25 embryo replicate.

[2] % increase (+) or decrease (-) compared to respective water control.

Table II. *Endogenous Amino Acid Pools*

Two hundred fifty mg of seeds were surface-sterilized and either extracted immediately or subjected to a 9-hr incubation in the presence or absence of GA_3 (100 μg/ml). Results expressed as μmol/250 mg seeds.

Amino Acid	Dry Seeds Pool Size	9 Hr H_2O Pool Size	% Dry Seeds decrease	% Dry Seeds increase	9 Hr GA_3 Pool Size	% Dry Seeds decrease	% Dry Seeds increase
Asp	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thr	0.795	0.713	10.3		0.724	8.9	
Ser	0.750	0.928		23.7	0.950		26.7
Glu	0.690	1.040		50.7	0.990		43.5
Pro	0.0	0.0			0.0		
Gly	0.248	0.125	49.6		0.154	37.9	
Ala	0.165	0.160	3.0		0.225		36.4
Cys	0.0	0.0			0.0		
Val	0.055	0.067		21.8	0.067		21.8
Met	0.011	0.016		45.5	0.016		45.5
Ile	0.037	0.042		13.5	0.038		2.7
Leu	0.035	0.045		28.6	0.043		22.9
Tyr	0.006	0.014		133.3	0.016		167.0
Phe	0.009	0.026		189.0	0.027		200.0
Lys	0.070	0.077		10.0	0.087		24.3
His	0.021	0.028		33.2	0.026		23.8
Arg	2.522	2.435	3.5		2.405	4.6	
Total (µmol)	5.404	5.717		5.79	5.768		6.73

flora, incorporation results may simply reflect contaminating bacterial or fungal activity (12). Our seeds were tested for surface bacteria and the effect on these of surface sterilization and antibiotic treatment. Surface viable colonies were examined for seeds imbibed for 12 hr, since this was close to the longest incubation period used in our radioactive experiments.

Untreated seeds of the 1970 harvest were heavily contaminated with bacteria after 12 hr of imbibition (2.32×10^6 colonies/100 mg seeds) but this was reduced 400-fold (6×10^3 colonies) if the seeds were first surface-sterilized, and a further reduction (7×10^2 colonies) occurred if the sterilized seeds were imbibed in 50 IU/ml penicillin/streptomycin (Biocult Laboratories Ltd., Glasgow). We consider it most unlikely that the observed GA_3-stimulated increase in [^{3}H]leucine incorporation by seeds was merely a consequence of hormone-induced growth and metabolism of the contaminating bacteria. There is no evidence that GA_3 has such an effect on bacteria, but more compelling evidence is that: (*a*) embryos themselves isolated from sterilized seeds and maintained in sterile conditions showed GA_3-enhanced incorporation of leucine into protein (Table I); and (*b*) that there was a rise in the polyribosome population of GA_3-treated seeds: the low amount of contaminating bacteria relative to the mass of the seeds could not have contributed in any measurable way to the polyribosome profile (Fig. 3). Surface sterilization reduces incorporation from 452 to 173 cpm/75 seeds in non-GA_3-treated seeds and from 768 to 292 cpm/75 seeds in GA_3-treated seeds. The degree of GA_3-enhanced incorporation by sterilized and unsterilized seeds was the same (69%). In relation to these results, we must also point out the possibility that the bleach used during our surface sterilization procedure (12) could also have penetrated into the endosperm and reduced the protein synthesis known to be carried out by this tissue (6).

Protocol for Extraction of Proteins. As outlined in "Materials and Methods," leucine incorporation was followed into a supernatant fraction collected from the seed homogenate following a clearing spin at 20,000*g*. It appears that the speed of this initial centrifugation was of prime importance for allowing a demonstration of GA_3-induced protein synthesis. In Figure 6, we show that the percentage difference in specific radioactivity of incorporation between seeds imbibed in GA_3 or water for 4 hr increased as a function of the speed at which the tissue homogenate was centrifuged. Protein precipitated from supernatants derived from GA_3-treated seeds and cleared at speeds greater than 15,000*g* (using extraction buffer at pH 7.6) showed that they had incorporated more leucine than the untreated control seeds. Below this clearing speed, the trend was reversed al-

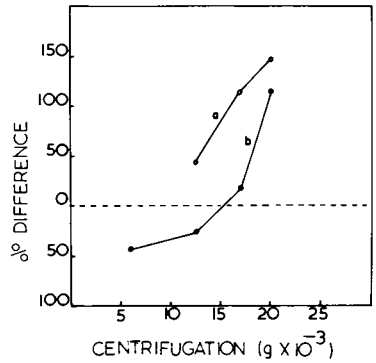

FIG. 6. Effects of pH of the extraction medium and the speed of the clearing spin of the seed homogenate on the detection of GA_3-induced protein synthesis. a: Extraction buffer pH 8.5; b: extraction buffer pH 7.6. Two replicates of 75 seeds were incubated in 10 μCi L-leucine 4,5-T in the presence and absence of GA_3 (100 μg/ml). Seeds were extracted as described in "Materials and Methods" except that the speed of the 15-min clearing spin and the pH of the extraction buffer were varied as noted. The ordinate is per cent modification of incorporation in water by GA_3.

though not always to the same magnitude as shown in Figure 6: sometimes the per cent difference remained closer to zero. When an extraction buffer at pH 8.5 was used, enhanced GA_3-induced protein synthesis was also noted when supernatants cleared at higher speeds were used. The actual values for leucine incorporation into the hot trichloroacetic-acid-precipitable protein fractions obtained from pH 8.5 homogenates cleared at various speeds are shown in Table III. The per cent difference in incorporation in homogenates of GA_3- or water-imbibed seeds appears to be due to a combination of factors, an enrichment in specific radioactivity of the GA_3-stimulated proteins with increasing speed of centrifugation, accompanied by a decline in the specific radioactivity of the control proteins. We find it difficult to explain the reason for this differential shift in specific radioactivity, although part of the reason could result from the effective removal from the supernatant (to the pellet fraction), at higher speeds, of some labeled protein common to both treatments.

The data shown in Figure 6 could be obtained for GA_3- and water-imbibed seeds from both the 1970 and 1971 harvests extracted at pH 7.6 and pH 8.5. Furthermore, we also obtained similar results using the tris-glycine (pH 8.4) extraction technique of Black and Richardson (6) adopted by Bewley and Black (4). It is interesting to note that their extraction technique involved a low speed clearing spin of the homogenate prior to

trichloroacetic acid precipitation, which would account for their lack of success in observing any GA_3-induced protein synthesis. Indeed, we could reproduce their results by an appropriate reduction in the speed at which the tissue homogenate was cleared.

We conclude from our results that GA_3 does promote protein synthesis in lettuce seeds at an early stage (after only 4 hr of imbibition) during germination. The ability to detect this change in protein-synthesizing capacity is dependent upon the extraction protocol, which is indicative that there is an apparent subtle distribution of the newly synthesized proteins within the cell supernatant.

Protein Extraction without Detergent. The regular protocol for protein extraction involved the addition of 1% DOC to the grinding medium to aid in the release of membrane- or organelle-associated proteins. Since it has been reported that GA_3-induced protein synthesis in hazel seeds could be detected only when this detergent was added to the grinding medium (15), we repeated our experiments without using it. From the results in Table IV we see that there was still GA_3-induced protein synthesis whether or not DOC was present (pH 8.5, cleared at 20,000*g*). More total protein was extracted when DOC was used and the associated reduction in its specific radioactivity was probably due to a dilution of labeled by unlabeled proteins released by the detergent. The reduction in specific radioactivity was greatest for proteins extracted from seeds incubated in the absence of GA_3 (see values in parentheses, Table IV), which could mean that more of the DOC-solubilized proteins are associated with, and freed from, the membranes of GA_3-treated seeds.

ABA Inhibition of GA_3-Promoted Seed Germination. Whole seeds imbibed on GA_3 + ABA incorporated less labeled leucine into protein than their uninhibited counterparts (Table V), although extensive protein synthesis occurred in these dormant seeds. When embryos were dissected from intact or pricked seeds after 4 hr of imbibition, they also had extensively incorporated leucine (Table VI), although ABA reduced protein synthesis below that promoted by GA_3. Pricked seeds allowed for more leucine uptake, but neither in these seeds nor in the intact seeds did ABA reduce uptake. Thus, ABA-inhibited protein synthesis was not a consequence of reduced precursor availability.

Table III. *Effect of Centrifugation Speed on Extraction of Labeled Protein*

Values are the mean of two replicate treatments of 75 seeds incubated under standard labeling conditions for 4 hr in 10 μCi L-leucine 4,5-T. Extraction was as described in "Materials and Methods" except that the first centrifugation step was modified as noted.

Centrifugation 10^{-3} g	Treatment	
	$-GA_3$	$+GA_3$
	cpm/mg protein	
12.5	487	697
17.0	384	827
20.0	366	902

Table IV. *Effect of Detergent on Extraction of Labeled Protein from a 20,000g Supernatant*

Treatment	Specific Activity[1] cpm/mg protein	Total Protein[2]
-DOC $-GA_3$	676.6	5.62
$+GA_3$	1095.1	6.04
+DOC $-GA_3$	366.1(47.1)	8.51
$+GA_3$	902.1(17.7)	8.52

[1] Each value is the mean of two replicate treatments of 75 seeds incubated under standard labeling conditions for 4 hr in 10 μCi L-leucine 4,5-T. Extraction was as described in "Materials and Methods," except that DOC (1% w/v) was omitted from the extraction buffer where indicated. Values in parentheses are per cent decrease *cf.* −DOC treatment.

[2] Estimated by the Lowry method (19).

Table V. *Protein Synthesis in Intact Lettuce Seeds Treated with GA_3 or GA_3 and ABA*

Two replicates of 75 seeds were incubated in 10 μCi L-leucine 4,5-T.

Imbibition time	Specific Activity cpm/mg protein		
	4 hr	7 hr	12 hr
GA_3 (0.29mM)	504	1952	4210
GA_3 (0.29mM) + ABA (0.04mM)	379	1201	3723

Table VI. *Protein Synthesis in Embryo Tissues: [^{3}H]Leucine Uptake and Incorporation*

Duplicate treatments of 30 intact or pricked seeds were incubated for 4 hr in 5 μCi L-leucine 4,5-T. Concentrations of ABA and GA_3: 0.04 mM and 0.29 mM, respectively. Twenty-five embryos were dissected following incubation, washed briefly with 10^{-3} M unlabeled leucine, and extracted. Free leucine was estimated as described in "Materials and Methods."

	Leucine Uptake	Change	Leucine Incorporation	Inhibition
	cpm/25 embryos	%	cpm/mg protein	%
Intact seeds + GA_3	4950		180.7	
Intact seeds + GA_3 + ABA	5360	+8.3	160.8	11
Pricked seeds + GA_3	9460		420.7	
Pricked seeds + GA_3 + ABA	9750	+3.2	283.4	32.6

ABA Inhibition of Embryo Germination and Its Reversal by Cytokinin. The use of embryos has several advantages: they respond in an all or none fashion to low concentrations of ABA and 6-BA; it is easier to extract polyribosomes from them than intact seeds (11); and there is no permeability barrier to retard uptake of organic chemicals (*e.g.* leucine) (18).

Changes in the polyribosome patterns in isolated embryos imbibed in germination-permitting or inhibiting conditions are shown in Figure 7. Embryos incubated in water had a greater polyribosome content before germination (which started at 9 hr) than the nongerminating ABA-treated embryos. An increase in the polyribosome population of ABA-treated seeds did occur up to the 12th hour of imbibition, but then declined. Embryos incubated in ABA + 6-BA showed only a slightly increased population of polyribosomes prior to 16 hr of imbibition, but this was considerably higher up to the point of germination at hour 28. At a time just prior to germination of ABA- + 6-BA-treated embryos, the increase in polyribosomes over the levels found in the ABA-inhibited embryos was approximately equal to the increase shown by the water-imbibed (germinating) embryos over the 8- to 10-hr imbibed ABA-inhibited embryos.

When cumulative *in vivo* incorporation of leucine into protein was followed in similarly treated embryos, the results in Figure 8 were obtained. Incorporation by the isolated embryos was considerably higher than that into intact seeds (Fig. 4) due to the lack of the endosperm barrier to uptake. Throughout the 30-hr incubation period, there was no change in total extractable protein (determined by the Lowry method [19]; data not presented). Abscisic acid-treated embryos incorporated less leucine into protein throughout the time course of the experiment. Between the 8th and 12th hr of imbibition, there was a decrease in specific radioactivity of the proteins in all treatments. This decrease may be due to increased turnover of proteins as growth of the water-imbibed embryo gets underway (Fig. 8a). It is not clear to us why this turnover should also occur about this time in the dormant ABA-treated embryos (Fig. 8b) or in those imbibed on ABA + CK (Fig. 8c) at least 12 to 16 hr before germination commences. Such an observation is not without precedent because isolated barley embryos also show a dramatic decrease in their rate of protein synthesis prior to, and just after, germina-

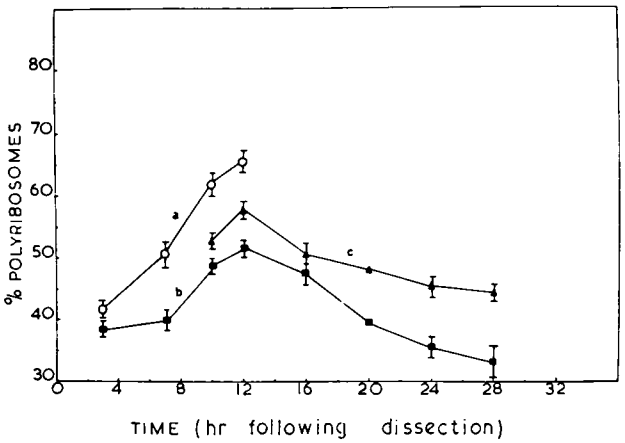

FIG. 7. Effect of ABA and 6-BA on the polyribosome content of isolated embryos. a: Embryos incubated in water; b: embryos incubated in 0.02 mM ABA; c: embryos incubated in 0.02 mM ABA + 0.01 mM 6-BA. Each point is the mean (±SE) of at least two determinations based on extractions of 100 embryos.

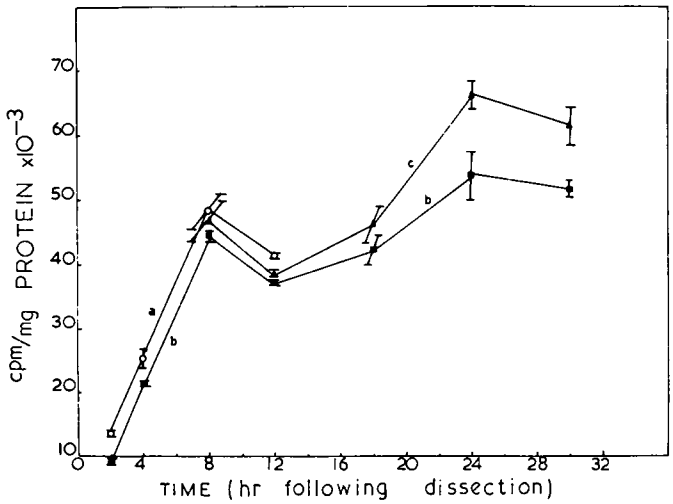

FIG. 8. *In vivo* incorporation of [^{3}H]leucine by isolated embryos. a: Embryos incubated in water; b: embryos incubated in 0.02 mM ABA; c: embryos incubated in 0.02 mM ABA + 0.01 mM 6-BA. Two replicates of 50 embryos were incubated in 25 μCi D,L-leucine 4,5-T (Amersham Searle, 28 Ci/mmole) and extracted for proteins as described in "Materials and Methods." Each point represents the mean ±SE.

tion (24). A similar decrease was also noted some 12 hr later (Fig. 8, b and c). Incorporation by embryos imbibed on water was not followed beyond 12 hr because growth had commenced by this time.

FURTHER DISCUSSION AND CONCLUSIONS

An early event during GA_3-induced germination is an increase in the protein synthetic capacity of lettuce seeds. Polyribosome populations increase early in the lag phase before germination and this is accompanied by increased incorporation of labeled precursor amino acid into protein. These results are in contrast to an earlier report (4), where no increased protein synthesis was detected. Extraction protocol is important for demonstrating the apparently subtle GA_3-induced changes in protein synthesis, however. Potential contributions to *in vivo* [^{3}H]leucine incorporation into seed protein by differential leucine uptake, amino acid pool shifts, endosperm involvement, and seed microflora are insufficient to account observed changes. Lettuce seeds, like a number of others (8, 9, 15), exhibit GA_3-induced protein synthesis prior to germination, above that level of synthesis being carried out by water-imbibed, dormant seeds.

The inhibitory effects on germination by ABA and the relief by 6-BA are also associated with early changes in protein synthesis by the embryo. ABA retards protein synthesis very early during imbibition (by the 4th hr), and thus ABA may act even before the seeds are completely hydrated (11). Although ABA never completely reduced the GA_3-enhanced protein synthesis back to the level observed for water-imbibed seeds (compare Fig. 4b and Table V), the reduction was considerable at all times of imbibition. Water-imbibed dormant seeds are capable of carrying out considerable protein synthesis; this is enhanced by GA_3 in those seeds which are going to germinate, and reduced again by ABA in inhibited seeds. This lends credence to the suggestion that there is some GA_3-induced protein synthesis necessary for germination, and reduction of this prevents germination. A qualitative study of protein synthesis is proposed to study this possibility.

The effect of 6-BA is to reverse the ABA-inhibited protein synthesis in embryos. Here too, the enhancement due to 6-BA is long before germination occurs and is maintained throughout the long lag phase. Whether the synthesis of the same proteins that were prevented by ABA occurs in the presence of CK is also a subject for future investigation.

Results shown here suggest that at least part of the actions of GA_3, ABA, and CK on lettuce seed germination are mediated directly or indirectly through modulation of protein synthesis. Since the physiological actions of ABA and CK are at a different site from that of GA_3 (5, 17) we must now determine if there are qualitative differences in protein synthesis affected by these three compounds which might bear this hypothesis out. Certainly, our preliminary results on RNA metabolism in germinating lettuce seeds show that ABA and ABA + 6-BA can alter RNA synthesis during early germination, whereas GA_3 (10), like red light (12), does not.

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