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# Amide-Linked Indoleacetic Acid Conjugates May Control Levels of Indoleacetic Acid in Germinating Seedlings of *Phaseolus vulgaris*[1]

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## ABSTRACT

**We have shown that amide-linked IAA (indole-3-acetic acid) conjugates accumulated to high levels during maturation of bean seeds (K. Bialek and J.D. Cohen [1989] Plant Physiol 91: 775–779). In the present study, we were interested in the fate of these and other IAA conjugates during seed germination. The content of amide-linked conjugates of IAA in cotyledons declined dramatically during the first hours of imbibition. The rate of decline slowed markedly during the period of the resumption of axis growth. The level of amide-linked IAA conjugates in cotyledons remained relatively high after almost 1 week of germination. The decline of IAA conjugates in cotyledons was followed by a steady increase in the content of both free and amide-linked IAA in the embryonic axes. Amide-linked IAA conjugates were also present in the axes cultured on agar after the cotyledons were removed, which suggests that de novo production of these IAA conjugates occurs in the axis of germinating bean seedlings. A comparison of relative amounts of free and conjugated IAA in the axes of intact seedlings and axes cultured on agar showed lower levels of free IAA and higher levels of conjugated IAA in much slower growing isolated axes. These results suggest a more general role for IAA conjugates in the control of seedling growth than simply to serve as a seed storage form of auxin.**

Seeds of higher plants are known to accumulate large amounts of IAA conjugates during maturation. The studies of maize (*Zea mays*) have shown that the IAA ester conjugates that accumulate in the storage tissue of kernels are practically the only source of free IAA for the growing seedling (3, 18, 20, 21). Legumes accumulate mostly amide-linked IAA conjugates in mature seeds (1, 5–7, 11, 12, 17). In bean (*Phaseolus vulgaris*), amide-linked IAA conjugates increase gradually in developing seeds (7). They represent approximately 80% of the total IAA pool in completely mature seeds. The amount of amide-linked IAA conjugates that accumulated in mature seeds was closely related to the amounts of free and ester-linked IAA that disappeared from the rapidly growing seeds. A series of immunologically related IAA peptides of 3 to 25 kD were the major IAA conjugates found in bean seeds (4, 5, 16).

The aim of this study was to analyze the fate of IAA conjugates in the cotyledons and axes of germinating bean seeds. Our results indicate that the axes of bean seeds may be autonomous with respect to the IAA supply from the cotyledons during germination. De novo synthesis of amide-linked IAA conjugates in the axes of germinating seeds suggests a role for these compounds in the regulation of the concentration of endogenous free IAA in bean seedlings.

## MATERIALS AND METHODS

### Plant Material

Sterilized bean seeds (*Phaseolus vulgaris* L., cv Bush Burpee, 1989 harvest, obtained from the Meyer Seed Co., Baltimore, MD)[2] were germinated aseptically at 26°C in the dark. Ten seeds were placed in each 100- × 80-mm Petri dish containing several layers of disposable laboratory towels and 40 mL of distilled water. After the specified time (0–6 d), the cotyledons and axes (including both shoots and roots) were removed separately from germinating seedlings and their fresh weights were determined. Seedlings were frozen in liquid nitrogen and freeze-dried. Dry weights of cotyledons and axes were determined from parallel samples. In experiments on the growth of seedlings with the cotyledons removed, the seeds were first imbibed aseptically in water for 24 h, and then the axes were removed aseptically from the seeds. The isolated axes were cultured on MS[3] medium solidified with 1.5% agar. Each experiment was performed twice with three replications in each experiment.

### Determination of Free and Conjugated IAA

The GC-MS-selected ion-monitoring method utilizing $^{13}C_6$-IAA as an internal standard (13) was used for the analysis of the content of free and conjugated IAA. Freeze-dried samples (50–100 mg) were ground in a mortar to a fine powder with a mixture of 65% isopropanol/35% 0.2 M imidazole buffer, pH 7.0, for free IAA determination, or they were directly hydrolyzed in 1 N (1 h, room temperature) and 7 N (3 h,

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[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply approval to the exclusion of other products or vendors that may be suitable.

[3] Abbreviation: MS, Murashige-Skoog.

100°C) NaOH for analysis of free plus conjugated portions of IAA. Hydrolysis with 1 N NaOH releases IAA from ester-linked IAA conjugates, and 7 N NaOH hydrolysis cleaves all conjugated forms of IAA. The latter was carried out in sealed Teflon vials purged with water-saturated nitrogen gas. An $O_2$-scrubbing cartridge was added to the gas line to trap any traces of $O_2$ in the system (6). Approximately 0.84 kBq of [$^3$H]IAA (803 GBq × mol$^{-1}$, Amersham) was added before homogenization or hydrolysis to aid in IAA peak detection during the purification procedure along with the $^{13}C_6$-IAA (50–100 ng/sample) internal standard. The samples were purified using solid phase extraction minicolumns and HPLC as described previously (10). Purified samples were methylated and analyzed by GC-MS-selected ion monitoring. The analyzed ions included the unlabeled and labeled quinolinium and molecular ions (m/z 130, 136, 189, and 195) of methylated IAA. The amount of free IAA in the plant tissue was calculated using a modified isotope dilution equation as described previously (13). The amount of ester-conjugated IAA was calculated by subtraction of free IAA from the free plus ester-linked IAA determined after 1 N NaOH hydrolysis. Similarly, amide-linked IAA was determined by subtraction of the free plus ester value from the total IAA determined after 7 N NaOH hydrolysis.

## RESULTS

Analysis of the contents of free and conjugated IAA in the cotyledons of germinating bean seedlings showed a decline of amide-linked IAA conjugates during the first 2 d of germination (Fig. 1A). More detailed analysis of amide-linked IAA conjugates in the first days of germination revealed that the most dramatic decrease of these compounds in the seeds occurred in the first 8 h of imbibition (Fig. 2). Ester-linked IAA conjugates were practically not detectable in germinating seedlings during the course of our experiments, although we detected them previously in our studies of developing bush bean seeds (7).

As germination progressed, the rate of decline of conjugated IAA in the cotyledons slowed down markedly, and the amide-linked IAA content remained relatively high even after almost 1 week of seedling growth. The decrease in content of amide-linked IAA in cotyledons (Fig. 1A) was parallel to the decrease in their dry matter (Fig. 3A). Consequently, the concentration of these compounds, when calculated on a dry weight basis, remained at a similar level during the course of our experiments (Fig. 1C). The content of free IAA, which was relatively low in the cotyledons of mature seeds, decreased even further during germination. The concentration of free IAA also decreased, except on the first day of imbibition, when the IAA level measured on a dry weight basis increased slightly (Fig. 1C).

Contrary to the results for cotyledons, the contents of both free and amide-linked IAA increased steadily in the axes of germinating seedlings (Fig. 4A). These increases in free and total IAA were parallel to the increases in both fresh and dry weight (Fig. 5A). However, when changes in concentrations of both free and conjugated IAA measured in the axes were expressed on a fresh or dry weight basis, the dynamics of these compounds during germination gave a somewhat different picture. The concentration of both free and conjugated IAA in the axes (expressed on a basis of their fresh weight) actually decreased in the first 2 to 3 d of germination, and after that time, they remained at almost the same level (Fig. 4B). This sharp decline of auxin concentration in bean axes was coincident with the rapid uptake of water in the first days of germination (Fig. 5B). When the concentration of

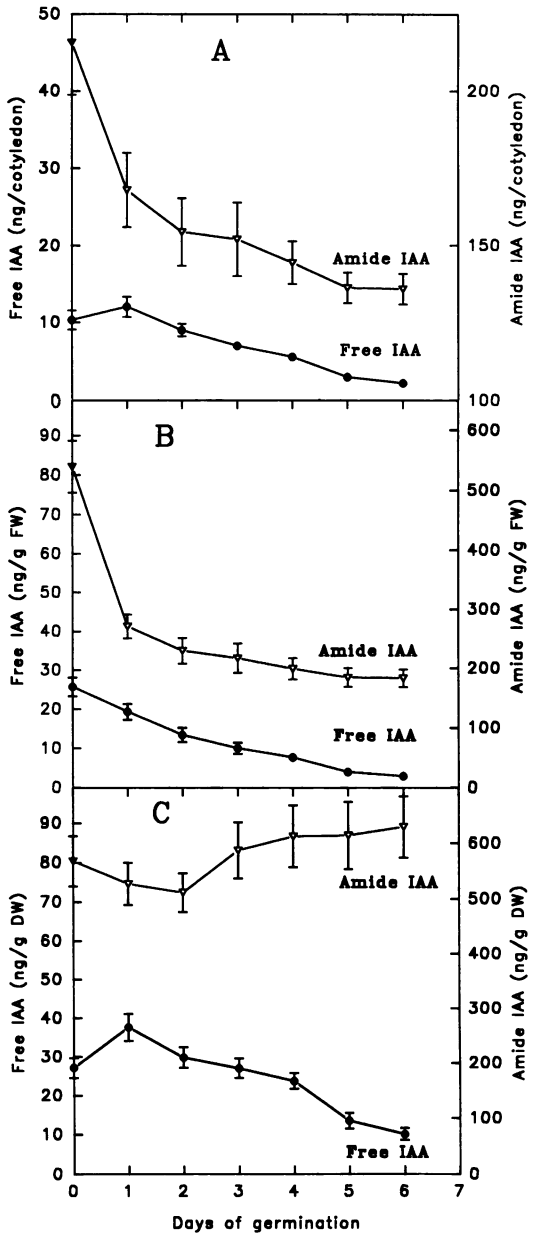


**Figure 1.** Changes in free and amide-linked IAA in the cotyledons of germinating bean seedlings expressed on a cotyledon (A) or fresh (B, fresh weight) or dry (C, dry weight) weight basis. Note almost parallel decline of the content of amide-linked IAA (A) and dry weight (Fig. 3 A), which resulted in similar levels of these compounds throughout the germination period when expressed on a dry weight basis (C). Vertical bars, when larger than the marked points, indicate the SD from the mean of four to six replicate samples.

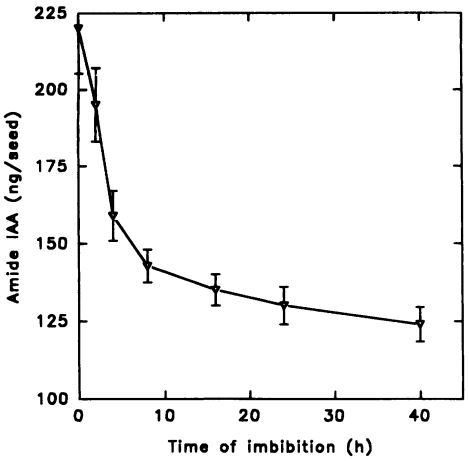


**Figure 2.** Changes in the content of amide-linked IAA during imbibition of bean seeds. Whole seeds were analyzed in this experiment. Each point is the mean of four replicates. Vertical bars show the SD from the mean.

these compounds was expressed on a dry weight basis, an initial increase in the level of both free and conjugated IAA was observed during the first 2 d of germination, which was followed by a decrease and then stabilization of their concentration (Fig. 4C).

We also evaluated the influence of removal of cotyledons on the changes in free and conjugated IAA in the axes during germination. The results of these experiments are presented in Table I. The growth rate of seedlings cultured on agar with their cotyledons removed was reduced during the time of the experiment in comparison to intact seedlings. This was accompanied by a reduction of the total IAA pool. After 6 d of germination, the fresh weight of such isolated seedlings and their total IAA content were approximately 10 times lower in comparison to the axes of intact seedlings. Thus, the concentration of total IAA expressed on a fresh weight basis was actually maintained at the same level in both intact and isolated axes.

Analysis of the contribution of free and conjugated IAA to the total IAA pool showed that, in spite of cotyledon removal, amide-linked IAA was present in the isolated axes, and it constituted even a higher percentage of total IAA than in the axes of intact seedlings. This was the result of a gradual increase in the content of conjugated IAA, whereas the content of free IAA remained at practically the same level. The changes in the contribution of free and conjugated IAA to the total IAA pool of germinating axes of both treatments are shown in Figure 6. The percentage of free IAA in the total IAA pool of axes of intact seedlings increased from 15.8% in dry seeds to 37.0% after 6 d of germination. In 6-d-old isolated axes, free IAA constituted only 12.5% of the total IAA pool.

## DISCUSSION

It is well established that ester conjugates of IAA, abundant in mature maize kernels, are the major source of auxin required for the growth of seedlings (3, 18, 20, 21). In maize, the IAA ester conjugates were shown to disappear during germination as they were mobilized to provide for the free IAA needs of the seedling (24). Disappearance of IAA ester conjugates from the seed and simultaneous appearance of free IAA during germination was shown for pine (22). However, data concerning changes in IAA conjugates during germination of other plants are limited, especially in cases in which amide-linked IAA conjugates are abundant, such as in the seeds of legumes (7, 11, 14).

Comparison of our results with those of other studies is difficult because data presented by others usually does not differentiate between the storage tissue of the seed and the embryo or embryonic axis. The levels of free and conjugated IAA also were typically expressed on a basis of fresh or dry weight of the whole seed. We have analyzed the levels of free and conjugated IAA separately in the embryonic axis and storage tissue of bean seedlings because such analyses tell us more about the sequence of changes and possible redistribution in the levels of these compounds during germination and seedling growth. In addition, results expressed on a fresh or dry weight basis of the whole seed may be misleading because dramatic changes in dry and fresh weights due to intensive water uptake and the use of reserves are not the same for the cotyledons and embryonic axis

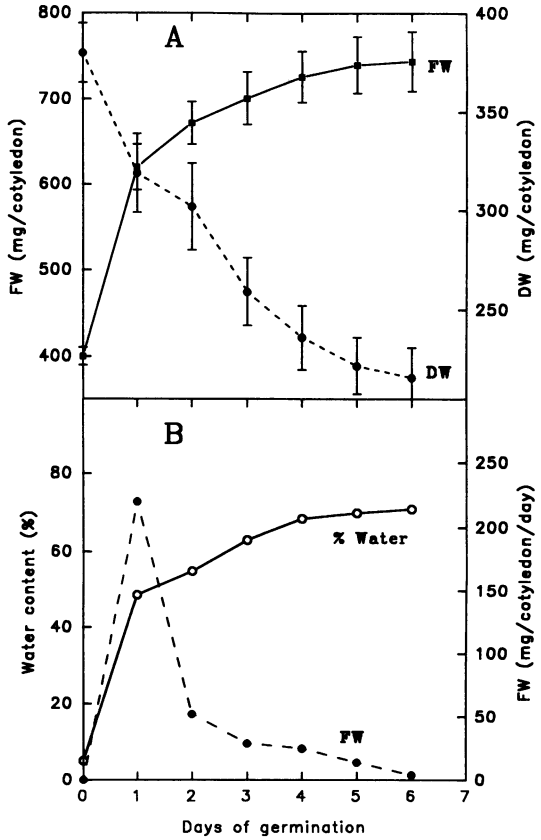


**Figure 3.** Dry (DW) and fresh (FW) weights in the cotyledons of germinating bean seedlings (A) as compared to changes in the water content and the rate of fresh weight accumulation (B). Vertical bars show SD from the mean of at least four replicate samples.

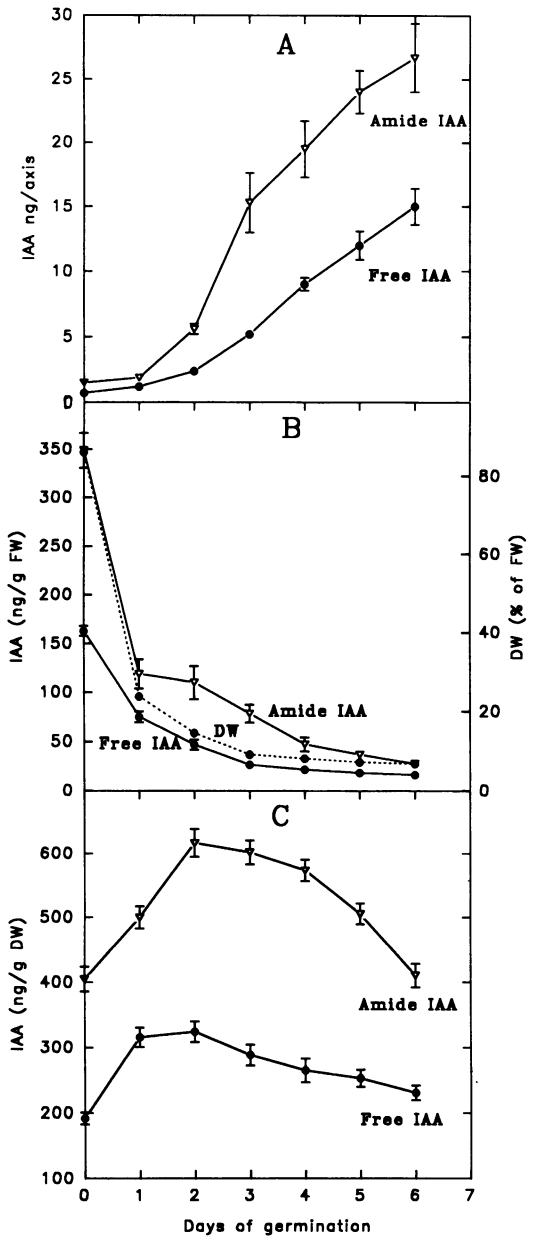


**Figure 4.** Changes in free and amide-linked IAA in the axes of germinating bean seedlings expressed on an axis (A) on fresh (B, FW) and dry (C, DW) weight basis. Note the relationship between the decline in the level of both free and conjugated IAA in the axes on the first day of germination, when data were expressed on a fresh weight basis, and the decrease in dry weight content (B). The decrease in the level of these compounds after 2 d of germination, when expressed on a dry weight basis (C), was related to a rapid increase in the dry weight of the axes (Fig. 5A). Vertical bars, when larger than the marked point, represent the SD from the mean of four to six replicate samples.

(compare Figs. 3 and 5). Our experiments show that the content of amide-linked IAA in cotyledons decreased in the first days of germination simultaneously with the decrease in dry weight. Similar patterns of changes in levels of IAA conjugates during germination were described before for maize and pine when whole seeds were analyzed (22, 24).

In our experiments, the decline in content of IAA conjugates in cotyledons was followed by a gradual increase of the total contents of both free and conjugated IAA in the embryonic axes. When these results were expressed on a fresh weight basis, the total IAA level in the embryonic axes declined dramatically (Fig. 4B). However, this was clearly related to the dramatic uptake of water in the first days of germination. During the first day of water imbibition, the percentage of dry weight in the axes decreased from 86 to 23% because of the intensive uptake of water. This tremendous increase in the water content of embryonic axes was reflected in a dramatic decrease in the total IAA concentration (including both free and conjugated IAA). Stabilization of dry weight in germinating seedlings was accompanied by a stabilization of the total IAA level. Therefore, one can assume that the production of IAA in bean seedlings is actually maintained at almost the same level. When our results concerning the changes in free IAA in the axes of germinating

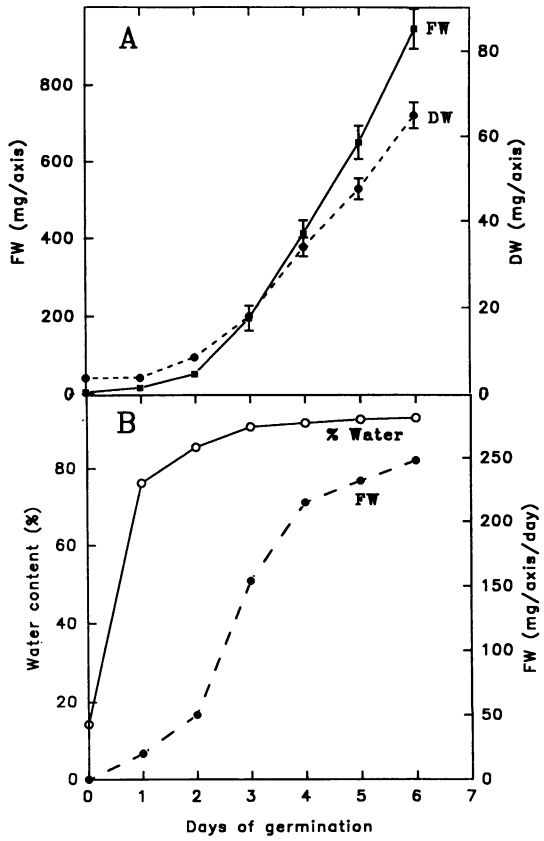


**Figure 5.** Dry (DW) and fresh (FW) weights of axes of germinating bean seedlings (A) as compared to the changes in the water content and the rate of fresh weight accumulation (B). Vertical bars show SD from the mean of at least four replicate samples.

**Table I.** *Contents of Free and Conjugated IAA in the Axes of Germinating Intact Bean Seedlings or Axes Isolated from the Seedlings and then Cultured on Agar*

The isolated axes were removed from the seeds, imbibed aseptically for 24 h, and then transferred to 1.5% agar supplemented with MS medium. Day of germination of isolated axes shows the age of seedlings; for example, 2 d germination means 1 d water imbibition and 1 d incubation on MS medium/agar. Data are the mean values from duplicate experiments with three replicates in each experiment. Numbers in parentheses are the SD from the mean.

| Days of Germination | Type of Axes | Fresh Wt of Axis$^{-1}$ | Amounts of IAA | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total | | Free | | Amide linked | |
| | | *mg* | *ng/axis* | *ng/g of fresh wt* | *ng/axis* | *ng/g of fresh wt* | *ng/axis* | *ng/g of fresh wt* |
| 0 | | 4.3 | 1.9 | 441.8 | 0.3 | 69.8 | 1.6 | 372.0 |
| | | (0.5) | (0.2) | (27) | (0.04) | (6) | (0.2) | (20) |
| 2 | Intact | 51.0 | 8.0 | 156.8 | 2.4 | 47.0 | 5.6 | 109.8 |
| | | (6) | (0.7) | (13) | (0.2) | (4) | (0.4) | (9) |
| | Isolated | 21.0 | 3.1 | 148.0 | 0.6 | 28.6 | 2.5 | 119.4 |
| | | (2.3) | (0.3) | (12) | (0.06) | (3) | (0.2) | (10) |
| 4 | Intact | 412.5 | 28.5 | 69.0 | 9.0 | 21.8 | 19.5 | 47.2 |
| | | (33) | (2.7) | (5) | (0.5) | (2.3) | (2.2) | (4.5) |
| | Isolated | 50.0 | 3.5 | 70.0 | 0.6 | 12.0 | 2.9 | 58.0 |
| | | (5.5) | (0.3) | (7) | (0.05) | (1.3) | (0.3) | (6) |
| 6 | Intact | 945.0 | 41.7 | 44.1 | 15.0 | 16.3 | 26.7 | 27.8 |
| | | (50) | (4) | (4) | (1.4) | (1.5) | (2.7) | (2.6) |
| | Isolated | 104.0 | 4.0 | 38.5 | 0.5 | 4.8 | 3.5 | 33.6 |
| | | (12) | (0.4) | (4.1) | (0.04) | (0.5) | (0.3) | (3) |

seedlings were expressed on a dry weight basis, they showed good agreement with data obtained for pine, maize, and bean, in which the IAA concentration increased in the first days of germination, reached a peak after 2 to 3 d, and then began to decline (22, 23).

We have shown recently that de novo biosynthesis of IAA was initiated in bean seedlings on the 2nd or 3rd d of germination (9). Thus, the initial increase of free IAA in the cotyledons and embryonic axes, when calculated on a dry weight basis, is probably related to the release of IAA from conjugates accumulated in the dry seed. In later stages of germination, when de novo biosynthesis of IAA, mostly from tryptophan, is becoming gradually a major source of free IAA (9), the IAA conjugates still present in the cotyledons have to be much less important in supplying auxin to the young bean seedling.

The data presented in this report show that the role of amide IAA conjugates in the bean seedling is not limited to the storage of free auxin in the seed. Our results suggest a more general regulatory role for these conjugates in bean seedling development. Strong support for such a conclusion comes from our experiments in which the cotyledons were removed and the axes cultured on agar supplemented with MS culture medium. We show that IAA conjugates were not only present in such axes but that they constituted a higher percentage of the total IAA pool than in the axes of intact seedlings (Table I and Fig. 6). Moreover, the concentration of total IAA (measured on a weight basis) was actually the same in both intact axes and axes growing without cotyledons. Only the relative proportion of free to conjugated IAA changed. Thus, the net accumulation of IAA was maintained at the same level, and the excess IAA, which was not utilized by the seedlings when the growth rate was drastically reduced after removal of the cotyledons, was conjugated. We

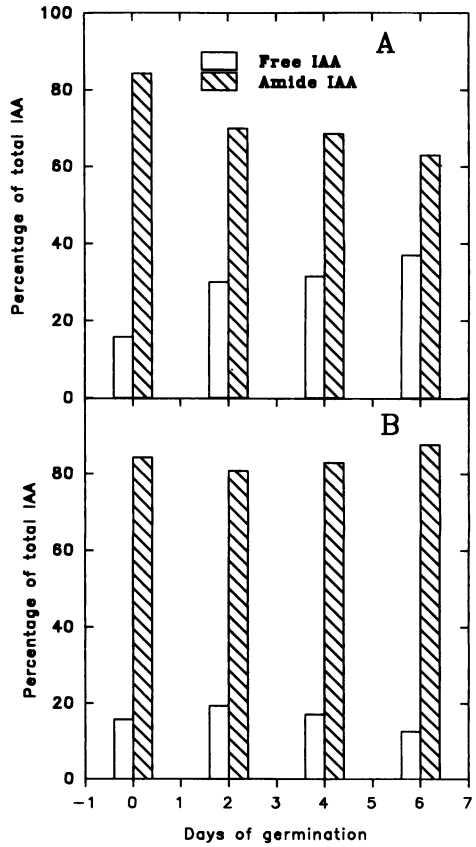


**Figure 6.** The contribution of free and amide-linked IAA to the total IAA pool during germination of intact (A) or isolated (B) bean axes.

showed previously that IAA amide-linked conjugates were hydrolyzed by bean seedlings when they were applied to the stem sections or studied in vitro (8, 15). Thus, both synthesis and hydrolysis of amide-linked IAA conjugates have been demonstrated in bean seedlings, and these conjugation/hydrolysis reactions could be involved in regulation of IAA levels in the plants growing under different conditions. Such a function for ester IAA conjugates in the control of IAA levels in maize seedlings exposed to different light conditions was first suggested by Bandurski et al. (2).

The presence of IAA-Asp was reported for the cotyledons and hypocotyls of 7-d-old soybean seedlings (11, 12). The level of IAA-Asp in such seedlings was significantly higher than the levels found in dry seeds (12). Moreover, the transport of $^{14}$C-labeled IAA-Asp applied to cotyledons of these seedlings showed that only a small percentage of label from the conjugate moved to the hypocotyls. Other studies have also shown that the rate of transport of IAA amino acid conjugates in bean stem sections was greatly reduced in comparison to that for the free acid (19). These data further support our findings that suggest IAA conjugates found in the axes of legumes in later stages of germination do not originate from the cotyledon reserves but, rather, are produced de novo in the hypocotyls.

The influence of endosperm removal on the levels of IAA and ester-linked IAA conjugates in germinating maize seedling was studied by Momonoki et al. (20). They observed a decrease of free IAA levels in seedlings in which the endosperm had been removed. They interpreted these results as further support for their hypothesis that ester IAA conjugates are the major, if not the only, source of free IAA for growing seedlings. At the same time, they noticed also a slight increase in the level of ester conjugates in the seedling when the endosperm was removed, but they found this change to be insignificant. However, considering that in their experiments the endosperm was removed after 4 d of germination and then the seedlings were incubated for only 1 d, they probably observed the same phenomenon we report here: de novo production of IAA conjugates can be accelerated in the seedlings when the growth rate is reduced after removal of the storage part of the seed.

In summary, the role of bean amide-linked conjugates of IAA is not limited to serving as a seed IAA reserve for use during germination. De novo production of amide IAA conjugates in the axes at the time when they resume intensive growth and begin their own IAA synthesis suggests a more general role for these IAA conjugates in the control of the seedling growth. We have shown that their role might be related to the homeostatic regulation of the steady-state concentration of IAA in the plant as required for different growth conditions.

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