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A Quantitative Estimation of Alkali-labile Indole-3-Acetic Acid Compounds in Dormant and Germinating Maize Kernels[1]

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Abstract. An estimate has been made of the quantities of alkali-labile esters of indoleacetic acid (IAA) in kernels of sweet corn (*Zea mays*). The amount is between 70 to 90 mg of IAA per kilogram of dry kernels. About one-half of the IAA is present as high molecular weight esters and the remaining one-half as esters of *myo*-inositol. Free IAA, which may have existed in the kernels, or may have resulted from ester hydrolysis during isolation or storage, amounts to between 1 to 10% of the esterified IAA. Five newly observed low molecular weight indoleacetyl compounds are described and their chromatographic behavior reported. The total IAA content of corn kernels and intact seedlings decreases during germination, declining to about 10% of the original content during 96 hr of germination. Difficulties in obtaining quantitative results and the possible physiological significance of these results is discussed.

Indole-3-acetic acid (IAA) is commonly accepted as a naturally occurring plant growth hormone. Nonetheless, most, and possibly even all, of the IAA in some tissues occurs as a complex "bound auxin". For example, most of the IAA of corn kernels, and all of the detectable IAA of corn shoots occurs as an alkali-labile complex (5). Methods for quantitative analysis of the bound auxins have not been developed. In previous publications from this laboratory (8, 10) the occurrence in corn kernels of 4 esters of indole-3-acetic acid (IAA) and *myo*-inositol have been reported. These were designated as the B group and include B_1 (indole-3-acetyl-1-0-*myo*-inositol), B_2 (indole-3-acetyl-2-0-*myo*-inositol), B_3 and B_4. B_3 and B_4 are arabinosides of B_1 and B_2 respectively. Earlier work (1, 5, 11) has established that corn kernels also contain a high molecular weight IAA complex, here designated as the A fraction.

This report is concerned with the estimation and partial characterization of the alkali labile IAA complexes in dormant corn kernels. The changes in concentration of these complexes during germination have been studied as a step towards clarification of their physiological role. The chromatographic properties of 5 newly observed esters of IAA are reported. A preliminary brief report of these data has been submitted (3).

Methods and Results

Extraction. Corn kernels (*Zea mays* L., cultivar, Stowell's Evergreen Hybrid, 1963 harvest) were ground to 20 mesh and stored at −20°. A 15 g sample was extracted with 60 ml of 50% aqueous acetone for 1 hr at room temperature with continuous agitation by a magnetic stirrer and then filtered. This procedure was repeated 4 more times. Each of the acetone extracts were then assayed for alkali-labile IAA compounds. The extracts were made 1 N with respect to NaOH, then, after 15 min at room temperature, they were acidified to pH 2.5 with 5 N H_2SO_4 and extracted 3 times with an equal volume of peroxide free ether. The ether was evaporated to dryness, the residue redissolved in 1 ml of 95% ethanol, 4 ml of Salkowski reagent (12) added and IAA estimated colorimetrically. The corn meal residue was extracted with 100 ml of N NaOH for 15 min at room temperature. This extract was acidified and treated as above. IAA complexes which are not extracted and hydrolyzed by this procedure would escape detection.

Colorimetric assays were corrected for interference by compounds which yield a yellow-brown color with Salkowski reagent. Optical density was measured at 482, 532, and 582 mμ to establish a base line between 482 and 582 mμ. The height of the 532 peak above the base line obtained by the equation

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$A_{532} - (A_{482} + A_{632})/2$, was compared to the same value for IAA standards.

As shown in Fig. 1, 5 extractions with aqueous acetone removed 95 % of the alkali-labile esters. The 100 % value, in the figure, is the total amount of IAA obtained by summing the IAA content of the successive extracts. In other experiments, it was found that a single extraction with 300 ml of 50 % aqueous acetone (per 15 g) for 5 hr gave the same or a higher extraction efficiency and thus this procedure was used for the experiments reported in this paper.

Fractionation Procedure. The fractionation procedure adopted is shown in Fig. 2. A sample of 15 g of ground corn kernels was extracted with 300 ml of 50 % aqueous acetone for 5 hr and filtered. The filtrate was concentrated to near dryness in a rotating film evaporator at a temperature below 45°. During this procedure some gummy material precipitated. Adhering liquid was recovered by washing the gum twice with 20 ml portions of distilled water. Suspended material was removed by centrifugation for 20 min at 25,000*g*, leaving a still cloudy extract. The extract was lyophilized, redissolved in 4 ml of distilled H_2O, placed on top of a 51 × 1.1 cm column of Sephadex G-10 and eluted with distilled H_2O to yield fractions 1 to 6. The precipitate was then extracted twice with 20 ml portions of 50% aqueous acetone and centrifuged. The residue is fraction 10. The combined supernatant fluids were chromatographed on a 52 × 1.1 cm column of Sephadex G-10 with 50 % aqueous acetone as eluent, yielding fractions 8 and 9. The remaining

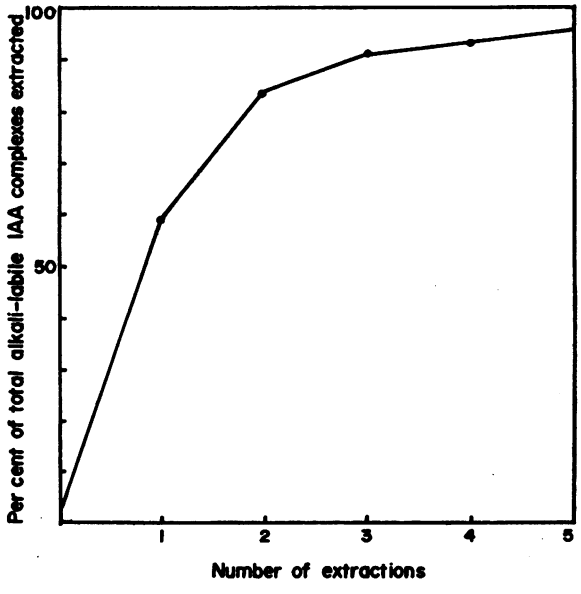

FIG. 1. Efficiency of extraction of IAA compounds from corn kernels. The 100 % value is the total amount of IAA obtained by summing the IAA content of the 5 successive extracts and the alkaline hydrolysate of the residue. The curve shows the cumulative yield.

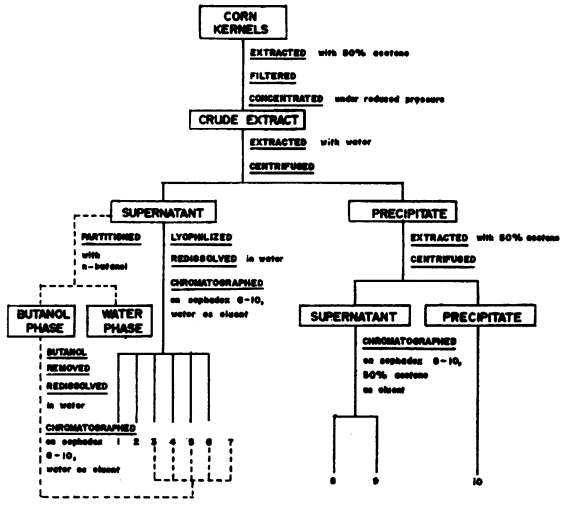

FIG. 2. Fractionation procedure for the characterization and quantitative estimation of IAA compounds in corn kernels. Fractions are numbered from 1 to 10 for convenience of discussion. Fractions derived from the supernatant fluid are deisgnated as B fractions, while those from the precipitate are called A fractions.

insoluble material was suspended in 50 ml of N-NaOH in 50 % aqueous acetone to hydrolyze any remaining esters. Elutes from the above columns, after alkaline hydrolysis, were assayed for Salkowski positive substances and the positive fractions were examined with 2 solvent systems by Silica gel thin layer chromatography after concentration by lyophilization.

In some experiments, the water extract was partitioned twice against an equal volume of *n*-butanol. The apparent partition coefficient was 0.2 (in the organic phase) for the total B group. The butanol phase was evaporated to dryness under reduced pressure, redissolved in distilled water and chromatographed on a Sephadex G-10 column with distilled water as eluent and yielded fractions 3 to 7.

Recovery of Authentic IAA. The following experiment was conducted to check for possible losses of IAA by destruction or absorption. To 15 g of ground corn kernels (1967 harvest) 800 μg of authentic IAA was added at the beginning of the extraction. After extraction, concentration and re-extraction with water, the water soluble fraction was divided into 2 equal portions. IAA (400 μg) was added to 1 portion and both were then assayed for free IAA. The same experiment was conducted with 4 day old seedlings from 15 g of corn kernels. The results are shown in table I. Authentic IAA, added before extraction, was lost to some extent, the highest loss being observed with germinated plants. As no free IAA was recovered from the water insoluble fraction, the loss observed was mainly due to IAA destruction. Even IAA added just before the assay was not recovered completely

Table I. *The Recovery of Added IAA*

Tissue	Addition of IAA 1st[1]	Addition of IAA 2nd[2]	Free IAA Recovered	Endogenous free IAA	Recovery from 1st Addition	Recovery from 2nd Addition
Kernels	μg	μg	μg	μg	μg	μg
Expt. 1	400	0	316	2	314	0
					↓	
Expt. 2	400	400	700	2	314 (79 %)	384 (96 %)
Seedlings (96 hr)						
Expt. 1	400	0	260	0	260	0
					↓	
Expt. 2	400	400	620	0	260 (65 %)	360 (90 %)

[1] The first addition of IAA was made before extraction.
[2] The second addition of IAA was made after concentration of the acetone extract and removal of the A fraction. The arrows indicate that it is assumed that the recovery of the first IAA addition is the same in Experiment 2 as was measured for Experiment 1.

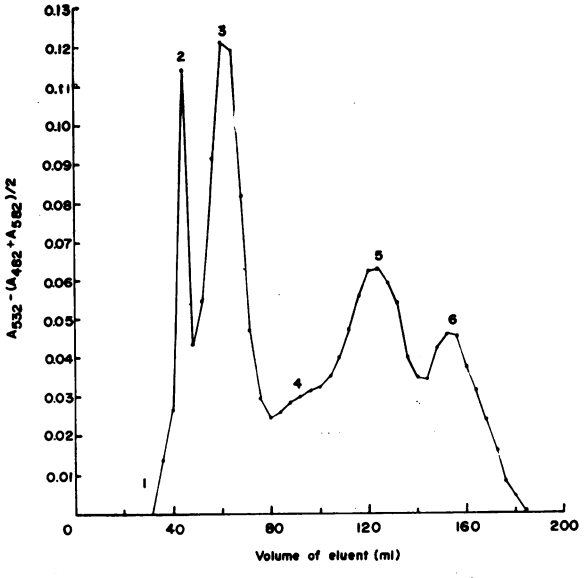

FIG. 3. Elution pattern of the water soluble fraction from Sephadex G-10 column using water as eluent. IAA was assayed as follows: To a 2 ml aliquot (out of 4 ml collected per tube) 0.5 ml of 5 N NaOH was added, and after 15 min, 4 ml of Salkowski reagent was added. The absorption at 482, 532, and 582 mμ were measured 30 min later. The numbers show the fraction number as described for Fig. 2.

suggesting complexing with some water soluble constituents. This adsorbed IAA could be released by mild alkaline treatment.

Qualitative Aspects. The elution pattern of the water soluble fraction from a Sephadex G-10 column is shown in Fig. 3, while Fig. 4 shows the elution pattern for the butanol extract. Several more lipophylic substances can be seen in chromatograms of the butanol extract that, owing to low concentration, could not be found in the total water soluble fraction.

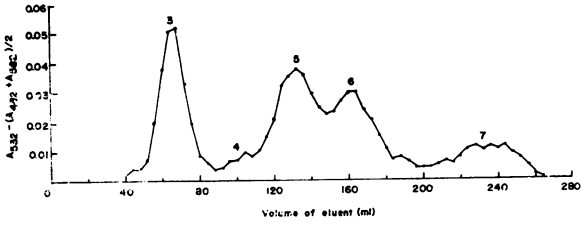

FIG. 4. Elution pattern of the butanol fraction from a Sephadex G-10 column with water as eluent. The method of IAA assay is as described for Fig. 3.

The elution pattern of the 50 % aqueous acetone soluble fraction is shown in Fig. 5. The ordinate represents the absorbancy in the Salkowski test, corrected as discussed above. The IAA containing fractions from column chromatography were examined by Silica gel TLC using a Salkowski reagent

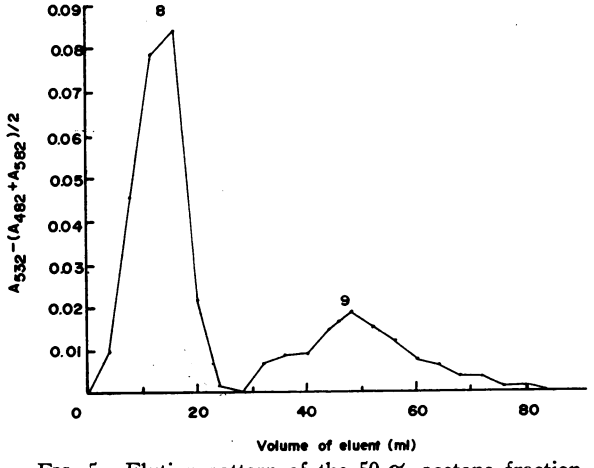

FIG. 5. Elution pattern of the 50 % acetone fraction from a Sephadex G-10 column with 50 % acetone as eluent. The method of IAA assay is essentially the same as that used for Figs. 3 and 4.

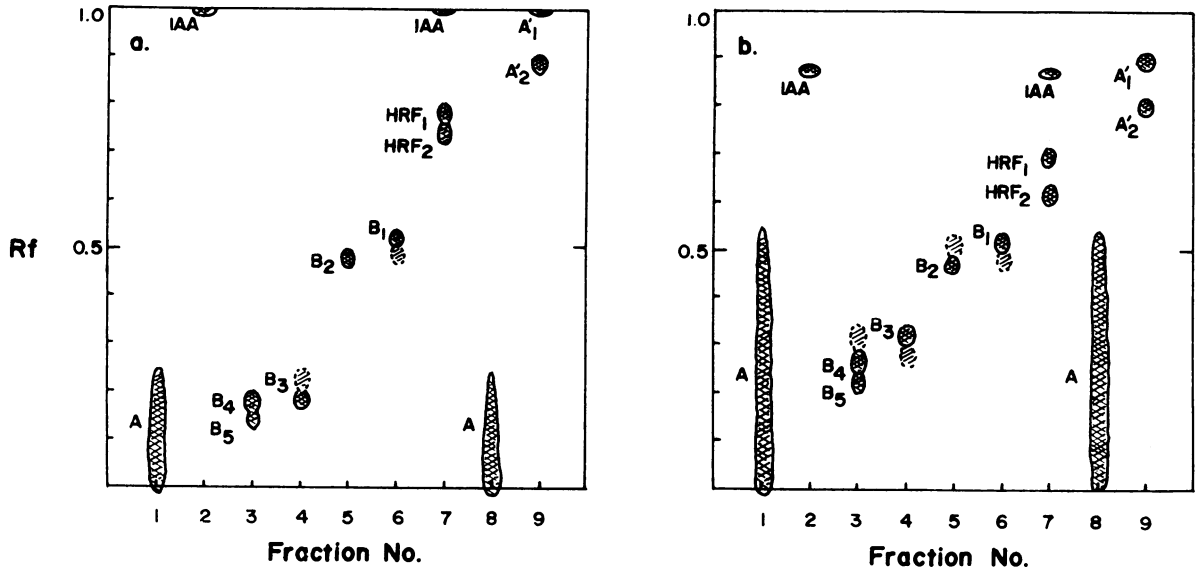

FIG. 6. TLC patterns of IAA compounds for each fraction from column chromatography. The plates used were from Brinkmann Instruments Inc. (Silica Gel F-254). Two solvents were used: a) ethyl acetate; methylethyl ketone; formic acid; water (5:3:1:1). b) ethyl acetate; methylethyl ketone; ethyl alcohol; water (5:3:1:1). A Salkowski reagent spray was used for visualization. Free IAA developed a pink color and the esters developed a purple color.

spray for visualization (Fig. 6). Fraction 1 contains a residual amount of A compound(s) which did not precipitate during concentration. Fraction 2 contains free IAA. Fraction 3 contains B_4 and a minor amount of an unknown IAA compound, B_5. B_5 can be separated from B_4 more effectively by paper chromatography (*n*-butanol:acetic acid:water, 5:1:2.2) and was shown to be an IAA ester. Fraction 4 contains B_3 and B_4, the isomer of B_3. Isomerization of B_3 to B_4 is minimized when neutral solvents are used for thin layer chromatography indicating, as would be expected, that acidic solvents favor isomerization of B_3 to B_4. Fraction 5 and 6 contain B_2 and B_1 accompanied by their isomers B_1 and B_2 respectively. Interconversion between B_1 and B_2 has previously been demonstrated (10). Fraction 7, obtained from the butanol extract, contains 2 IAA compounds designated as HRF_1 and HRF_2 and free IAA. These 2 new compounds also release IAA after mild alkaline hydrolysis. Fraction 8 corresponds to the A compound(s). Fraction 9 contains 2 more lipophylic, alkali-labile IAA compounds designated A'_1 and A'_2, contaminated with a minor amount of the B group. It is uncertain whether fraction 10 (A") is a compound or a group of compounds different from the A fraction. No extensive studies have been made of fraction 10 but it is difficult to redissolve in acetone-water whereas the A fraction is readily redissolved.

In addition to the above compounds corn kernels also contain some other Salkowski positive substances. However, the speed of color development and stability and tint of the color developed is so different from that of IAA compounds that these interfering compounds are easily recognized.

Remarks on the Behavior of IAA. As will be seen in Fig. 6, peak 2 of Fig. 3, and peak 7 of Fig. 4, contain free IAA. IAA is eluted quickly or slowly depending upon other substances in the extracts. From the water soluble fraction of corn kernels harvested in 1967, free IAA is eluted with $B_4 - B_5$, and from that of 2 day old germinated plants free IAA is eluted with B_3. Authentic IAA neutralized with NaOH to pH 6.1 is eluted with fraction 2, but when acidified to pH 3.1, it is eluted far later than the fraction 7 position. IAA dissolved in 50% aqueous acetone comes between these extremes. These results are summarized in Fig. 7. Dissociation of the imidazole-*N* and the carboxyl group of IAA and the resultant changes in solubility of IAA in complex mixtures explains this behavior. Buffering of the column, while helpful, unfortunately reduces the already weak adsorptive capacity of the Sephadex column and also interferes with subsequent examination by TLC. Thus, it was found necessary to correct for free IAA in the various fractions as discussed below.

Quantitative Aspects. Several difficulties, mentioned briefly above, were encountered in the quantitative estimation of these many IAA compounds. Due to incomplete separation of fractions during column chromatography, several estimations are reported as the sum of 2 fractions, for example, $B_4 + B_5$ and A and other "A" fractions. In addition some fractions, for example, the HRF fraction, are present in such small amounts that they were

Table II. *Quantitative Estimation of IAA Compounds in Corn Kernels*

The value for free IAA has been corrected for losses during analysis using the experimentally determined factor of 1.27 from table I. The values for all other compounds are not corrected.

	1963 Harvest			1967 Harvest		
	mg IAA/kg	Percent		mg IAA/kg	Percent	
Total	88.5	(100)		72.6	(100)	
Free IAA	10.5	11.9		0.5	0.7	
A group	34.9	38.4	(100)	42.2	58.2	(100)
A + A'	29.2	83.7		39.4	93.4	
A''	5.7	16.3		2.8	6.6	
B group	43.1	48.7	(100)	29.9	41.2	(100)
B_1	6.2	14.4		3.1	10.4	
B_2	11.3	26.2		7.0	23.4	
B_3	4.7	10.9		3.7	12.4	
B_4 + B_5	20.9	48.5		16.1	53.8	

neglected for quantitative purposes. The inconsistent behavior of free IAA made a correction mandatory. Thus each fraction from column chromatography was examined by TLC. For those fractions containing IAA, the IAA content was estimated by acid ether extraction, and corrected for. Difficulties due to free IAA decomposition could be corrected for but, owing to lack of standards, a correction cannot be applied to the esters.

Concentration of IAA and IAA Esters in Corn Kernels. Table II shows the results of a quantitative analysis of dry corn kernels harvested in 1963 and 1967. Considering the fluctuation which might be expected from different ripening conditions, the content of the components and their distribution are rather constant. The only striking difference is that 1963 corn contains a higher amount of "free" IAA.

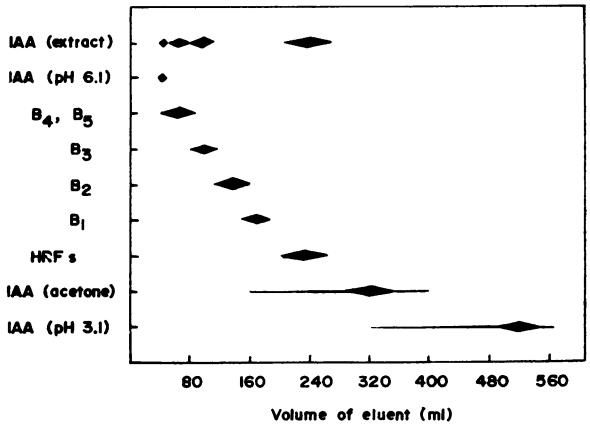

FIG. 7. Elution of free IAA from a Sephadex G-10 column as influenced by preparative conditions. IAA (extract) refers to putative IAA in extracts from dry or germinated corn, described in — Remarks on the Behavior of IAA. IAA (pH 6.1) and IAA (pH 3.1) were prepared by adding NaOH or H_2SO_4 to a solution of authentic IAA. The peaks for the IAA compounds are located for convenience of comparison. HRFs refers to HRF_1 + HRF_2.

Changes in Concentration During Germination. Fig. 8 shows the changes in amounts of the IAA compounds during germination of 1967 harvest corn. Each lot of 15 g of corn kernels was sterilized with 4% formaldehyde solution, washed with water for 4 hr and germinated on moist paper in the dark at 27°. After 24, 48, and 96 hr, the entire plants were harvested and homogenized in a mortar with 50% aqueous acetone, correcting for the water in the plants. The homogenates were extracted for 5 hr with a total volume of 300 ml of 50% aqueous acetone. The content of total IAA, A group and B group all decreased rapidly and linearly. "Free" IAA accumulated at 24 to 48 hr to a small extent. The order of rate of decrease was A > B and $BB_{3,4,5} > B_{1,2}$ but with only a slight difference. B_3 showed some increase between 24 and 48 hr corresponding to the rapid decrease in $B_{4,5}$ during the same time period. Until the 48 hr period, there was no substantial change in the TCL pattern. At 96 hr a considerable contamination with Salkowski positive material was observed. One of these compounds slowly developed a purple color with the Salkowski reagent and was assumed to be the corn sweet substance (6).

Discussion

It has been known for years (1, 2, 4, 5) that sweet corn kernels contain an extraordinarily large amount of IAA as an alkali-labile bound form. The IAA content reaches a maximum 1 to 3 weeks after fertilization and then decreases (2). Various figures have been obtained for the total IAA content of dormant corn kernels, ranging from 13 mg/kg by Haagen-Smit *et al.* (4) to 200 mg/kg by Avery *et al.* (2); Our results of 73 to 88 mg/kg are rather close to the value of 60 to 70 mg/kg reported by Avery *et al.* (1). Since the older data were based on bioassays and ours on colorimetry and since the condition of hydrolysis employed by various workers

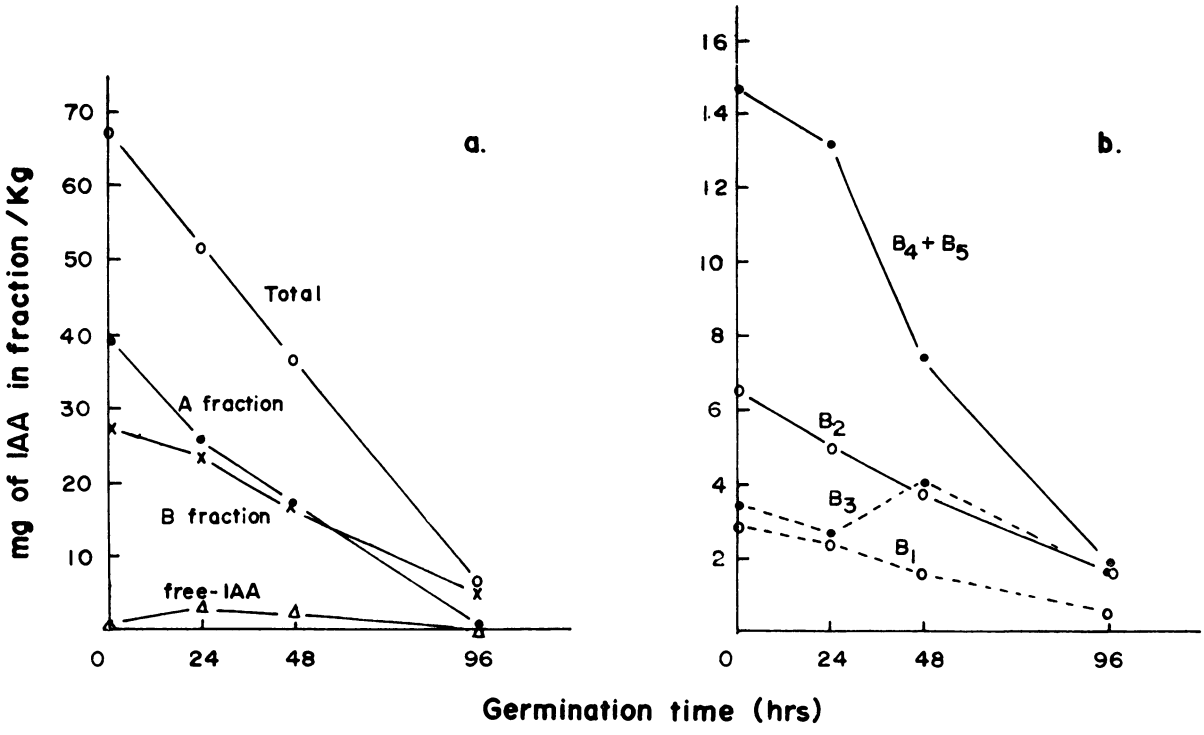

FIG. 8. Changes in concentration of IAA compounds during germination. IAA values were obtained for entire plants and expressed on the basis of the original dry weight of kernels. The figure on the left (a) shows the changes which occur in total IAA, A and B fraction IAA and free IAA and the figure on the right (b) shows the changes occurring in the B group. Free IAA values were corrected for recovery by factors ranging between 1.27 (0 hr) and 1.54 (96 hr), as derived from the data of table I.

differ with regard to alkali strength, time and temperature this seems a reasonable agreement.

The occurrence of free IAA in corn kernels has frequently been reported (*cf.* 1). Since, as is shown in this paper, free IAA may amount to only 0.7 % of the total IAA, it is difficult to determine whether it is a real constituent or an artifact of storage and isolation procedures. Free IAA is released from its conjugates during the isolation procedures employed and, in fact, some IAA is liberated from compounds of the A and B groups during preparation and during storage even at −20°. It is however, equally possible that some portion of the IAA is left free during maturation or is liberated from its conjugates during storage of the kernels. The variation in content of free IAA observed here might support this idea. Our data are however, insufficient to decide whether the small amount of free IAA which we observe in mature corn kernels is, in fact, a constituent or is an artifact of extraction.

In this work, we report the occurrence of 5 new IAA compounds (B_5, HRF_1, HRF_2, A'_1 and A'_2). The chemical structures of these IAA complexes have not yet been determined. It is only known that they are alkali-labile and thus, presumably, are esters, and that they yield IAA upon hydrolysis. Their chromatographic properties suggest that B_5, HRF_1 and HRF_2 are related to the IAA inositols. The A fraction as well as the A″ fraction seems to be a mixture of several compounds. However, studies of Takano *et al.* (11) and Takano and Hayashi (unpublished) indicate that compounds of the A fraction are unstable to the conditions of thin layer chromatography and thus we may be observing degradation fragments. The A compounds yield indole-acetamide upon treatment with ammonia demonstrating that the carboxyl group is esterified (Takano and Hayashi, unpublished). Thus, the chemical relationship between the A and B groups is unknown except that both are esters of IAA and the A group compounds are of higher molecular weight.

The physiological function of both the A and B series of IAA esters is completely unknown. Nonetheless, the occurrence in nature of esters of indole-3-acetic acid and *myo*-inositol seems, to us, significant (3). Many speculative hypothesis are possible including a direct function in maturation and/or germination of kernels; functioning as a labile storage form; or, functioning as a transport form capable of movement to the coleoptile tip. Our present data do not permit conclusions. Recently, however, Nicholls (personal communication) has isolated B_2 from the aleuron layer of wheat seeds indicating that the IAA-inositols are not restricted to corn.

We observed a rapid decrease in concentration of all of these esters, as estimated by the Salkowski test, during germination. According to Hemberg (7), the total auxin content of corn kernels, as measured by biological activity following wet ether extraction, does not decrease during germination. Such extracts might, of course, contain other auxins (4) and thus our data cannot be directly compared to Hemberg's data. Meudt (9) has reported that *in vitro* oxidation of IAA produced intermediate products which are Salkowski negative but very active biologically. To the extent that our results can be compared to Hemberg's, it would seem that the Salkowski negative product(s) formed from these esters is biologically active. There are no available data on the fate of the inositol moiety.

Quantitative determination of IAA and indoleacetyl esters in plant tissues is difficult. For example, losses occur during isolation and purification. We have attempted to estimate such losses for free IAA by the addition of relatively large amounts of additional IAA, as in table I. Such corrections may not be accurate since small amounts of IAA may be lost while larger amounts would be recovered. Hamilton *et al.* (5) added ^{14}C-labeled IAA to homogenates of corn seedlings to permit a correction for losses of IAA during isolation. These workers (in addition to observing the occurrence of an alkali-labile IAA complex in seedling tissue) found that recoveries of free ^{14}C-labeled IAA was increased by adjustment of the original extract to an alkaline pH prior to the first ether extraction. We have confirmed this and conclude that alkaline-ether extraction improves recoveries of free IAA possibly by removal of impurities that preclude extraction of IAA into ether from acid solution or by salt effects that modify the partition of IAA between the aqueous and ether phases. Owing to the importance of an accurate determination of the concentrations of free IAA and IAA esters in plant tissue, we are continuing our studies.

Acknowledgment

We take this opportunity to thank Dr. P. B. Nicholls who supplied the samples of IAA-inositol esters.

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